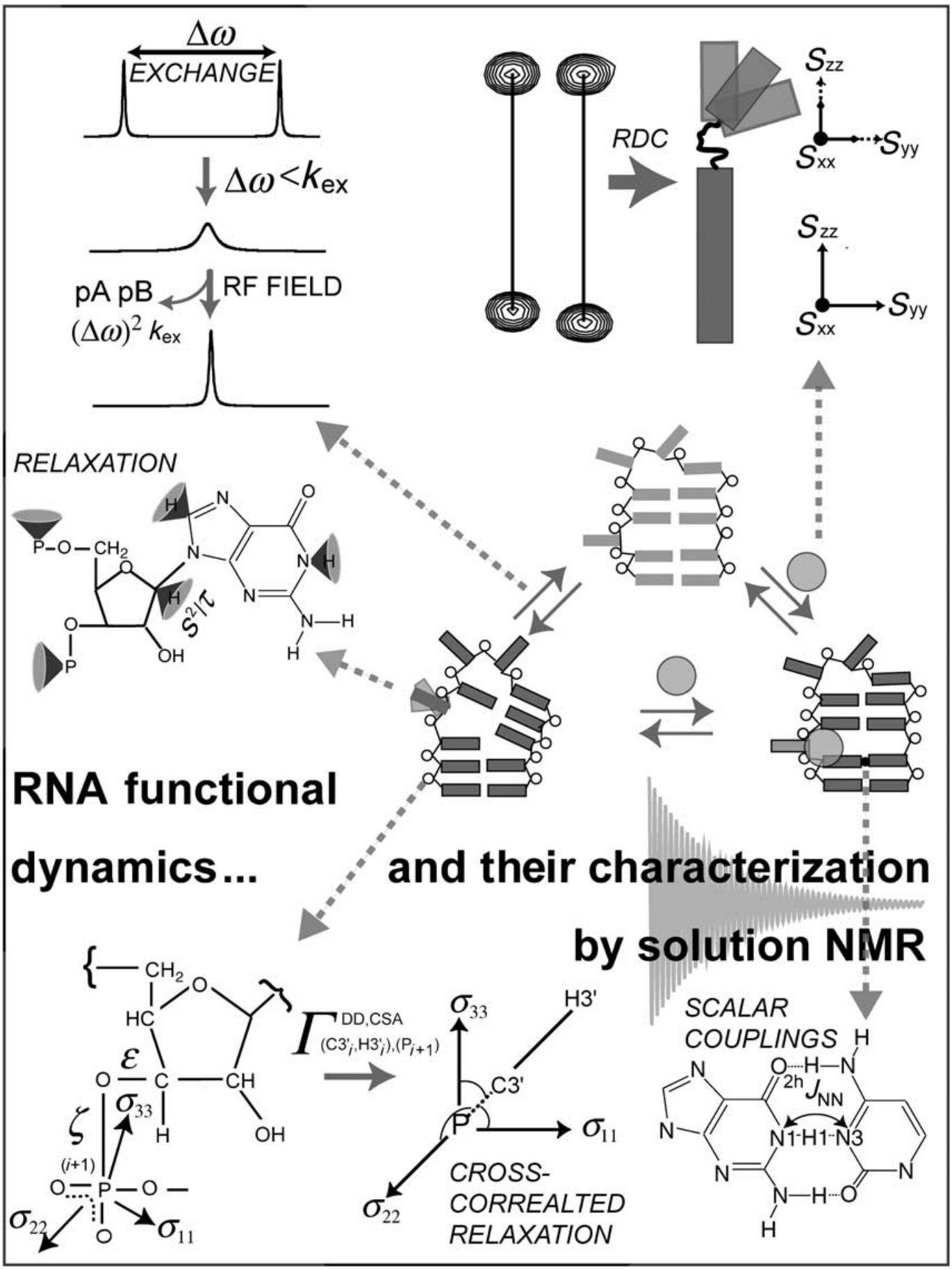

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Dynamics-Based Amplification of RNA Function and Its Characterization by Using NMR Spectroscopy

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The ever-increasing cellular roles ascribed to RNA raise fundamental questions regarding how a biopolymer composed of only four chemically similar building-block nucleotides achieves such functional diversity. Here, I discuss how RNA achieves added mechanistic and chemical complexity by undergoing highly controlled conformational changes in response to a variety of cellular signals. I examine pathways for achieving selectivity in these conformational changes that rely to different extents on the structure and dynamics of RNA. Finally, I review solution-state NMR techniques that can be used to characterize RNA structural dynamics and its relationship to function.

Introduction

The last two decades have witnessed an explosion in the number of cellular roles ascribed to RNA and many more are likely to be uncovered in the near future. RNA can store and relay genetic information, recognize proteins, small molecules, and other RNAs with high specificity, and carry out a range of sophisticated catalytic reactions.[1–5] It is involved in regulation of gene expression at the transcriptional and translational levels, RNA splicing, processing, and modification, and protein synthesis and transport.[2,6–8] The advent of genome sequencing is increasingly aiding the discovery of a universe of small microRNAs[9,10] and interfering RNAs[11,12] that underlie an entirely new genetic regulatory network. The search for such noncoding RNAs hidden in intergenic DNA sequences has only just begun and even the long-standing belief that proteins are the main chemical agents and gene products of living cells is now being questioned.[13]

That RNA is involved in such a variety of functions is quite remarkable considering that it is only composed of four chemically similar building-block nucleotides. Although RNA can fold into more complicated structures than once thought,[14–16] these static structures cannot fully account for RNA's functional diversity. Rather, much of RNA's functional diversity appears to derive from dramatic conformational changes that are either self-induced[17] or, more typically, triggered upon binding to cellular cofactors such as proteins, small molecules, divalent ions, and other RNAs.[1,3,18,19] RNA's structural coverage appears to be more limited in scope in the absence of such cofactors and it is often impossible to deduce the fate of an RNA structure following complexation. Rather, the ubiquitousness of RNA conformational change and its strong coupling to function implies that conformational flexibility in the free RNA can also code for its biological activity. As an extension of this idea, dynamics may generally amplify RNA's functional versatility beyond what is possible based on a stationary structural framework composed of only four building block nucleotides. The goals of this review are to 1) advance dynamics-based amplification of function as a ubiquitous property of RNA, 2) examine its molecular basis, and 3) discuss how NMR spectroscopy can be applied towards its characterization.

Amplifying RNA Function by Conformational Dynamics

The function of most RNAs involves dramatic changes in RNA conformation.[1,3,18,19] Although conformational changes are observed in the functions of most biomolecules, what is particularly striking in the case of RNA is the range of cellular signals that can induce such structural changes, its occurrence in diverse functional contexts, and the shear magnitude of the conformational changes, which can involve large transformations at both the secondary- and tertiary-structure levels.

The functional value of RNA conformational change

The most well characterized RNA structural changes are those associated with the recognition of cellular cofactors. These binding-induced structural changes appear to serve two roles. First, they can allow RNA to access conformations that may otherwise be difficult to stabilize, thereby expanding RNA's structural and hence functional coverage. Second, cofactor-contingent RNA structural changes can be used as a sensory switch for temporally regulating RNA-mediated processes. Consider for example the conformational changes associated with RNA–protein recognition.[1,3,18] A common RNA structural rearrangement involves global reorientation of helical domains about linker motifs that often comprise important components of the recognition site (Figure 1a). As linker motifs such as junctions and bulges often interrupt the remote placement of

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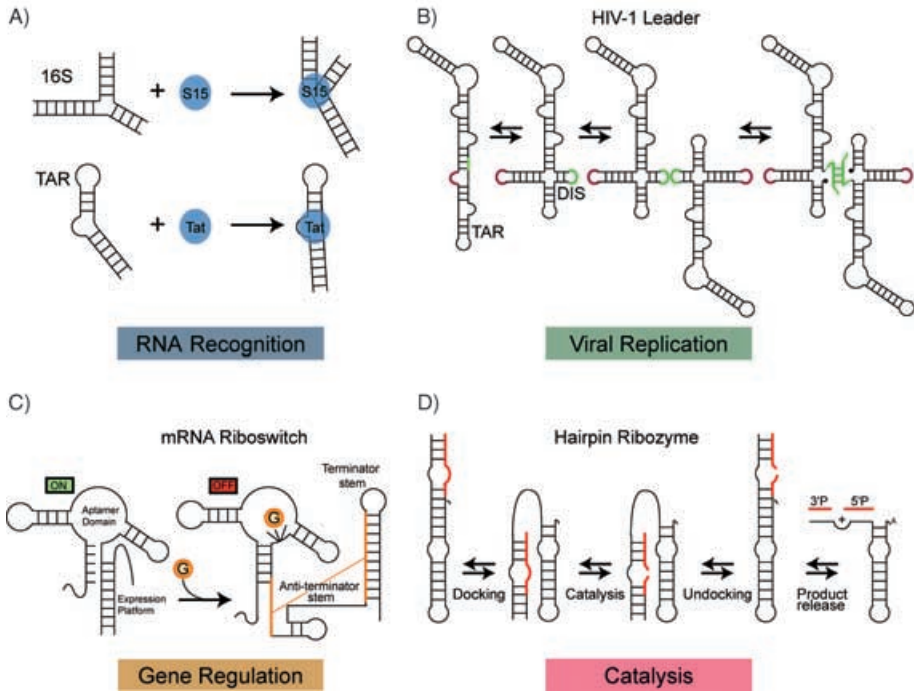

Figure 1. The role of conformational change in RNA function: A) in the recognition of 16S ribosomal and TAR RNA by their cognate protein targets S15 and Tat, respectively; B) in regulation of the timing of replication and packaging functions in HIV-I; C) in regulation of gene expression by allosteric riboswitches (the guanine-specific riboswitch from the *xpt-pbuX* operon of *Bacillus subtilis*[178] is shown); D) in catalysis by the hairpin ribozyme[70] (the substrate is shown in red).

phosphate groups in A-form helices, high negative charge density can accumulate at these sites. The free RNA conformation often minimizes such unfavorable electrostatic repulsion by avoiding coaxial alignment of helical domains.[20–23] However, once presented with a basic protein target, the coaxially aligned RNA conformation can become more favorable in the protein-bound state (Figure 1a). Examples of this mode of conformational adaptation are the recognition of the three-way junction 16S ribosomal RNA (rRNA)[23–27] by the ribosomal protein S15 and the bulge-containing stem-loop HIV-1 transactivation response element (TAR) by the transactivator protein Tat (Figure 1a).[21,22,28] In both cases, binding to divalent ions such as Mg^{2+} also leads to a similar coaxial alignment of domains.[23,29–31] As RNA lacks "positively charged nucleotides", the high negative charge density in such coaxially stacked architectures would be difficult to neutralize and hence stabilize in the free RNA without assistance from positively charged cellular cofactors.

In both of the above examples, the protein-induced RNA conformational switch serves an additional functional purpose. The binding of ribosomal protein S15 to 16S rRNA initiates the ordered assembly of the central domain in the 30S ribosomal subunit.[32] The conformational change in the 16S rRNA induced by S15 binding (Figure 1a) is required in this case to allow subsequent binding of the S6 and S18 ribosomal proteins. Similarly, the conformational change in HIV-1 TAR induced by binding to Tat (Figure 1a) is required for cooperative binding of other proteins and proper assembly of a ribonucleoprotein (RNP) complex, which ultimately activates viral transcription.[33–35] Thus, sequential changes in RNA conformation can help direct the order of hierarchical RNP assembly.[27]

Not all RNAs undergo conformational adaptation upon protein recognition. One example is recognition of the prefolded E-loop-containing helix IV from the *Escherichia coli* 5S rRNA by the ribosomal protein L25.[36] Here, the L25 protein undergoes conformational adaptation, as is also observed for many other RNA-binding proteins.[1,3,18] Similarly, RNA can undergo different degrees of conformational adaptation upon recognition of small therapeutic compounds.[37,38] For example, while recognition of the aminoglycoside antibiotic neomycin B leads to dramatic changes in the HIV-1 TAR conformation,[39] almost no conformational changes are observed upon recognition of the small organic molecule acetylpromazine.[40] In general, the largest RNA conformational changes appear to be observed when positive moieties on a cognate target stabilize RNA conformations with high negative charge density that would otherwise be unstable in the unbound RNA.

Changes in RNA conformation can also serve to temporally regulate viral processes. An example is the untranslated "leader" RNA located at the 5′-end of the HIV-I genome (Figure 1b).[41] The leader RNA contains a host of regulatory ele-

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ments that exert distinct functions during various stages of the viral replication cycle. There is evidence that the leader RNA undergoes multiple structural rearrangements from an extended thermodynamically stable conformation, which is probably the structure needed for translation, to a multibranched conformation that can allow genome dimerization and packaging (Figure 1b).[42] This conformational switch, which can be triggered by the chaperone nucleocapsid protein (NcP) and/or divalent ions, may temporally regulate viral replication and packaging functions.[42,43]

Self-induced RNA conformational transitions[17] which do not require cellular cofactors are also believed to play a role in RNA-mediated translational[44,45] and transcriptional regulation[46] as well as in the proper folding of ribozymes.[47–53] Such self-induced transitions are made possible by the fact that secondary motifs such as hairpins can fold at rates faster than RNA synthesis during transcription, thereby resulting in kinetically trapped intermediates. Once synthesis is complete and downstream RNA "trigger regions" are made available, these metastable structures can interconvert into the more thermodynamically stable, often biologically active, conformation. Self-induced transitions have also been observed in small (<35 nucleotide) artificially designed RNAs that can fold into more than one conformation.[17,54]

Changes in RNA conformation also form the basis for the regulation of gene expression by metabolite-sensing allosteric messenger RNA (mRNA) riboswitches (Figure 1c).[55,56] These mRNAs regulate the transcription and/or translation of genes responsible for producing metabolites by directly binding the metabolite molecules themselves. Riboswitches are generally comprised of a natural ligand-binding aptamer domain and an expression platform whose conformation affects gene expression (Figure 1c). Binding of metabolites to the aptamer domain elicits a structural rearrangement in the expression platform that generally turns off gene expression by either 1) forming an antiterminator stem which prevents transcription elongation (as shown in Figure 1c) or 2) altering the accessibility of the Shine–Dalgarno sequence during translation. Recent X-ray crystal structures[57,58] and NMR studies[59] of the aptamer domain from guanine- and adenine-sensing mRNAs indicate that, much like the situation in artificially evolved counterparts,[60] ligand binding induces folding of an otherwise unstructured RNA aptamer, thereby resulting in a complex three-dimensional RNA structure in which the ligand is completely engulfed within the RNA architecture. How the ligand-induced folding of the aptamer domain is allosterically communicated to the expression platform will probably be the subject of many future investigations. Other examples of ribosensors include mRNA thermosensors, which regulate gene expression in response to changes in temperature by undergoing temperature-dependent conformational changes.[61]

Changes in RNA conformation can also arise from protonation/deprotonation of base residues.[62–64] Recently, a proton-coupled conformational switch was reported in the dimerization initiation site (DIS) RNA from HIV-1.[65] The DIS is one of the regulatory RNA elements in the HIV-1 leader RNA that plays a role in noncovalently linking two strands of genomic RNA during viral assembly (Figure 1b). This is achieved by an autocomplementary loop located in the DIS that promotes formation of a homodimer through loop–loop kissing interactions (Figure 1b). As part of the HIV viral maturation process, the RNA chaperone NcP is believed to catalyze conversion of the metastable kissing dimer into the more thermodynamically stable duplex. By using NMR spectroscopy, evidence has been provided that protonation of an adenine base in the loop of the kissing dimer leads to increased local structural dynamics at the loop–loop helix, which in turn is accompanied by an increase in the rate of NcP-catalyzed kissing-to-duplex conversions.[65]

Conformational dynamics is also widely implicated in the catalytic functions of ribozymes.[66] For example, changes in interhelical angle and phase are believed to switch the hammerhead ribozyme between its cleavage and ligation activities.[67] In contrast, local conformational changes involving ejection of a catalytic metal ion following cleavage by the hepatitis delta virus ribozyme are believed to suppress the reverse ligation reaction, thereby rendering the ribozyme a dedicated nuclease.[68] Dynamics can also set up reaction groups for catalysis and play a role in substrate binding and product release.[66,69] For example, studies involving single-molecule fluorescence resonance energy transfer (smFRET) on the hairpin ribozyme[70] show that substrate binding begins with an extended "undocked" RNA conformation, which subsequently converts into a catalytically active "docked" state (Figure 1d). Following cleavage, the RNA returns to the undocked state and the products are released (Figure 1d). Here, changes in the RNA conformation play a role in helping to deliver/release substrates/products to and from the active site.

A number of themes regarding RNA conformational transitions emerge from the above examples. First, changes in conformation are widely observed in a variety of RNAs. Second, the conformational changes can serve diverse functions. They can aid formation of structural elements needed for recognition and catalysis, allow temporal regulation of biochemical events, and enable activation of RNA-mediated processes in a manner contingent on various cellular signals. Third, there are versatile ways to induce conformational changes in RNA. These include binding to cellular cofactors, changes in physiological conditions such as pH value and temperature, and even RNA synthesis itself. Finally, the conformational changes can be dramatic, resulting in total transformations in the RNA structure.

The link between equilibrium dynamics and RNA conformational change

The ubiquitous role of conformational changes in RNA function has led to the quest for a molecular understanding regarding how these transitions occur. The biological fidelity of any conformational switch is contingent upon it having a level of selectivity. The situation in which diverse RNA conformations are stabilized by arbitrary cellular signals must be avoided. Strategies for achieving this selectivity can in turn depend on the specific RNA function.

Two non mutually exclusive pathways for achieving RNA conformational changes have been proposed to account for RNA adaptive recognition.[1,3,18] *Induced fit* is often used to describe binding-induced transitions towards RNA conformations that are otherwise only marginally populated in the free state. In contrast, *tertiary capture* is often used to describe situations in which a cofactor recognizes and binds an RNA conformation that is transiently and dynamically sampled in the free state. The underlying difference between these pathways, which can be generalized to include RNA transitions induced by any cellular signal, is the extent to which the free RNA can dynamically sample the bound conformation, thereby leading to a link between RNA dynamics and function. In what follows, I examine whether distinct RNA functions favor different pathways and I explore strategies available to RNA for ensuring selectivity in the conformational change. While the discussion will focus on RNA conformational changes induced by binding to cofactors, extensions can be made to conformational changes induced by other cellular signals.

In some cases, the bound RNA conformation itself plays the primary role in activating a biological process. Here, it can be advantageous to minimize dynamical sampling of the bound state in the free RNA, as this could lead to activation of a biological process independent of cellular signals. One example is mRNA riboswitches in which it is the RNA structure and not the bound ligand that is believed to turn off gene expression (Figure 1 c). In this case, dynamic access to the RNA bound state and a tertiary-capture mode of recognition could lead to premature turning off of gene expression, even in the absence of high ligand concentrations. Another example is the structural isomerization of DIS (Figure 1 b). Spontaneous transitions between kissing and duplex dimers could temporally decouple structural isomerization from viral maturation. In these cases, the RNA bound state would ideally only become significantly populated following a cellular signal.

RNA appears to have evolved two strategies for meeting the above requirements. In one case, the RNA bound conformation is only sampled following binding to a cellular cofactor, because it is otherwise energetically unfavorable (Figure 2 a). To the extent that the bound RNA conformation is unstable in the absence of cofactors, so will the cofactor binding energy have to increase to ensure selective stabilization of the RNA bound state (Figure 2 a). Here, the bound RNA conformation must have structural properties that enable it to forge intermolecular interactions with a cellular cofactor that are overwhelmingly larger than corresponding interactions with other competing

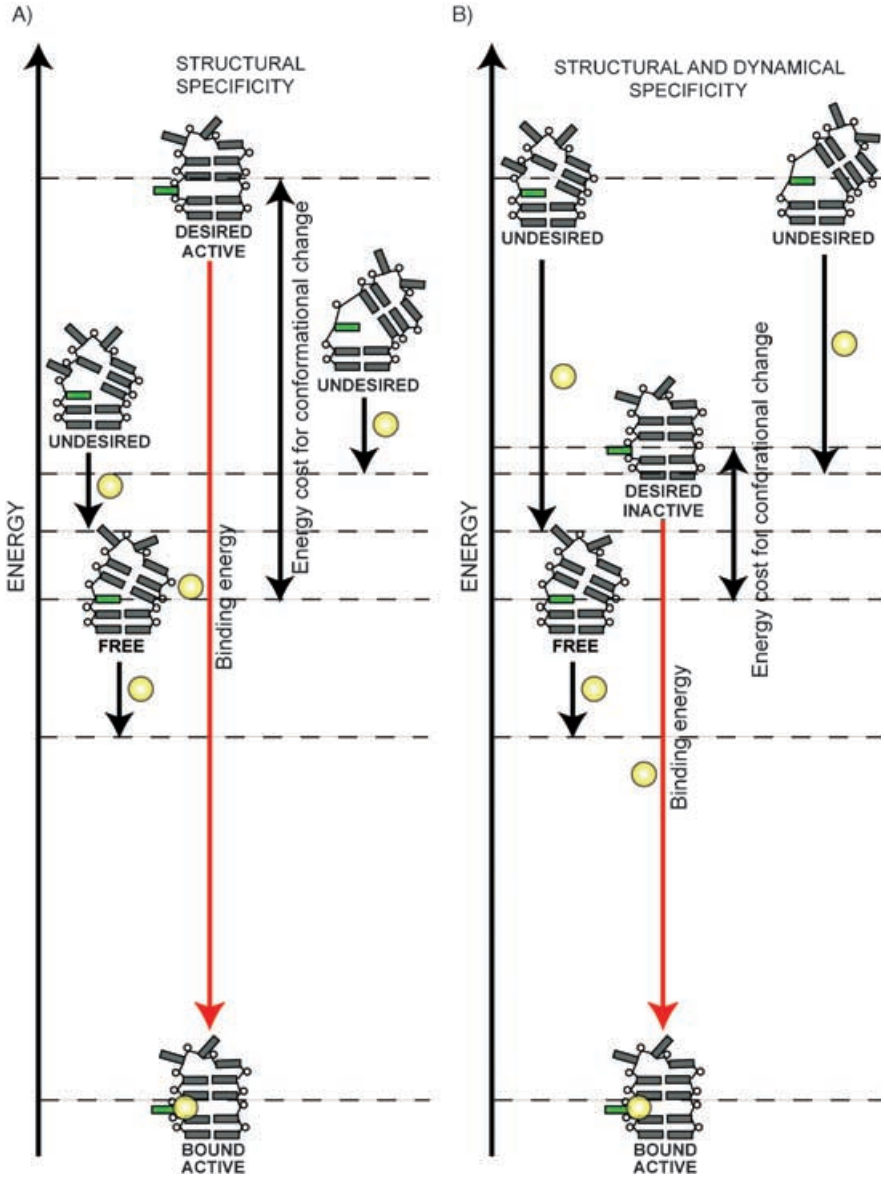

Figure 2. Achieving selectivity in binding-induced RNA conformational change. A) Selectivity based on structure can be achieved by having one RNA conformer with overridingly higher binding energies with a specific cofactor compared to all other conformers in the free ensemble. This pathway ensures that the RNA bound state is only populated following a cellular signal. B) Selectivity based on dynamics can be achieved by having the RNA sequence code for selective stabilization and destabilization of desirable and undesirable conformers, respectively, in the absence of cofactors. This pathway can afford selectivity in the RNA conformational switch without the requirement for high interaction energies.

RNA conformers in the free ensemble. Hence, in this case, the RNA structure governs the selectivity of the conformational change ("structural selectivity"; Figure 2a). Indeed, preliminary studies indicate that this is the case for the guanine- and adenine-sensing mRNA riboswitches in which numerous intermolecular contacts with the ligand ensure that an otherwise partially structured RNA adopts the bound conformation only upon ligand binding.[57–59] A second RNA strategy involves kinetically trapping the free RNA conformation. Here, the thermodynamically favored bound state only becomes appreciably populated following a cellular signal that rescues the free RNA from the kinetic trap. The rugged energy landscape underlying RNA folding[71] lends itself to such conformational switches. Examples include most self-induced RNA structural transitions,[17] as well as the NcP-catalyzed structural isomerization of DIS from a kinetically trapped kissing dimer to the thermodynamically favored duplex form (Figure 1b).

In other cases, the bound RNA conformation is not sufficient to activate a biological process; this requires additional cellular cofactors. An example is the conformational changes associated with RNP assembly in which the RNA bound state can be biologically inactive in the absence of the protein component (Figure 1a). For example, the protein-bound TAR conformation primarily serves as a scaffold for assembling proteins, including a cyclin-dependent kinase 9 (Cdk9) which is believed to activate viral transcription by phosphorylating RNA polymerase.[33–35] In these cases, dynamic access to the bound state and a *tertiary-capture* mode of recognition would not necessarily lead to premature biological signals. Compared to *induced fit*, this mode has the advantage of reducing the amount of binding energy required to change the RNA conformation, thus alleviating requirements for having highly selective and stabilizing intermolecular interactions with cofactors (Figure 2b). Rather, a degree of selectivity in the RNA conformational change can be achieved by specifically stabilizing the RNA bound conformation relative to other competing conformers in the free ensemble ("dynamical selectivity", Figure 2b). Interestingly, evidence for dynamic access to bound states has been reported for protein-binding RNAs. For example, by using smFRET analysis, it was shown that that the coaxially aligned Mg^{2+}-bound state of the 16S RNA (Figure 1a), which is structurally similar to the protein-bound state, is dynamically accessible in the free state, with Mg^{2+} shifting a preexisting equilibrium.[29] Similarly, studies of NMR residual dipolar couplings (which will be reviewed in subsequent sections) also provide evidence that TAR RNA (Figure 1a) can dynamically access the linear protein-bound state.[72] More generally, there is growing evidence that RNA has a unique propensity to fold into distinct yet dynamically interconverting conformations and one could argue that this property sets RNA apart from most globular proteins. Even RNAs as short as 18–21 nucleotides can fold into 2 well-defined yet distinct and appreciably populated conformers.[17,54] Furthermore, an RNA sequence has been designed that folds into two very different conformations that code for two distinct activities.[73]

From the above discussion, one can propose the following link between pathways for RNA conformational change and function. In cases where a change in RNA conformation is in itself sufficient to activate a biological process, *induced fit* is the favored mechanism for achieving regulation (Figure 2a). Here, selectivity in the conformational change can be achieved either by having highly specific cofactor–RNA interactions or by rendering an energetically favored RNA conformation inaccessible through kinetic traps. In cases where the presence of the cofactor is required for activating a biological process, *tertiary capture* can be a viable mechanism that potentially alleviates requirements for having highly specific and stabilizing intermolecular interactions between the RNA and the cofactor (Figure 2b). In this case, selectivity in the RNA conformational change can be achieved in part by the RNA energy landscape and the degree to which the RNA dynamically samples the bound conformation relative to other competing conformers in the free ensemble (Figure 2b). The latter is one example by which dynamics may compensate for RNA's limited structural coverage and hence its ability to achieve selective conformational transitions based purely on highly specific intermolecular interactions.

Elucidating the molecular basis for RNA conformational change and function

It follows from the above discussion that understanding how an RNA sequence codes for a particular function at a molecular level will often require both structural description of the conformational changes underlying RNA function and characterization of RNA dynamics. In addition to illuminating pathways underlying changes in RNA conformation, dynamics studies can provide insight into transiently sampled RNA conformations that may not directly participate in function but that may be opportune targets for developing RNA-targeting therapeutics.[38,74] The success of such studies clearly hinges on having the ability to describe RNA dynamics with high structural and temporal resolution. The development of techniques capable of providing such information is one of the most important objectives in structural biology.

Among many techniques that can be applied towards the characterization of RNA structural dynamics, including X-ray crystallography,[75] single-molecule[76] and ensemble fluorescence spectroscopy,[77] hydroxyl radical "footprinting",[78] EPR spectroscopy,[79] and molecular dynamics simulations,[80] NMR spectroscopy holds unique promise, as it allows atomic-level characterization of both structure and dynamics over biologically relevant timescales (ps–s) under physiologically relevant solution conditions. Although shortcomings in traditional methodology had precluded realization of NMR spectroscopy's full potential as a technique for probing RNA structural dynamics, recent advances have addressed many of these limitations. In what follows, the scope, recent advances, and challenges ahead of NMR-based characterization of RNA functional dynamics are reviewed.

Probing RNA Conformational Change by Using NMR Spectroscopy

The application of NMR spectroscopy in high-resolution structure determination of RNA enjoys a number of advantages over X-ray crystallography. First, NMR spectroscopy is applicable under a wide range of physiologically relevant solution conditions. This is particularly valuable when one is interested in comparing RNA structures in the presence/absence of various cofactors or under different conditions of temperature and pH value. Second, NMR spectroscopy does not suffer from potential artifacts arising due to crystal-packing forces,[81] which can be particularly problematic when trying to characterize changes in RNA conformation. However, until recently, these advantages were not fully realized owing to difficulties in solving RNA structures with sufficient precision and accuracy. NMR approaches for structure determination have traditionally relied on short-range distance constraints derived from nuclear Overhauser effects (NOEs) supplemented by local angular constraints derived from scalar couplings. Not only are such local constraints ineffective at defining global features of extended nucleic acids, but the paucity of protons and the severe spectral overlap in nucleic acids generally limits the number of derivable constraints. The resulting uncertainty in structures can often be so large as to preclude effective characterization of changes in RNA conformation induced by various factors.

Recent developments in NMR methodology have addressed many of these limitations.[82–87] A key development involves residual dipolar couplings (RDCs),[88–90] which can be measured in great abundance in partially oriented RNAs and which provide highly complementary long-range constraints on bond vector orientation.[83,86,91] The discovery of *trans*-hydrogen-bond-mediated scalar couplings[92,93] has also opened an avenue for directly probing hydrogen-bonding interactions in nucleic acids.[94,95] Measurements of cross-correlated relaxation rates are increasingly providing access to novel angular constraints.[96–98] Innovations in pulse-sequence design and labeling approaches continue to push the size limit of RNA that can be targeted by NMR spectroscopy.[85,99–101] In what follows, I review examples in which RDCs and *trans*-hydrogen-bond NMR methods have provided unique insight into changes in RNA conformation.

An early example is a study demonstrating the use of RDCs in characterizing conformational changes in RNA that are induced by binding to aminoglycosides.[102] The target of this RDC study was an RNA derived from the decoding site of the 16S rRNA to which binding of aminoglycosides in the ribosome context results in misreading of the genetic codes and inhibition of translocation. Previous NMR structures indicated that binding to paromomycin and gentamicin leads to local rearrangements in two adenosine residues located in an asymmetric loop. The RDCs measured in these adenosines exhibited clear differences in the free and antibiotic-bound RNA forms, thereby providing direct signatures of structural changes that are induced by the small molecules.

The enhanced quality of structures determined with the aid of RDCs has allowed more judicious comparison of nucleic acid structures determined under solution conditions by using NMR spectroscopy with counterpart structures determined by X-ray crystallography.[86,91,103–105] One example is the hammerhead ribozyme. Previous high-resolution X-ray crystal structures determined under high-salt conditions did not correlate well with mutational and modification data, and various conformational changes were proposed that would alter the X-ray crystal structure to the catalytically active conformation.[106–108] Analysis of RDCs measured in the hammerhead ribozyme revealed an RNA global conformation that was significantly more extended than its X-ray crystal structure counterpart[105] but was in good agreement with previous solution-state FRET studies.

A series of studies employed RDCs and *trans*-hydrogen-bond NMR spctroscopy to probe changes in the HIV-1 TAR RNA (Figure 3a) conformation induced by various cofactors.[31,72,109] TAR RNA is comprised of two Watson–Crick stems that are linked by a trinucleotide bulge (Figure 3a). Previous NMR studies had

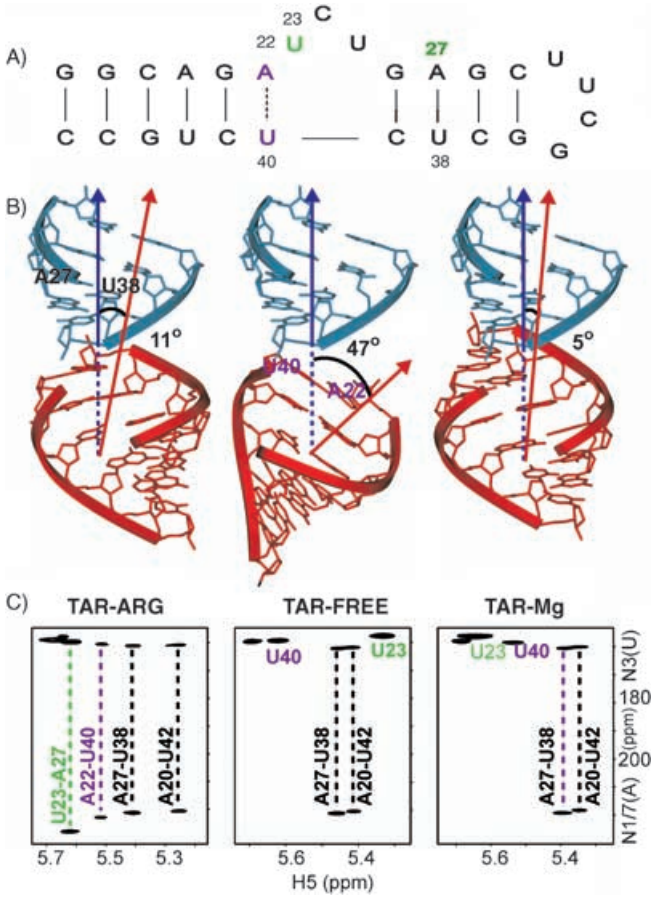

Figure 3. Cofactor-induced conformational changes in HIV-1 TAR RNA determined by using residual dipolar couplings and *trans*-hydrogen-bond NMR spectroscopy. A) The secondary structure of the HIV-1 TAR RNA sequence in which the wild-type loop is replaced with a UUCG counterpart. B) The global alignment of helical domains determined by using RDCs for TAR in the free state (TAR-FREE)[72] and when bound to Mg^{2+} (TAR-Mg^{2+})[31] or the ligand argininamide (TAR-ARG).[109] Stem II (in blue) is superimposed in all three cases. C) *Trans*-hydrogen-bond NMR spectroscopy[92,93,95] detection of ^{2h}J(N,N) couplings in A–U base pairs by using the H5(C5)NN experiment[109] in the free TAR state (TAR-FREE) and when bound to Mg^{2+} (TAR-Mg^{2+}) or argininamide (TAR-ARG).

established that when bound to the ligand argininamide or to peptides derived from its cognate protein target Tat, TAR adopts a global conformation characterized by coaxial stacking of the two stems.[28,110,111] By contrast, NMR spectroscopy[22,111] and transient electric birefringence[21] studies showed that, in the absence of ligands, TAR adopts a distinct global conformation in which the two domains are inclined by $\approx 45°$ relative to one another. However, the NOE-based global structure of free TAR[22] was poorly defined and yielded a family of low-energy structures in which the interhelical angle varied between 36–137°. An X-ray crystal structure of TAR in the presence of divalent ions was subsequently reported which differed from the NMR structure of free TAR in that the two helices were also coaxially stacked.[30]

By providing high-precision long-range orientational constraints, RDCs could be used to effectively examine the average TAR global conformation in the absence and presence of the above cofactors (Figure 3b). For free TAR, a bent global conformation with an average interhelical angle between 44–54° was determined.[72] By contrast, a coaxially aligned average TAR conformation was determined not only in the presence of the ligand argininamide[31] but also in the presence of Mg^{2+} (Figure 3b).[109] The latter result suggested that the differences between the X-ray crystal and NMR structures of TAR can be attributed to the absence of divalent ions in the latter rather than to crystal-packing forces in the former.

It was previously shown that a base triple involving a reverse Hoogsteen hydrogen bond between bulge residue U23 and an A–U base pair in the upper stem forms only in the protein-bound TAR[28,112] and not in free[22] or divalent-ion-bound[30] TAR. *trans*-Hydrogen-bond NMR experiments further revealed that hydrogen bonding between the Watson–Crick residues A22 and U40 at the junction of domain I only occurs (within limits of detection) in the argininamide-bound TAR and not in either free or Mg^{2+}-bound TAR (Figure 3c).[109] Weak or no hydrogen-bonding interactions between A22–U40 at the junction of the bulge may contribute to global motions between the two domains in free TAR which were also observed based on RDCs, as will be discussed in a subsequent section.[72] It is also noteworthy that distinct TAR conformations have also been reported when the RNA is bound to the aminoglycoside neomycin B[39] or the small molecule acetylpromazine.[40] It is remarkable that an RNA molecule as simple as TAR carries such a stunning ability to undergo local and global conformational adaptation in response to various cofactors. An understanding of the molecular basis for this structural plasticity will be important in the rational design of anti-HIV therapeutics targeting TAR.[113]

RNA Dynamics by NMR

Over the last decade, many NMR methods for studying protein dynamics have matured to a point that allows almost routine application.[114–116] By contrast, the study of RNA dynamics by NMR spectroscopy is far from routine. This largely reflects unique difficulties associated with applying NMR methods that have primarily been developed and tailored for proteins to the study of nucleic acids. The growing interest in RNA dynamics will probably rejuvenate interest in tackling some of these methodological limitations. In this section, I provide a brief update regarding NMR methods that can be used to probe dynamics in nucleic acids. Notable techniques that are not covered include studies of base-pair opening and closing dynamics by using imino-proton exchange (Figure 4a)[84,117] and measurement of dihedral fluctuations by using scalar couplings (Figure 4b),[82,118] both of which have been reviewed elsewhere. Here, I focus on NMR techniques based on spin relaxation (Figure 4c–e) and RDCs (Figure 4f) for which substantial developments can be anticipated in the near future. The goal is not only to highlight examples that illustrate the types of information that can be retrieved from each technique but also to survey the problems that need to be overcome in each case.

Fast (ps–ns) librational motions by spin relaxation

The measurement of autocorrelated longitudinal (T_1) and transverse (T_2 or $T_{1\rho}$) relaxation rates, along with heteronuclear NOEs, is the most widely used approach for measuring fast (ps–ns) internal motions in biomolecules by NMR spectroscopy.[115,119–121] The relaxation rates are typically analyzed by using the Lipari–Szabo model-free formalism,[122] which yields information about internal motions in the form of a generalized order parameter, S^2, which ranges between 1 for maximally restricted motions and 0 for minimally restricted motions, and a correlation time (τ) describing the effective timescale of the motions (Figure 4c).

Although measurements of ^{13}C, ^{15}N, and ^{31}P relaxation rates can in principle provide information about the amplitudes and timescales of fast (ps–ns) local librational motions in the base, sugar, and backbone moieties of RNA (Figure 4c), the unique properties of nucleic acids continue to pose challenges to such applications. Unlike the situation in proteins, there is a shortage of ^{15}N–(^1H) spin pairs in RNA and the most interesting of these (for example, in loops and bulges) are often nondetectable due to exchange broadening. By contrast, ^{13}C relaxation comes with a number of added difficulties. Large homonuclear $^1J_{CC}$ scalar couplings and ^{13}C–^{13}C dipolar interactions can complicate measurements of ^{13}C relaxation rates. At the same time, interpretation is made difficult by having highly asymmetric base ^{13}C chemical-shift anisotropy (CSA) tensors, which can have principal values that are noncollinear with the corresponding ^{13}C–^1H dipolar interactions. Experimental ^{13}C CSA values have only recently been reported for model mononucleotide crystals through the use of solid-state NMR spectroscopy[123] and remain to be validated and cross-referenced with values reported based on theoretical calculations.[124,125] A recent study illustrated how dynamic conclusions can vary depending on the choice of base ^{13}C CSA values.[126] Some of the ^{13}C CSA values, including those of C1′ and C3′ in the ribose moieties, can vary significantly depending on local conformation.[124,127] Further complications arise from the anisotropic tumbling of extended nucleic acids, which requires specification of a diffusion tensor, the experimental determination of which can be difficult owing to the poor orientational disper-

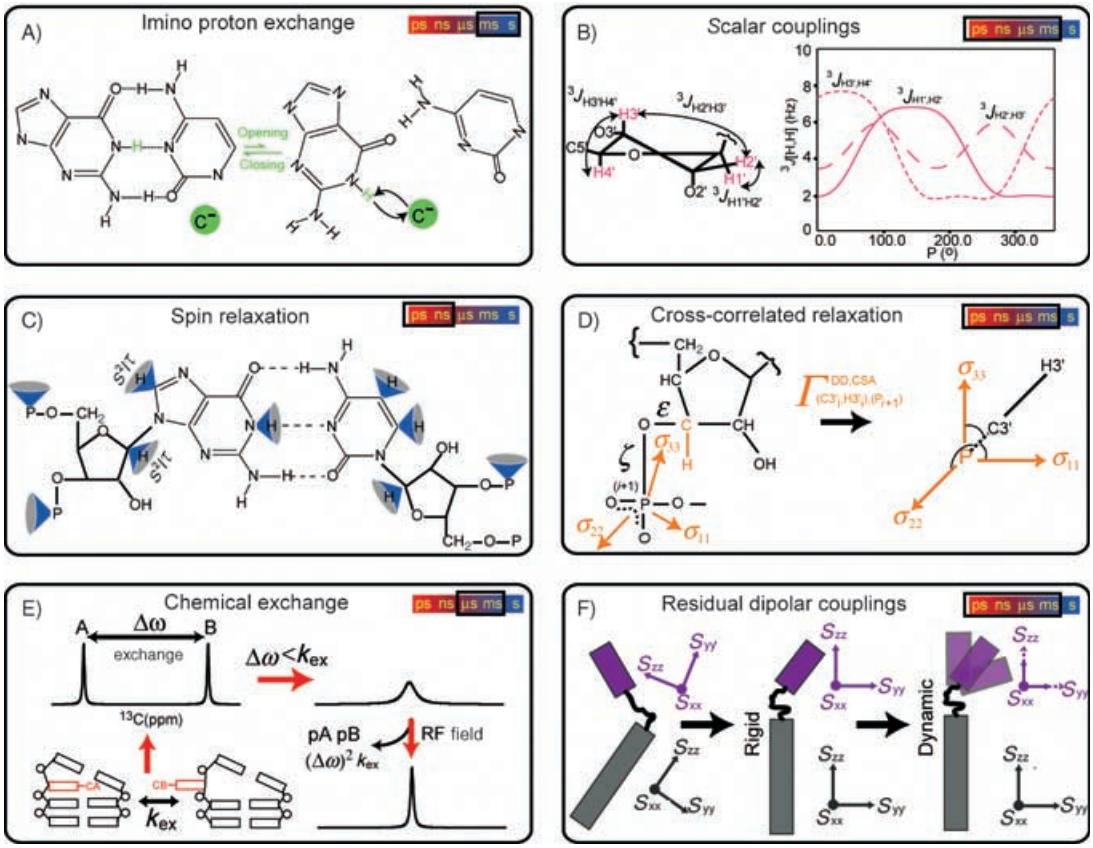

Figure 4. Techniques for probing RNA dynamics with NMR spectroscopy. A) Base-pair opening and closing dynamics at the ms–s timescales can be probed by measuring imino-proton exchange rates as a function of catalyst (C^-) concentration. B) Dihedral fluctuations over $<$ ms timescales can be characterized by measuring multiple scalar coupling interactions with unique Karplus dependencies on the dihedral angle. The example shown is the probing of conformational averaging in the ribose pseudorotation angle based on multiple $^3J_{HH}$ couplings. C) The amplitude and timescale of fast (ns–ps) librational motions involving ^{13}C, ^{15}N, and ^{31}P nuclei can be characterized by using measurements of relaxation rates. D) Cross-correlated relaxation can be used to characterize dynamics involving reorientations between pairwise anisotropic interactions. The example shown is for ^{31}P CSA and ^{13}C–^1H dipole–dipole cross-correlated relaxation, which can be used to probe the structure and dynamics of the phosphodiester backbone. E) Slow fluctuations (µs–ms) that lead to changes in chemical shift can be probed by using relaxation dispersion and CPMG NMR experiments. In ideal cases, this can provide information about the population of two states (p_A and p_B), the timescale of the exchange process (k_{ex}), and the difference in chemical shifts for the two states ($\Delta\omega$). F) Residual dipolar couplings measured in partially oriented RNAs can be used to probe motions occurring over submillisecond timescales including collective motions of helical fragments.

sion of ^{13}C–(^1H) interaction vectors.[128] Anisotropic tumbling can also require additional parameters to interpret relaxation rates in terms of motions.[129] Finally, global motions in modular RNAs can lead to time-dependent changes in the overall molecular tumbling, thereby leading to a breakdown in the decoupling approximation between internal and overall motions that is at the crux of the Lipari–Szabo formalism.[122,130–132]

The above illustrates the added difficulties associated with applying NMR spin relaxation to study dynamics in nucleic acids as compared to proteins. Solutions to some of the problems associated with measurements of relaxation rates have been proposed and recent advances may soon overcome some of the problems associated with data analysis. For small RNAs ($\tau_c < 4$ ns), relaxation contributions from ^{13}C–^{13}C dipolar–dipole interactions in ^{13}C–(^1H) spins is small and can safely be neglected. Under favorable conditions, low-power selective spin-lock fields can also be used to suppress the effects of $^1J_{CC}$ couplings and homonuclear ^{13}C–^{13}C Hartmann–Hahn magnetization transfer during Carr–Purcell–Meiboom–Gill (CPMG) spin locks.[133–135] Although still a laborious process, fractional ^{13}C labeling with appropriate ^{13}C–^{13}C filters can also be used.[136] The measurement of RDCs can aid in the determination of anisotropic diffusion tensors. The RDCs can improve the quality of the RNA structures determined by NMR spectroscopy, thereby reducing requirements for having relaxation rates measured for optimally dispersed interactions,[135] and can facilitate computation of diffusion tensors based on the overall RNA molecular shape.[137] Validation of reported ^{15}N and ^{13}C CSA tensors may be possible in the future, based on measurements of residual chemical shift anisotropy contributions in partially aligned RNAs, contributions that have successfully been measured for ^{31}P in DNA.[138]

Perhaps an even greater problem, which has yet to fully unveil itself, will be the interpretation of spin relaxation rates under conditions in which collective motions of RNA domains lead to modulations in the overall diffusion tensor and thus a breakdown in the decoupling approximation between internal and overall motions. Recent NMR spin relaxation studies provide direct evidence for such collective motions in RNA. By using ^{15}N relaxation measurements, it was demonstrated that

one stem loop in a 101-nucleotide RNA that contains the core encapsidation signal in the Moloney murine leukemia virus genome tumbles semiindependently of two other stem loops, which in turn appear to tumble as a single unit.[101] The examination of the validity of the decoupling approximation in different types of RNAs should be an immediate objective of future studies.[130–132]

Notwithstanding the above difficulties, a number of consistent trends regarding dynamics in RNA and nucleic acids in general have emerged from application of ^{15}N and ^{13}C spin relaxation. The generalized order parameters measured for bond vectors in base moieties in duplex helices tend to have values of around $S^2 \approx 0.8$.[126,139–141] Lower values are often observed in sugar moieties and terminal bases[142–144] and in nonhelical residues such as loops and bulges.[145,146]

A number of studies have also provided insight into the heterogeneity of RNA dynamics. One study employed ^{15}N and ^{13}C relaxation measurements to examine dynamics in free and argininamide-bound HIV-2 TAR RNA.[147] HIV-2 TAR differs from its HIV-1 counterpart by having two rather than three bulge nucleotides (Figure 3a). Interestingly, bulge residue U23, which undergoes a large conformational change upon complexation, also exhibited a high reduction in local mobility following complexation. A more recent study employed base (C8/6) and ribose (C1′) ^{13}C relaxation rates to examine the dynamics of a UUCG tetraloop.[126] The derived residue-specific dynamics correlated well with the network of interactions underlying the stability of the UUCG motif. Interestingly, for a given residue, ribose mobility was observed to be correlated with the corresponding base mobility. There has also been some progress in measuring ^{31}P relaxation rates as a direct probe of backbone dynamics. By using new NMR pulse sequences for enhancing the sensitivity of ^{1}H–^{31}P HSQC experiments,[148] ^{31}P relaxation rates (R_2) measured in a simple DNA duplex indicated that the backbone mobility increases in going from the center of the duplex to the terminal end.[149]

Another emerging approach for probing RNA conformational dynamics relies on measurements of cross-correlation between pairwise anisotropic interactions (Figure 4d).[97,150] There are unique advantages to this methodology, including the facts that the effect scales linearly with molecular weight, that multiple pairwise interactions can be targeted for measurements, and that, when the angular dependence of these pairwise interactions is taken into account, the motional sensitivity can extend from ps to ms timescales. To date, cross-correlated relaxation measurements in RNA have primarily been employed as structural reporters providing insight into the conformation of sugar moieties,[127,151,152] backbone,[153] glycosidic angles,[98,154] and hydrogen-bonding interactions.[155,156] Most of these studies also provide evidence for internal local fluctuations. Due to their dependence on the orientation of anisotropic tensors, both relative to one another and relative to the anisotropic diffusion tensor, cross-correlated relaxation rates carry exquisite information about molecular fluctuations. While this is a great asset, it also can render data analysis more difficult. Further investigations into CSA values in nucleic acids and developments in analysis frameworks that can combine multiple cross-correlation relaxation rates[157] as well as other information may allow more complete extraction of the dynamics information contained in these measurements.[97,150]

Probing slow (μs–ms) motions by using NMR chemical exchange

Slow motions (on the μs–ms timescale) leading to modulations in isotropic chemical shifts can be probed by using NMR relaxation dispersion and CPMG experiments.[114,116] The data are typically analyzed with the assumption of a two-state exchange model and, under favorable circumstances, this can allow determination of the population of the two states (p_A and p_B), the timescale of the exchange process ($1/\tau_{ex} = k_{ex} = k_{a'b} + k_{b'a}$), and the difference in chemical shift between the two states ($\Delta\omega$; Figure 4e). The latter contains information about the nature of the structural fluctuations, but the extraction of this information in practice remains compromised by difficulties in directly relating chemical shifts to molecular conformation. Slow collective motions of intact domains may also be difficult to probe with this methodology as such motions can occur without leading to substantial changes in the local surrounding spins in a given domain.

A subset of limitations that affect application of spin relaxation in studies of fast RNA dynamics is applicable in relaxation dispersion and CPMG NMR experiments, namely, the lack of ^{15}N probes and large $^{1}J_{CC}$ scalar coupling and associated TOCSY magnetization transfer pathways in experiments targeting ^{13}C nuclei. Nevertheless, a few studies have demonstrated the applicability of these techniques for the investigation of RNA dynamics. For example, measurements of the power dependence of $R_{1\rho}$ relaxation rates in base C2, C8, and C6 resonances revealed slow (10^{-5}–10^{-3} s) fluctuations in the catalytic leadzyme RNA.[69] A significant $R_{1\rho}$ value power dependence was observed for many noncanonical residues, including some that were located in the catalytic active site. In a previous study, the authors had shown that N7 in an adenine residue located in the catalytic internal loop had an unusual pK_a value of 6.5, with a protonation/deprotonation lifetime $\tau_{ex} = 31 \pm 8$ μs.[158] Importantly, a two-state analysis of the power dependence of both the C2 and C8 sites in this residue yielded very similar exchange rates ($\tau_{ex} = 40 \pm 1.8$ and 47 ± 18 μs, respectively), a result indicating that the $R_{1\rho}$ measurements are reporting on the same protonation/deprotontaton process. Other residues in the catalytic site exhibited different exchange rates, thereby indicating that they are involved in distinct dynamical processes. In practice, the slowest motional timescale accessible by power dependence of $R_{1\rho}$ measurements is limited by the power dissipation in the probe, although slower motions (tens of millisecond) may be accessed through application of off-resonance spin-locking fields or CPMG-based methodology.[114]

CPMG-based relaxation dispersion experiments have also been used to measure chemical exchange contributions to ^{31}P nuclei along the backbone of a simple DNA duplex.[149] Exchange contributions were observed for ^{31}P sites adjacent to adenine and/or thymine residues. With the assumption of a two-state model for the exchange process, lifetimes between

0.58 ± 0.04 and 5.45 ± 0.13 ms were determined, values which are in good agreement with the corresponding A–T base-pair lifetimes, estimated by using imino-proton exchange measurements. These results provide important insight into speculated couplings between base-pair opening dynamics and motions along the phosphodiester backbone and suggest that backbone motions that may play a key role in activating collective domain motions in RNA will increasingly become accessible to NMR characterization in the future.

Probing segmental motions by using residual dipolar couplings

The measurement of RDCs has not only enhanced the ability to determine RNA structures by using NMR spectroscopy but has also opened a new avenue for characterization of the amplitude and direction of reorientational motions over a wide range of timescales ($<$ ms) (Figure 4 f).[72,91,159–161] This includes collective motions of intact molecular domains that can be difficult to probe with other NMR techniques and that, as discussed throughout this review, are a recurrent theme in RNA conformational transitions (Figure 4 f).

Different approaches have been applied to extract dynamics information from RDCs in applications involving nucleic acids.[91] Most have focused on deriving information about global motions of domains, although some studies have also exploited the enhanced quality of RNA structures that can be derived with the inclusion of RDCs to infer the presence of local flexibility. The analysis of RDCs in terms of global dynamics generally requires information about the local structure of individual domains. Here, RDCs are used to determine domain-specific order tensors describing average domain alignment relative to the magnetic field. Superposition of domain-specific order tensor frames then allows determination of relative domain orientation, while comparison of domain-specific principal order parameters can be used to derive information about the amplitudes and directions of interdomain motions (Figure 4 f).[72,91,160,162,163] In particular, rigidly held domains should always report identical order parameters.[160] However, provided that internal motions do not affect global alignment and that one domain dominates overall alignment, domain–domain motions can lead to differences in the principal order parameters in a manner dependent on the amplitudes and directions of motions.[72,160,164]

Order-tensor-based analyses of RDCs are particularly well tailored for RNA because its modular architecture often allows dissection into constituent substructures such as A-form helices, for which a reasonable local conformation can often be assumed a priori.[72,163,165] However, the assumption that internal motions do not lead to changes in global alignment, which is similar to the decoupling approximation invoked in the Lipari–Szabo model-free analysis of spin relaxation rates, can break down when two domains having similar propensities for alignment undergo relative motions.[164] For example, two identical domains will always report equivalent principal order parameters regardless of interdomain motions. Hence, correlations between motions and alignment can lead to underestimation of motional amplitudes derived by using RDCs as well as the determination of conformations that do not reflect the true average conformation sampled in solution. As a result, similar domain-specific principal order parameters should not be interpreted as conclusive evidence that the domains are rigid with respect to one another. Given that many RNA constructs targeted by NMR spectroscopy have domains of comparable size, there is a critical need to examine the potential occurrence of such correlations in different RNA contexts.

Another general problem in the analysis of RDCs is that determination of accurate order tensors requires an accurate description of the local structure of fragments and, similarly, accurate refinement of a structure based on RDCs requires proper calibration of the order tensor. It has been shown that structural noise in a given fragment can lead to errors in derived order tensor parameters, thus compromising the validity of conclusions reached regarding structural dynamics.[166] Protocols have been introduced that strike a balance between the use of RDCs in refining the local structure of a molecular fragment on the one hand and the determination of order tensor parameters on the other. Application of such a procedure to the theophylline-binding RNA led to identical principal order tensor parameters for each of its two domains, a result indicating that the two domains are held rigid relative to one another.[167] A similar result was obtained for two domains in the iron-responsive element (IRE) RNA.[168]

By increasing the number of measured RDCs, one can carry out order tensor analyses on smaller and smaller molecular fragments for which an accurate conformation can be assumed a priori. It was demonstrated that by measuring an appropriate number of RDCs in nucleotide bases, it is possible to derive information regarding base–base orientation and dynamics.[169] More recently, in an application involving a 24-mer RNA stem loop,[170] an NMR experiment was introduced that allows simultaneous measurements of five RDCs ($^1D_{C1'H1'}$, $^1D_{C2'H2'}$, $^1D_{C1'H2'}$, $^1D_{C2'H1'}$, and $^1D_{H1'H2}$) in ribose sugars. This made it possible to determine order tensors for individual ribose moieties in A-form helices by assuming a standard C3′-*endo* ribose ring-pucker conformation. While the principal order parameters were similar for residues within the helix, a reduction of $\approx 15\,\%$ was observed for the terminal nucleotide, a result that was attributed to terminal end-fraying and hence motional averaging.

One RDC study has revealed global motions in RNA in an application involving HIV-1 TAR.[72] As discussed previously, TAR adopts a bent average interdomain conformation (interhelical angle $\approx 45°$) in the free state and a more coaxially aligned conformation in the presence of either Mg^{2+} or mimics of the Tat protein such as argininamide. A central question is the extent to which the bound coaxially aligned conformations are dynamically sampled in the free TAR ensemble. The RDC-derived principal order parameters for the two domains in free TAR were significantly different ($\approx 40\,\%$); this indicates that the two helices undergo substantial motions relative to one another (Figure 5 a). Interpretation of the principal order parameters by using a cone motional model yielded rigid-body interhelical motions with amplitudes of $46° \pm 4°$. This provided evidence

that TAR may dynamically access the coaxially aligned argininamide/Mg^{-2+}-bound conformations in the free state (Figure 5b). In contrast to the free TAR ensemble, the domain-specific principal order parameters were very similar for TAR bound to Mg^{2+} or argininamide (Figure 5a); this indicates that recognition in these cases is accompanied by a total arrest of global motions (Figure 5b).

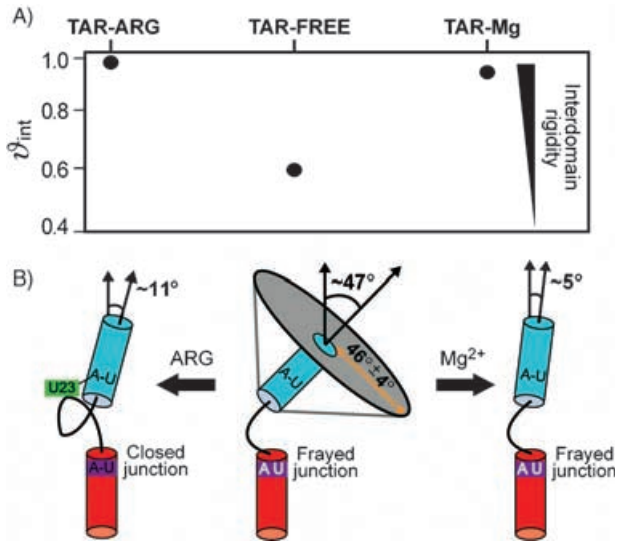

Figure 5. Global dynamics characterized by using residual dipolar couplings for TAR in the free state (TAR-FREE) and when bound to Mg^{2+} (TAR-Mg^{2+}) or the ligand argininamide (TAR-ARG). A) The generalized degree of internal order corresponding to the ratio of the principal order parameters for the two domains ($\vartheta_{domain\,I}/\vartheta_{domain\,II}$) for TAR-FREE, TAR-$Mg^{2+}$, and TAR-ARG. B) Dynamic access to bound states by the free TAR conformation. A summary of the structure and dynamics of TAR determined in the presence of different cofactors by using residual dipolar couplings and *trans*-hydrogen-bond NMR spectroscopy is shown.

A recent study on free TAR that exploits measurements of RDCs under spontaneous magnetic-field-induced alignment[89,171–176] attempted to address some of the concerns associated with having correlations between motions and overall alignment.[164] This approach relies on comparison of the magnitude of χ-tensors measured experimentally by using RDCs with values predicted by structure-based calculations of χ-tensors. The degrees of order calculated for the individual domains were very similar, a result indicating that the two domains have similar alignment propensities. However, the experimentally determined degree of alignment was attenuated by $\approx$55% relative to the values calculated with the assumption of a static structure. By using a framework for analyzing cone motions between domains, which accounts for correlations between motions and alignment, the results were interpreted as independent evidence for substantial directionally unbiased motions between the two domains in TAR. It is important to note that structure-based computation of nucleic acid χ-tensors requires accurate values for individual base χ-tensors. A recent study has raised questions concerning the appropriate base χ-tensor values that should be used[177] and this will be an important area of future investigation.

Summary and Future Perspective

Consider the problem of having to build functional diversity into a biopolymer. An easy approach would be to employ an expanded chemically diverse building-block alphabet that can allow the precise design of complex structures with diverse chemical reactivity. However, as the molecular alphabet increases, the selection of sequences that code for a productive structure becomes more difficult (many more sequences may code for meaningless structures) and additional cellular machinery must be dedicated to regulate the synthesis of both the building blocks and the biopolymer. An alternative approach is to employ a more limited building-block alphabet that codes for controllable molecular flexibility. One sequence that can fold into two functionally distinct conformations in a manner that is coupled to variations in the cellular environment may carry more than twice as much functional aptitude than its single conformation counterpart. This provides a mechanism for enhancing functional diversity beyond the limits accessible by well-defined three-dimensional structures. The challenge in this case is to avoid chaos by ensuring that a good fraction of the molecular flexibility is dedicated to productive biological causes. RNA appears to have evolved a remarkable ability to strike an optimal balance between structural and dynamic parameters in achieving this task. A future objective, which can be considered as an extension of studies into the RNA-folding problem, will be to understand how an RNA sequence codes for structural dynamics that can give rise to conformational switches. This will in turn require techniques for characterizing RNA conformational dynamics with high spatial and temporal resolution. As hopefully illustrated in this review, NMR spectroscopy can make important contributions to this endeavor. In order to achieve the greatest impact, existing methodological limitations in the study of dynamics in nucleic acids need to be critically addressed and a consensus built around well-defined protocols that can help to streamline such NMR investigations.

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Keywords: conformational adaptation · NMR spectroscopy · RNA sensors · RNA · structure–function relationships

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