Domain-elongation NMR spectroscopy yields new insights into RNA dynamics and adaptive recognition

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ABSTRACT

By simplifying the interpretation of nuclear magnetic resonance spin relaxation and residual dipolar couplings data, recent developments involving the elongation of RNA helices are providing new atomic insights into the dynamical properties that allow RNA structures to change functionally and adaptively. Domain elongation, in concert with spin relaxation measurements, has allowed the detailed characterization of a hierarchical network of local and collective motional modes occurring at nanosecond timescale that mirror the structural rearrangements that take place following adaptive recognition. The combination of domain elongation with residual dipolar coupling measurements has allowed the experimental three-dimensional visualization of very large amplitude rigid-body helix motions in HIV-1 transactivation response element (TAR) that trace out a highly choreographed trajectory in which the helices twist and bend in a correlated manner. The dynamic trajectory allows unbound TAR to sample many of its ligand bound conformations, indicating that adaptive recognition occurs by “conformational selection” rather than “induced fit.” These studies suggest that intrinsic flexibility plays essential roles directing RNA conformational changes along specific pathways.

Keywords: adaptive recognition; induced fit; tertiary capture; functional dynamics; NMR

INTRODUCTION

Ever since it was shown by X-ray crystallography that tRNA^{Gln} undergoes changes in the relative alignment of its helical stems upon binding to *Escherichia coli* glutamyl-tRNA synthetase (Rould et al. 1989), it has become abundantly clear that RNA structures ubiquitously undergo adaptive conformational changes on binding to cognate targets, including ligands, small molecule therapeutics, metabolites, proteins, and other RNAs (Hermann and Patel 2000; Williamson 2000; Leulliot and Varani 2001; Al-Hashimi and Walter 2008). These changes not only optimize intermolecular interactions, but they also serve to sense, process, and transmit chemical information. For example, structural changes triggered by ligand binding form the basis by which many RNA riboswitches both sense and respond to environmental factors in regulating gene expression (Mandal and Breaker 2004; Montange and Batey 2008; Serganov and Patel 2008). Multistep RNA structural

transitions can help order the sequence of transactions underlying assembly of ribonucleoproteins and catalysis (Liu et al. 2007; Stone et al. 2007; Williamson 2008; Woodson 2008). Structural transitions can also be used to inhibit the activity of RNA. For example, the antibiotic activity of aminoglycosides is largely derived from their ability to flip out two internal loop adenines in the ribosomal A-site (Fourmy et al. 1996), which serves to disrupt decoding and the fidelity of translation (Vicens and Westhof 2003; Hermann 2006).

Over the past two decades, there has been great interest in understanding the mechanism by which RNA structures undergo adaptive recognition (Hermann and Patel 2000; Williamson 2000; Leulliot and Varani 2001; Al-Hashimi 2005). On the one hand, these studies are motivated by a desire to understand how RNA structures change in response to specific cellular signals while avoiding promiscuous transitions from taking place. On the other hand, having the ability to predict how RNA structures change adaptively upon binding to small molecules is key for rationally designing RNA-binding therapeutics. Two non-mutually exclusive mechanisms have been used to explain RNA adaptive recognition (Fig. 1; Frankel and Smith 1998; Williamson 2000; Leulliot and Varani 2001). The *induced-fit*

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.1806909>.

FIGURE 1. Schematic representation of induced-fit and conformational selection/tertiary capture mechanisms for RNA (R) adaptive recognition upon binding to cofactors (C).

model, which was introduced by Koshland in the 1950s to explain the much lower rates of protein enzyme catalysis observed with substrate analogs (Koshland 1958), was used to explain most of early observations of RNA adaptive recognition. Here, it is assumed that the RNA bound conformation is not significantly populated in the absence of the binding partner, either because it is energetically unfavorable or because it is separated from the ground state by very large kinetic barriers, and that binding partners are required to effect the RNA conformational change (Fig. 1). However, there were also studies that provided experimental evidence that binding partners do not induce the RNA conformational change but rather capture pre-existing RNA conformations (Fig. 1). An early example of RNA *conformational selection* or *tertiary capture*, which was first introduced by Foote and Milstein (1994) to explain kinetic data on a set of antigen–antibody interactions, was the observation that complexes formed by complementary RNA stem-loops are stabilized upon binding to the ColE1 Rom protein (Eguchi and Tomizawa 1990). Weeks and Cech (1995, 1996) also invoked tertiary capture to explain adaptive recognition during the assembly of CBP2-group I intron ribonucleoprotein complex, in which proteins bind rapidly and capture transiently formed RNA tertiary conformations along the assembly pathway.

Whether by induced fit, conformational selection, or both, a complete understanding of how RNA structures change adaptively requires the characterization of RNA's intrinsic structural flexibility at the atomic level (Leulliot and Varani 2001; Al-Hashimi 2005; Al-Hashimi and Walter 2008; Hall 2008). Regulatory RNAs must encode—at some level—flexibility that allows their structures to change specifically along functionally productive pathways. This flexibility is imprinted in the thermal fluctuations of RNA structure over picosecond-to-second timescales. The past decade has witnessed great developments in biophysical

techniques for characterizing internal motions in RNA. These include ultrafast spectroscopy (Xia 2008), computational molecular dynamics simulation (Chen 2008; Hall 2008; Mackerell and Nilsson 2008), single molecule techniques (Li et al. 2008), time-resolved hydroxyl radical footprinting techniques with millisecond resolution (Sclavi et al. 1998; Shcherbakova et al. 2006), and both solution and solid-state nuclear magnetic resonance (NMR) (Furtig et al. 2007; Getz et al. 2007a; Shajani and Varani 2007). In this mini-review, we focus on recent developments in solution NMR spectroscopy that involve elongating RNA targets, are making possible the quantitative site-specific characterization of dynamics over picosecond-to-millisecond timescales, and are helping unveil the rich complexity of RNA flexibility at atomic resolution and its role in conformational adaptation.

DOMAIN ELONGATION: DECOUPLING INTERNAL AND OVERALL MOTIONS IN NMR STUDIES OF RNA DYNAMICS

Consider the task of reconstructing thermal motions of a complex biomolecule at atomic resolution over biologically relevant timescales based on experimental data; compared with solving the structure of a single snapshot, one requires a factor of $\sim 10^{12}$ more data to solve a one-second dynamic trajectory with picosecond resolution. Measuring such a mind-boggling amount of data is currently impossible by any experimental technique. By allowing measurements of not one but many complementary interactions on a per site basis, NMR spectroscopy offers a unique opportunity to obtain a glimpse into the time–space complexity of RNA dynamics. NMR spectroscopy has greatly contributed to our understanding of protein dynamics and its role in folding and function (Bruschweiler 2003; Kern and Zuiderweg 2003; Palmer 2004; Mittermaier and Kay 2006; Henzler-Wildman and Kern 2007). However, most NMR methods for dynamics characterization have been developed in application to proteins, and they often cannot be directly applied to RNA, which has distinct spectroscopic and hydrodynamical properties (Al-Hashimi 2005; Latham et al. 2005).

NMR data, such as spin relaxation rates, which can be used to probe motions occurring at picosecond-to-nanosecond timescales (Lipari and Szabo 1982), and residual dipolar couplings (RDCs) (Tolman et al. 1995; Tjandra and Bax 1997), which can be used to probe motions over sub-millisecond timescales (Tolman et al. 1997; Getz et al. 2007a), fundamentally probe the reorientation of anisotropic spin interactions, which are centered on a given nucleus, relative to the external magnetic field. For example, spin relaxation rates and RDCs report on fluctuations that change the orientation of a given C–H bond relative to the external magnetic field. In solution NMR, reorientation of the C–H bond is dominated by overall rotational

diffusion of the biomolecule. However, internal motions can also contribute—albeit by a smaller amount—to the reorientation of C-H bond and thereby influence spin relaxation rates and RDCs. The internal and overall motional contributions to NMR measurements can be disentangled provided one can assume that they are not correlated to one another. While this so-called “decoupling approximation” (Lipari and Szabo 1982) has been shown to hold to a high degree of validity for many globular proteins (Peti et al. 2002; Tolman 2002), studies over the past decade have shown this not to be the case for highly flexible RNAs (Zhang et al. 2003, 2006; Showalter and Hall 2005; Musselman et al. 2007). In RNA, collective movements of A-form helices linked by flexible junctions can occur at timescales approaching overall rotational diffusion and cause large deformations in the overall hydrodynamic molecular shape and therefore overall reorientation (Fig. 2A). The ensuing motional couplings make it difficult, if not impossible, to separate the internal and overall motional contributions and thus to extract the desired quantitative information regarding the internal dynamics.

In our laboratory, we have developed a domain-elongation strategy for decoupling internal and overall motions in RNA (Zhang et al. 2006). In this approach, a terminal helix is elongated using a stretch of Watson–Crick base pairs,

designed to adopt an A-form helix structure (Fig. 2A). This renders the overall shape of the elongated RNA, and therefore overall reorientation, far less sensitive to internal motions occurring in other parts of the molecule. By slowing down the overall rate of molecular tumbling, the elongation serves to broaden the timescale sensitivity of spin relaxation measurements, which is limited to internal motions occurring at timescales faster than overall tumbling. The elongation also predefines overall motions to be axially symmetric with principal direction oriented along the elongated axis. This further simplifies the characterization of the overall motions and the quantitative interpretation of NMR data in terms of internal motions. A key feature of the domain-elongation strategy is that it allows computation of measured NMR spin relaxation and RDC parameters, for any given dynamic trajectory, regardless of its complexity. This proves to be particularly useful in comparing NMR data with molecular dynamics simulations (Musselman et al. 2007; Frank et al. 2009).

An isotopic labeling strategy was devised in order to avoid unmanageable NMR spectral overcrowding arising due to elongation residues (Zhang et al. 2006). The strategy involves the preparation of two constructs in which stretches of unlabeled A-U (E-AU-RNA) and G-C (E-GC-RNA) base pairs are employed for elongation using otherwise uniformly

FIGURE 2. Domain elongation and NMR spin relaxation reveal nanosecond motions in HIV-1 TAR RNA (Zhang et al. 2006). (A) Collective motions of helices can lead to coupled changes in the overall rotational diffusion (D_{zz}) and/or partial alignment (S_{zz}) of the RNA, which can be decoupled by domain elongation. (B) TAR conformational adaptation upon binding to different ligands and small molecules. (C) Resolving a hierarchical network of local and collective motional modes in TAR RNA. Shown are normalized sugar and base C-H resonance intensities (peak heights) measured from nonconstant time two-dimensional ^1H – ^{13}C HSQC spectra E-TAR and TAR. (D) The intrinsic dynamics underlies the conformational adaptation. Correlation plot between the mean-angular-difference ($\langle \Delta\theta \rangle$) in the orientation of sugar and base C-H bond vectors across eight different HIV-1 TAR structures and the corresponding free E-TAR normalized resonance intensities.

$^{13}\text{C}/^{15}\text{N}$ -labeled G-C and A-U nucleotides, respectively. The two constructs thus allow acquisition of NMR data over the entire RNA target while keeping elongation residue NMRs “invisible.” Of key importance is that the elongation does not affect the structural and functional integrity of the studied RNA. Thus far, comparison of nonelongated and elongated NMR spectra for a variety of RNA contexts under free and ligand or metal bound conditions suggest that elongation does not affect the structural, dynamical, and functional integrity of the targeted RNAs (Zhang et al. 2006; Getz et al. 2007b; Shajani et al. 2007; Sun et al. 2007; Bardaro et al. 2009).

DOMAIN-ELONGATION AND SPIN RELAXATION MEASUREMENTS EXPOSE NANOSECOND INTERNAL MOTIONS IN RNA

Domain elongation was first used to study the dynamical properties of the transactivation response element (TAR) (Muesing et al. 1987), which is a stem-loop located at the 5'-end of the HIV-1 RNA genome that plays essential roles in various steps of the viral replication cycle. Early studies by Williamson, Puglisi, and Varani established that TAR is a highly adaptable molecule that undergoes large structural rearrangements upon binding to peptides and ligands derived from the TAR cognate target the transactivator protein Tat (Puglisi et al. 1992; Aboul-ela et al. 1995, 1996). Subsequently, structures were reported for TAR bound to Mg^{2+} ions (Ippolito and Steitz 1998) and five different small molecule inhibitors of the TAR-Tat interaction (Faber et al. 2000; Du et al. 2002; Davis et al. 2004; Murchie et al. 2004). These TAR structures superimpose with an all-atom root-mean-square deviation of 4.7 Å and differ both in the global orientation of helical domains (interhelical angle ranging between $\sim 5^\circ$ and $\sim 47^\circ$) and local structure of the binding pocket comprising bulge and neighboring residues (Fig. 2B). A key question that has important implications on the rational design of TAR binding anti-HIV therapeutics is whether various ligands induce distinct TAR conformations via induced-fit or bind pre-existing conformations from a dynamical ensemble by conformational selection.

Domain-elongation NMR spectroscopy was used to obtain insights into the dynamical properties of HIV-1 TAR (Zhang et al. 2006). Despite the agreement between NMR spectra of elongated and nonelongated TAR, the spin relaxation properties of elongated TAR (E-TAR) exposed complex motional modes that evade detection in the nonelongated construct due to motional couplings. This complex motional manifold appeared as large variations in the resonance intensities of E-TAR, which, ignoring chemical exchange, report the net dynamics of a given site relative to the applied magnetic field. Unusually high intensities were observed for bulge and neighboring residues, indicating that the interhelix interface is highly

flexible (Fig. 2C). The consistently higher intensities observed for the short helix compared with the elongated helix provided evidence that the helices move collectively across the flexible interface (Fig. 2C). These motions were not resolved in spectra of nonelongated TAR due to motional couplings. A similar pattern of intensities was resolved in a different HIV-1 element, SL1, indicating that these nanosecond local and collective motional modes are a general feature of RNA architecture. Model-free analysis of ^{15}N relaxation measured in E-TAR and E-SL1 confirmed the presence of collective helix motions with amplitudes corresponding to spin relaxation order parameters of $S_s^2 = 0.68 - 0.83$ (the value of S^2 ranges between 0, for maximum motions, and 1, for rigidity) that move with time constants ($\tau_s = 1.4\text{--}1.9$ nsec) approaching the hydrodynamically predicted time constants for their overall rotational diffusion.

How do the motions uncovered in TAR relate to its ability to adopt different conformations and thereby bind to different small molecules and ligands? Interestingly, a correlation was observed on a site-by-site basis between the observed dynamics at a given site, as measured qualitatively based on resonance intensities, and the amplitude of the rearrangements the site undergoes upon binding to different ligands (Fig. 2D). These results provided evidence that TAR codes for intrinsic dynamics that allows its conformation to change functionally and adaptively.

VISUALIZING SUBMILLISECOND HELIX MOTIONS IN HIV-1 TAR USING DOMAIN ELONGATION AND RDCs

The spin relaxation data suffer from two limitations. First, sensitivity is limited to motions occurring at timescales faster than overall tumbling, i.e., tens of nanoseconds. Second, while insights can be obtained regarding the amplitudes of motions, it is generally difficult to reconstruct the spatial characteristics of the underlying trajectory, which are needed, for example, to establish whether unbound TAR can dynamically sample its ligand bound conformations. The measurement of RDCs can fill this gap (Tjandra and Bax 1997; Prestegard et al. 2000). They are sensitive to motions occurring at submillisecond timescales and, when internal motions are decoupled from overall alignment, as afforded by domain elongation, they can readily be interpreted in terms of a dynamical distribution of bond vector orientations relative to an alignment or order tensor describing overall alignment. RDCs are particularly powerful at characterizing rigid-body motions, such as collective motions of helices in RNA (Al-Hashimi et al. 2002; Musselman et al. 2006). In this case, collections of RDCs measured throughout the helix can be analyzed in concert, thus sharpening the spatial resolution with which the underlying dynamic trajectory can be characterized (Tolman et al. 1997).

Domain elongation greatly simplifies the dynamical interpretation of RDCs. In particular, the elongations render RDCs dependent on the angle (Ω) between bond vectors and the internal elongated axis and not a detached external magnetic field (Zhang et al. 2007). For a given elongated RNA, RDCs can be used to probe interhelical bending (β) and twisting (α) motions of the short helix; however, they are insensitive to twisting motions (γ) around the axially symmetric elongated helix (Fig. 3A). To resurrect this vanishing sensitivity, and thereby measure collective helix motion complete three-dimensional (3D) rotational sensitivity, a second elongated construct is prepared in which the short helix is now elongated. In this construct, the RDCs can probe variations in the angles β and γ , but not α (Fig. 3A). Thus, assuming that the two different elongations do not perturb the motions, the measurement of RDCs in the two elongated samples makes it possible to characterize interhelix motions with complete 3D rotational sensitivity.

Domain elongation and inverse domain elongation coupled with RDC measurements were used to visualize interhelical motions in HIV -1 TAR. An order tensor analysis of RDCs revealed super large amplitude helix motions ($\vartheta_{\text{int}} \sim 0.45$ and 0.47 , for EI-TAR and EII-TAR, respectively) that significantly exceed the amplitudes observed previously by spin relaxation ($\sqrt{S_s^2} = \vartheta_{\text{int}} \sim 0.86$) (Zhang et al. 2006). Given the broader timescale sensitivity of RDCs (<0.1 msec) compared with spin relaxation (<19 nsec) and absence of significant exchange broadening in and around the bulge (Zhang et al. 2006; Dethoff et al. 2008), the motions most likely occur at the nanosecond–microsecond timescale.

The RDCs measured in the two elongated TAR samples were used to construct interhelical ensembles consisting of up to three conformations (Zhang et al. 2007). No single interhelical conformation could be determined that satisfied the measured RDCs within experimental precision; likewise, no two-state ensemble could be determined that satisfied the measured RDCs even when allowing for asymmetric populations. However, three-state ensembles with equal populations could be identified that satisfied the measured RDCs close to within experimental precision. Cluster analysis of these ensembles revealed that the three conformers consistently trace out a spatially choreographed trajectory in which the two helices twist and bend in a spatially correlated manner (Fig. 3B). Thus, although the helix motions have very large amplitudes, they are not spatially random. Strikingly, the seven distinct ligand bound TAR conformations fell along various positions of this dynamic trajectory, indicating that ligands most likely capture pre-existing TAR conformations by conformational selection rather than induce new ones by “induced fit” (Fig. 3C).

OTHER APPLICATIONS AND DEVELOPMENTS IN DOMAIN-ELONGATION NMR SPECTROSCOPY

A growing number of studies have used domain-elongation and spin relaxation measurements to uncover local motions that are slower than overall tumbling (so-called supra- τ_m motions) in diverse RNA contexts. For example, domain elongation exposed near- τ_m local motions in an extruded uridine bulge residue in the P4 helix of RNase P

FIGURE 3. Visualizing collective helix motions in TAR RNA using domain elongation (Zhang et al. 2007). (A) Domain and inverse domain elongation for visualizing interhelical motions with 3D rotational sensitivity to twisting (α and γ) and bending (β) motions. (B) Transitions between the three TAR ensemble conformers give rise to correlated twisting and bending dynamics. (C) Comparison of the three TAR ensemble conformers (in green) and ligand bound TAR conformations (in gray). Subconformers along the linear pathway linking three ensemble conformers are shown in light green and the direction of the trajectory is shown in orange arrows.

that completely evades detection in nonelongated P4 (Getz et al. 2007b). Likewise, it helped uncover local motions in two guanine internal loop residues in HIV-1 SL1 that are activated upon Mg^{2+} binding and that may play an important role in adaptively binding the nucleocapsid protein (Sun et al. 2007). Domain elongation also revealed an unexpected increase in local flexibility of RNA residues located within a binding site induced by binding of U1A protein (Shajani et al. 2007). A similar increase in mobility was observed in the TAR bulge on binding argininamide and other ligands (Bardaro et al. 2009). Domain elongation has also helped resolve near- τ_m local motions within the wild-type TAR apical loop that are superimposed onto slower microsecond–millisecond motions (Dethoff et al. 2008). Together, these studies have also helped establish the generality of collective helix motions across a variety of junctions. Results so far suggest that the amplitude of interhelical motions increase with the length of the interhelical linker. New methods have also been introduced that permit quantitative measurement and analysis of sugar and base ^{13}C spin relaxation data in large elongated RNAs, thus significantly increasing the probes available to quantitatively characterize motions, particularly for functionally important internal loops and bulges for which ^{15}N relaxation measurements are generally not feasible (Hansen and Al-Hashimi 2007).

There have also been developments in the application of domain elongation and RDCs. Theoretical frameworks have been introduced to extract the maximum information contained in RDCs measured from differentially elongated RNA samples, which also make it possible to establish the correlated nature of motions involving more than two helices (Fisher et al. 2008). An approach has also developed to approximately reconstruct spatially complex continuous motions of chiral domains, such as helix motions in RNA, using multialignment RDCs (Fisher and Al-Hashimi 2009). This study showed that it is theoretically possible to experimentally reconstruct salient features of a multisegment continuous interhelical motional trajectory obtained from a molecular dynamics (MD) simulation of TAR. More recently, a new strategy has been developed that combines RDCs measured in elongated RNA with computational MD simulations (Frank et al. 2009). This approach was successfully used to generate all-atom structural ensembles of HIV-1 and HIV-2 TAR with timescale sensitivity extending up to milliseconds. The ensemble allowed visualization of local motions in the internal bulge and neighboring residues that activate the collective interhelical dynamics and provided further support that unbound TAR can dynamically sample its ligand bound conformation. Together, these studies suggest that the synergistic combination of NMR experimental data and computational MD simulations will make it possible to meet the challenge of acquiring the parameters needed to construct atomic movies of RNA structures in action.

CONCLUDING REMARKS

By providing quantitative insights into the amplitudes, timescales, and spatial choreography of motions with timescale sensitivity extending from picoseconds up to milliseconds, domain-elongation NMR spectroscopy is greatly contributing toward our understanding of RNA dynamics and its role in function. It is now clear that motions in RNA are as complex as those observed in proteins, featuring local and collective motional modes spanning picosecond–second timescales and that trace out spatially complex trajectories. There is a need to classify the various types of motional modes so as to better understand the relationship between motions and RNA structure on the one hand, and motions and RNA function on the other. For example, it is tempting to speculate that changes in interhelical orientation that do not feature changes in secondary structure, including those observed for tRNA^{Gln} on binding to glutamyl-tRNA synthetase two decades ago, universally occur by conformational selection rather than induced fit. Domain-elongation NMR spectroscopy will undoubtedly play an important role uncovering these dynamic characteristics of RNA structure at atomic resolution. When combined with other developments in time-resolved NMR spectroscopy for directly monitoring RNA structural transitions at atomic resolution (Furtig et al. 2007) and solid-state techniques for high-resolution spatiotemporal characterization of motions (Miller et al. 2006; Olsen et al. 2008), the future of NMR in studies of RNA dynamics appears very promising.

ACKNOWLEDGMENTS

This work was supported by NIH and NSF grants to H.M.A.-H. (RO1 AI066975-01 and MCB 0644278). Q.Z. is a Baltimore Family Fellow of the Life Sciences Research Foundation. We thank present and past members of the Al-Hashimi laboratory for their contributions to various aspects of domain-elongation NMR spectroscopy reviewed here. The Al-Hashimi laboratory acknowledges collaborations with the laboratories of Professor Ioan Andricioaei (UC Irvine) and Professor Carol Fierke (University of Michigan).

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RNA 2009 15: 1941-1948 originally published online September 23, 2009

Access the most recent version at doi:[10.1261/rna.1806909](https://doi.org/10.1261/rna.1806909)

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