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Towards structural classification of long non-coding RNAs

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Abstract

While long non-coding RNAs play key roles in disease and development, few structural studies have been performed to date for this emerging class of RNAs. Previous structural studies are reviewed and a pipeline is presented to determine secondary structures of long non-coding RNAs. Similar to riboswitches, experimentally determined secondary structures of long non-coding RNAs for one species may be used to improve sequence/structure alignments for other species. As riboswitches have been classified according to their secondary structure, a similar scheme could be used to classify long non-coding RNAs.

Keywords

RNA; long non-coding RNA; non-coding RNA; RNA structure; RNA biochemistry

Long non-coding RNAs (lncRNAs) have emerged as important players in development, epigenetics, stem cell biology, plant biology, RNA processing, hormone response, cancer and brain function [1–17]. Preceded by the widespread identification of non-coding RNAs in general [18, 19] long non-coding RNAs were shown to have high specificity to tissue type and developmental stage [20, 21] (also see [22] and references therein). One of the earliest known lncRNAs is Xist (X chromosome inactivation stimulated transcript), responsible for X chromosome inactivation during development [23]. More recently, several lncRNAs have been shown to be critical in HOX gene systems during development [1]. The ½sbs-lncRNA controls mRNA decay by hybridizing with mRNA to form a platform for STAU1 protein binding, triggering degradation of mRNA [6]. Other lncRNAs are required for p21 activation [24], stem cell reprogramming [25] and stress response [26].

Although the physiological relevance of many of the reported (>20,000) lncRNAs has not been determined, many lncRNAs have been shown to possess important, visible phenotypes [27]. In addition to Xist, required for dosage compensation, the Braveheart lncRNA has been shown to be required for lineage commitment in cardiomyocytes [2]. FENDRR lncRNA is required for heart, lung and gastrointestinal development [28]. Linc-brn1b is required for neocortex development [28]. The COOLAIR lncRNA is required in *A. thaliana* for cold-timed flowering [4]. Additionally, the NEAT1 lncRNA has the clear phenotype of being critical for paraspeckle formation [29–31].

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While lncRNAs span a wide range of physiological contexts and functions, they have several common characteristics, including length (>200 nts), alternative splicing, poly-adenylation, low abundance, lack of protein product, and low sequence identity. Many studies have been performed to identify new lncRNAs, determine their protein partners, and determine their functions (*via* loss of function knock down and knock out experiments). However, few studies have examined their mechanism at the atomistic level [32]. In the past few years, researchers have been laying the foundation for structure-function studies. Genome-wide studies of RNA secondary structure have been performed, revealing the lncRNAs tend to be more structured than mRNAs, but less structured than ribosomal RNAs [33–39]. Detailed secondary structure studies of complete, intact single lncRNA systems show that some lncRNAs are hierarchically structured with sub-domains containing modular RNA secondary structure motifs [40–42]. Studies of Malat-1 and related lncRNAs show that the 3'-end forms a triple helix, protecting it from RNase degradation [14, 43, 44]. Recent studies have elucidated lncRNA-protein interactions, emphasizing the need for detailed structural studies and mechanistic studies at the molecular and atomistic level [45, 46].

LncRNAs tend to have low sequence identity and are often described as non-conserved. We note that some of the most well-studied non-coding RNAs (miRNAs and rRNAs) have very high sequence identity (>78% in nucleic acid sequence identity) [47]. In contrast, many other important classes of non-coding RNAs have relatively low sequence identity (nucleic acid sequence identity of ~ 50%−65%), but secondary structures that are conserved across thousands of sequences. For example, riboswitches, which regulate metabolism in bacteria, typically have sequence identities of only 50%−65%, but have secondary structures conserved across thousands of species [47]. The U2 and U4 spliceosomal RNAs have sequence identities < 60% but secondary structures conserved for > 9000 sequences. The 5S ribosomal RNA has sequence identity of ~ 60% but secondary structure conserved over 229,000 sequences. The group I intron has decidedly low sequence identity (~ 36%) but structure conserved across 60,000 species [47].

RNAs with low sequence identity are difficult to find using conventional search algorithms such as BLAST. However, knowledge of secondary structure dramatically enhances the search success. In the case of riboswitches, the RNA secondary structure was determined for a single species using *in vitro* chemical probing of the RNA in cell-free reconstituted systems[48–55]. Next, this structure was used as a fingerprint to find the structure in thousands of other species, despite the low sequence identity [56]. The secondary structures determined from cell-free systems by chemical probing were verified by X-ray crystallography [57–61].

To determine the RNA secondary structure of lncRNA molecules, we follow similar strategies to those used to determine the original 16S rRNA secondary structure [62–64] and the riboswitches [65] (Fig. 1). Namely, we perform chemical probing experiments to determine nucleotides that are highly mobile and likely to reside in looping regions, as well as those nucleotides with low mobility, likely to participate in Watson-Crick base pairs. To cope with the large RNA size, we employ 3S (Shot-Gun Secondary Structure), which probes the entire RNA first and then probes shorter segments of the RNA in successive rounds of probing [40, 66]. By matching signals of short segments with full RNA experiments, we

identify modular sub-domains, for which a secondary structure is often readily discernable. The resulting secondary structure can be used to improve existing phylogenetic sequence alignments, and, in principle, can be used to find instances of the lncRNA not previously found in other species. In our studies, we typically begin with either alignments generated by genome browser, or alignments using synteny. We then use the initial secondary structure to improve these sequence alignments, focusing on alignment of helical regions. Covariance analysis helps to validate each helix. Next, we use the helices with the most covariant base pairs to further improve the sequence alignment. This process can be performed iteratively, with improved or validated helices enabling improved sequence alignments, and improved sequence alignments enabling more accurate covariant measures.

To demonstrate this principle, we consider the 873 nt steroid receptor RNA activator lncRNA in humans (SRA-1). This lncRNA co-activates the hormone response in human T-47D cells and co-immunoprecipitates with a large number of important proteins, including several hormone receptors (estrogen receptor, progesterone receptor, androgen receptor, glucocorticoid receptor and thyroid receptor) [67–70]. Binding assays in *in vitro* cell-free reconstituted systems have shown strong binding to the pseudouridinylase Pus1p, estrogen receptor, thyroid receptor, the sex reversal factor DAX-1, and the epigenetic factor SHARP. While the primary function of SRA-1 is to co-activate the hormone response, a speculated secondary function involving the binding of SRA-1 to its cognate protein SRAP has recently been shown not to occur (SRA-1 does not bind to SRAP) [71].

Our previous study demonstrated that SRA-1 contains four modular secondary structure sub-domains, each containing multiple secondary structure motifs (Fig. 2). The secondary structure was consistent with four different probing techniques (SHAPE, DMS, in-line, and RNase V1). Base pair flips with respect to species were found in the vast majority of helices. Binding studies have shown that SHARP binds to the helix 12 / helix 13 (H12/13) domain [72].

Here, to demonstrate the utilize of secondary structure determination, we use the secondary structure of domain IV to improve the phylogenetic alignment of SRA-1 from the Ensembl dataset, in a similar manner to that used by Breaker and co-workers to improve alignments for riboswitch ncRNAs [65]. Figure 3 shows the alignment from Ensembl and the improved alignment of domain IV based on the secondary structure derived form chemical probing. The domain IV secondary structure is present in many of the sequences and covariant base pairs are observed.

This strategy can, in principle, be used to identify orthologs of lncRNAs in other species. Before classifying a lncRNA as a non-conserved RNA, we recommend that the secondary structure be studied and used to search other genomes, in addition to performing BLAST style searches. We note that *in vivo* probing studies provide important information validating the *in vitro* structures. *In vitro* studies are important to establish the *ab initio* structure because the probing signal *in vivo* may to be obfuscated by multiple proteins binding to the RNA, as suggested recently (Fig. 4) [11, 12]. In addition, there are few known cases where an *in vitro* structure of an intact, individual RNA has been shown to differ from its corresponding *in vivo* structure. For example, the vast majority of crystallographic structures

of RNAs, which are of course determined *in vitro*, have either (i) been validated *in vivo*, or (ii) not been disproven *in vivo*. In the case of riboswitch RNAs, crystallographic data strongly support initial secondary structures determined by chemical probing techniques discussed above. Overall, structure-function studies of lncRNAs are in their infancy and represent an open area of research. Studies of larger lncRNAs (10–100 kB) may open a new area of structural biology and have the potential to reveal novel RNA and RNP mechanisms. Three-dimensional studies of smaller lncRNAs are also an exciting area, especially in light of the combination of specific and non-specific RNA-protein interactions thought to be involved in lncRNP complexes [12].

While there are few existing experimentally determined secondary structures of lncRNAs, the future determination of the precise and detailed secondary structure of many lncRNAs may allow immediate classification into type I: highly structured RNAs with sub-domains and complex structural motifs, such as multiway junctions, type II: loosely structured RNAs with multiple stem-loops, but lacking hierarchical domain structure and complex motifs, and type III: unstructured, disordered RNAs, which lack secondary structure. Further classification can proceed upon discovery of many lncRNAs with complex structure in terms of the specific structural motifs that organize the RNA.

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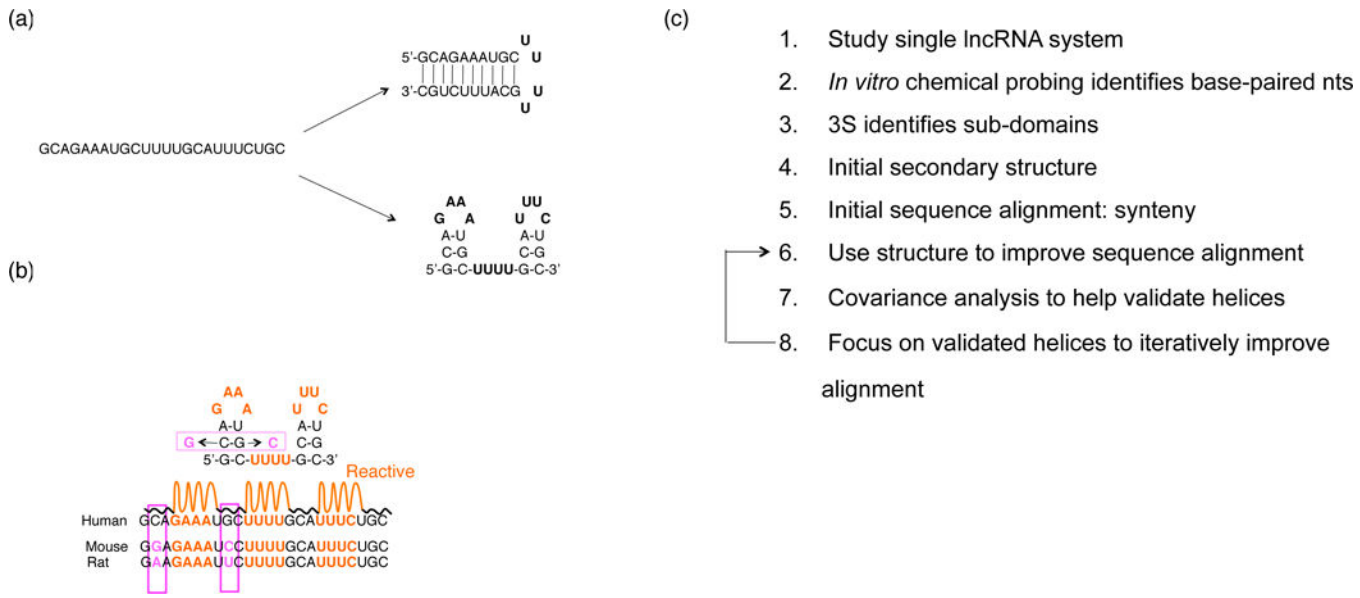


Figure 1.

RNA secondary structure determination. (a) One sequence may be consistent with multiple folds. (b) Chemical probing reactivity data helps to lift degeneracy between folds. Multiple sequence alignment help identify covariant base pairs (pink). (c) Pipeline to determine secondary structures of long non-coding RNAs.

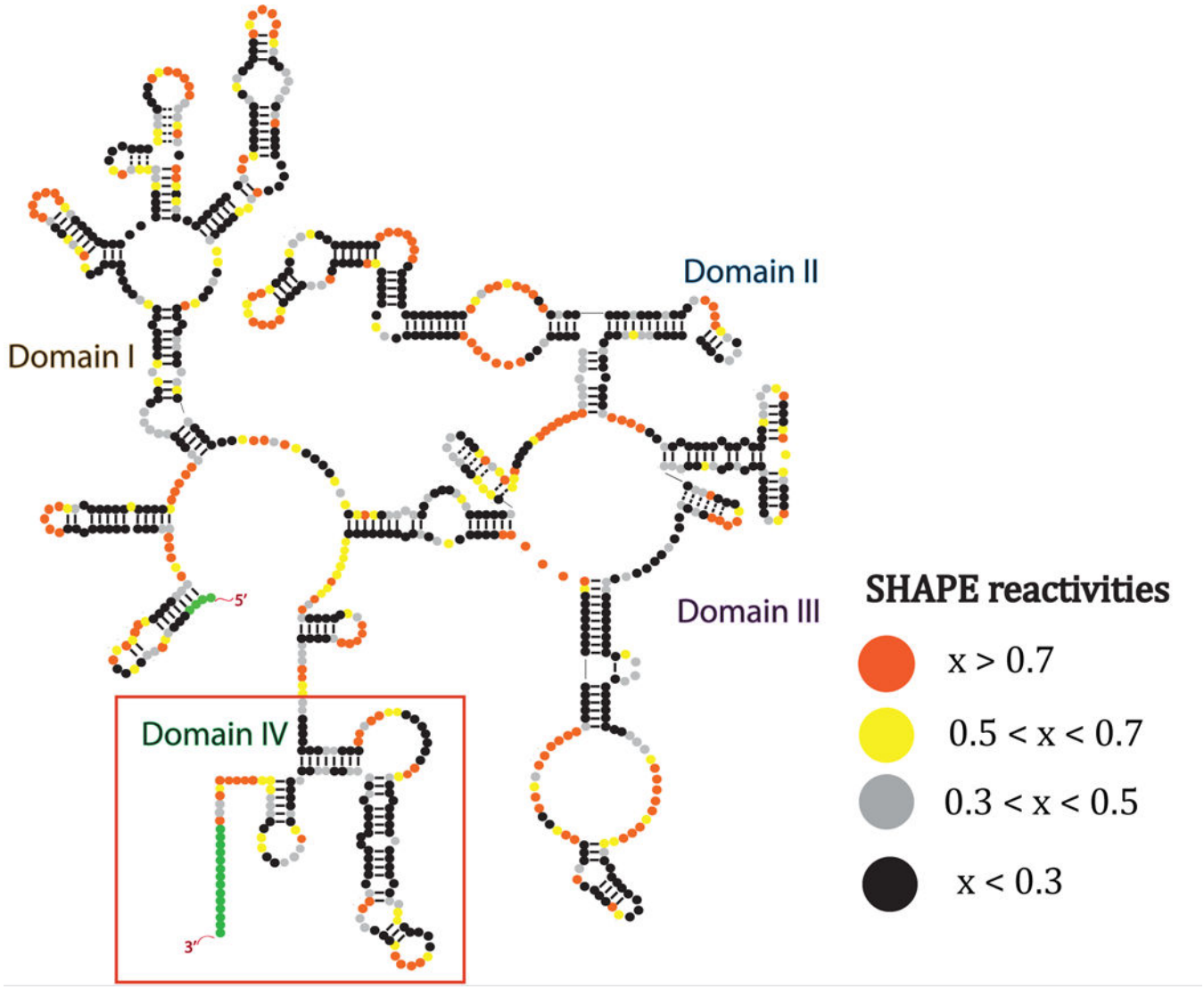
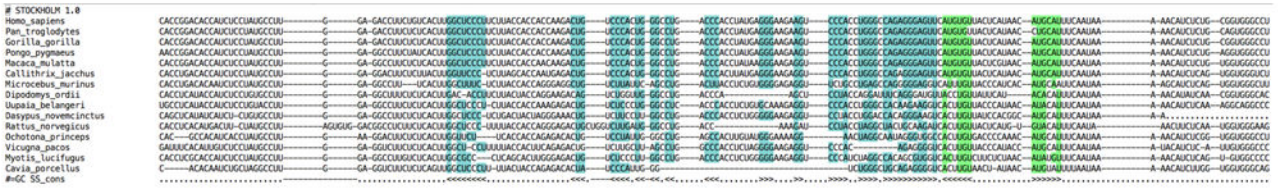


Figure 2.

Secondary structure of steroid receptor RNA activator, as determined by 3S, consists of four sub-domains. The chemical probing reactivity data helped to improve structure/sequence alignments for domain IV.

(a)



(b)

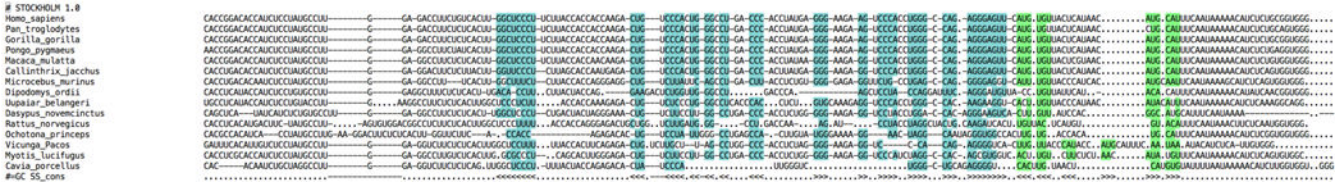


Figure 3.

Example of sequence alignment for domain IV of SRA. (a) Original alignment from Ensembl database. (b) Improved alignment using knowledge of secondary structure.

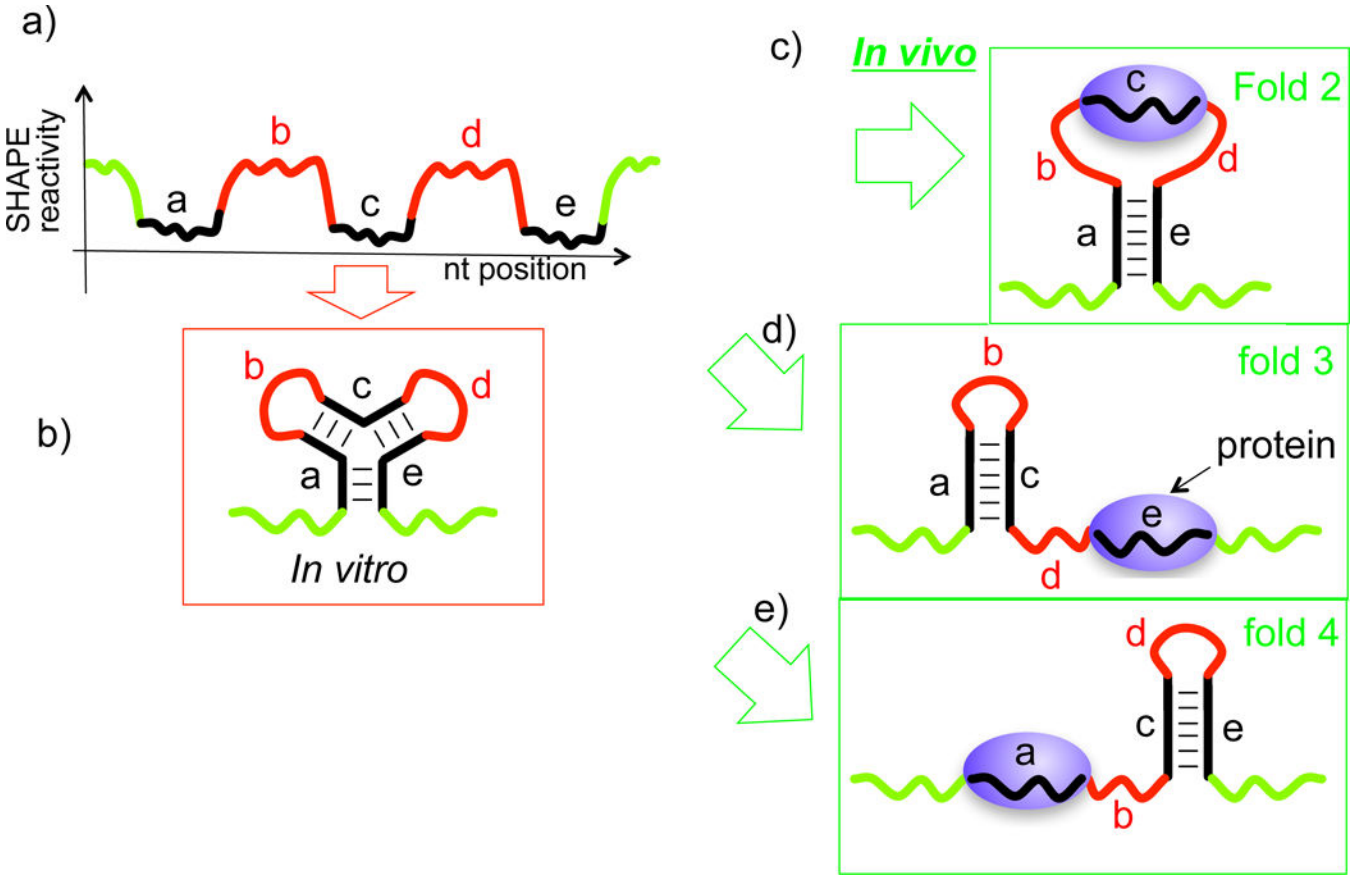


Figure 4.

In vivo probing signals may be obfuscated by protein binding, making it more difficult to distinguish configurations. (a) Schematic of SHAPE probing signal for three-helix junction. (b)-(d) Other configurations that may be consistent with signal (a).