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Riboswitches: natural SELEXion

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Abstract. Advances in our knowledge of the structure and chemistry of RNA have been harnessed in the process known as SELEX to develop artificial RNA-based molecules that can act as enzymes and ligand binders performing a wide variety of functions. The discovery of riboswitches, natural RNA aptamers involved in genetic regulation, offers a basis of comparison between the artificial selection and the natural selection of structured RNAs for small-molecule recognition. The guanine riboswitch structural determination allows us to draw conclusions regarding the apparent increased complexity of the riboswitch aptamers compared to their in-vitro-selected cousins.

Key words. RNA; riboswitch; aptamer; X-ray crystallography; structure.

The combination of heritable information and the ability to form complex functional structures in RNA has given rise to powerful in vitro selection methods, often referred to as SELEX, that have yielded a wide variety of catalysts (ribozymes) and ligand binders (aptamers) [1, 2]. This technology is being increasingly harnessed in the development of biosensors, enzymes and molecular biological tools [3]. Recently, sequences were discovered in the 5'-untranslated region of bacterial mRNAs consisting of aptamers coupled to a secondary structural rearrangement creating a form of genetic regulation called riboswitches [reviewed in refs 4, 5]. Riboswitch aptamer domains, like their SELEX counterparts, bind small molecules and metabolites, controlling expression of the mRNA in a cis fashion. Found to control over 2.0% of all known genes in the *Bacillus subtilis* genome [6], riboswitches act in the absence of protein cofactors to regulate at either the transcriptional or translational level. In transcriptional regulation, metabolite binding to the mRNA dictates the formation of an antiterminator or terminator stem that signals the polymerase to continue or abort synthesis of the mRNA. Determination of the structure of the guanine riboswitch aptamer domain bound to hypoxanthine and guanine [7, 8] allows us to draw comparisons of ligand recognition between natural and artificial aptamers with an eye toward how SELEX methods can be modified to yield RNAs that resemble their natural kin.

One of the most striking features of natural aptamers is that they contain more complex secondary structure, and presumably, tertiary architecture than those obtained through in vitro selection. An extreme example of this is the vitamin B_{12} aptamer obtained by selection which is a simple pseudoknot [9] and the biological vitamin B_{12} aptamer that extends over 200 nucleotides with conserved residues distributed throughout a complex proposed secondary structure [10–12]. Evidence of tertiary architecture is clearly found in the S-adenosylmethionine (SAM) riboswitch which contains a conserved kink-turn motif [13], a sequence element associated with ribosomal RNA, small nuclear RNAs and spliceosomal RNA [14]. The crystal structure of the guanine riboswitch reveals that highly phylogenetically conserved nucleotides outside the ligand binding pocket form a loop-loop interac-

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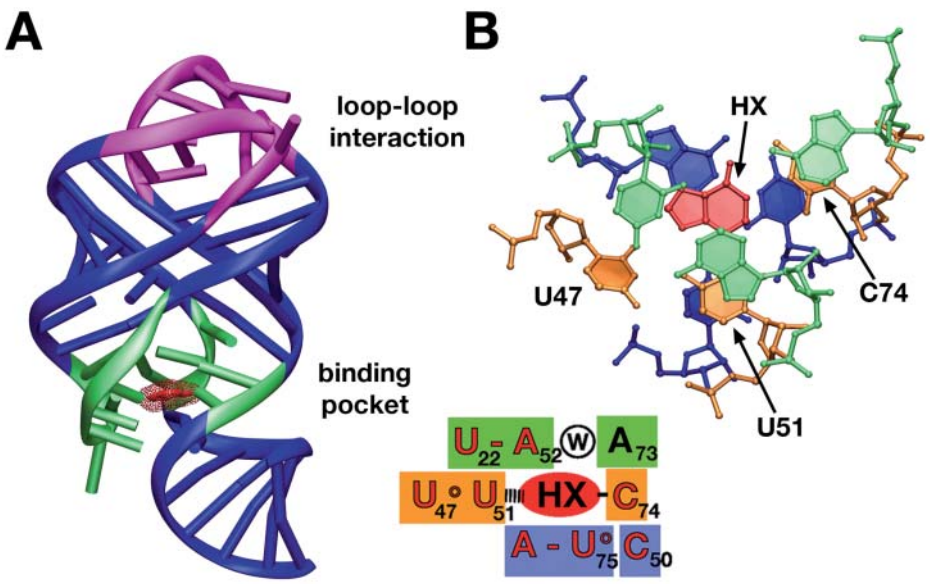

Figure 1. (A) Global structure of the guanine riboswitch bound to hypoxanthine. The loop-loop tertiary interaction indispensable for ligand binding is pictured in magenta. Bases involved in forming the three-way junction of the binding pocket are shown in green. (B) Base stacking in the binding pocket of the guanine riboswitch. Notice how neither the nucleotides above (green) nor below (blue) are involved in strong base-stacking interactions with hypoxanthine (red).

tion (fig. 1A). Despite not contributing directly to ligand binding, the terminal loops are absolutely essential for ligand recognition [7]. In-line probing of this riboswitch demonstrated that these loops are structurally ordered in the absence of guanine, unlike the ligand-binding pocket [6]. Thus, the global fold of the guanine riboswitch is preorganized by a tertiary interaction via the two terminal loops while local structure around the binding site remains disordered. In contrast, a majority of in vitro selected aptamers bind their ligand solely through internal or terminal loop motifs imbedded within a single stem-loop without the use of tertiary architecture, as defined by the packing of multiple helices. In part, this is due to the limited size of the randomized libraries used as the initial starting pool (often around 40 nucleotides) in SELEX and an experimental bias to characterize simple sequence motifs that emerge from selection.

Does the increased complexity of natural aptamers facilitate their ability to interact with ligands? One explanation is that it significantly improves the affinity of the riboswitches for their appropriate ligand. While some riboswitches indeed display low nanomolar affinity for their ligands, many bind with affinities similar to aptamers obtained by in vitro selection. For instance, the adenine riboswitch binds adenine with an equilibrium dissociation constant of ~300 nM [15], analogous to the binding strength of the theophylline aptamer, an in vitro selected purine binder [16]. Another possible reason for architecture that globally organizes the riboswitch may be related to its mechanism of gene regulation. Many riboswitches act on the level of transcription using rho-independent termination where stem-loop motifs downstream of the aptamer direct RNA polymerase to either continue or abort transcription of the mRNA [17]. This requires that the ligand binds to the mRNA very rapidly, allowing the antiterminator/terminator switch to adopt the appropriate configuration prior to the polymerase escaping the regulatory element. Thus, the riboswitch is at least partially under kinetic control. This presents a problem, as many RNAs use an induced-fit mechanism to bind proteins and ligands, typified by slow bimolecular association rates (10^4–10^5 $M^{-1}s^{-1}$) [18, 19]. Global preorganization of the RNA may allow riboswitches to bind with sufficiently fast kinetics to beat the polymerase. However, studies by Crothers and coworkers on the FMN riboswitch [20] and our group on the purine riboswitch (unpublished data) clearly indicate that natural aptamers bind their ligands on a comparable timescale to that of the theophylline aptamer [21]. Preorganization does not facilitate binding kinetics; instead, riboswitches appear to contain sequence elements that stall the polymerase, giving the RNA sufficient time for slow events such as ligand binding and secondary-structure rearrangement to occur [20]. From a biophysical perspective, the natural aptamers do not appear to enjoy a clear thermodynamic or kinetic superiority over their in vitro selected counterparts.

The complexity of riboswitch aptamers is likely linked to a second feature of ligand binding that differs from in-vitro-selected aptamers. In the structure of the guanine riboswitch, the three-way junction binds the nucleobase in a fashion that buries 97% of the ligand surface area. This explains the observation that the binding pocket is locally disordered while the RNA is globally ordered; the ligand can only access the binding site while the RNA is in an open conformation. As a consequence of the complete burial of the ligand, the riboswitch recognizes

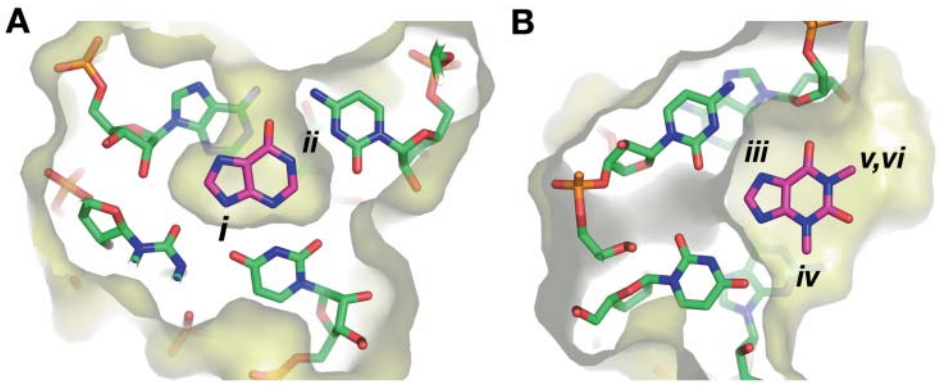

Figure 2. Comparing the ligand recognition face of the guanine riboswitch bound to hypoxanthine (A) to that of the in-vitro-selected theophylline aptamer (B). Additions to the 9-position of hypoxanthine (denoted by i) like those that occur in the nucleotide phosphate derivatives of the nucleobase, or substitution of adenine (ii) altering the hydrogen bonding donor/acceptor pattern in the Watson-Crick binding face, results in at least a 50,000-fold loss in affinity. The theophylline aptamer discriminates between theophylline and caffeine, which differ by the addition of a methyl group at the N7 position (iii). The affinity of the RNA for caffeine is 10,900-fold less than for theophylline as a consequence of this single methyl group. However, this aptamer binds 3-methylxanthine (iv), 1-methylxanthine (v) and xanthine (vi), three derivatives that differ in additions to the six-membered ring of the purine, with affinities varying from 6.3- to 28-fold less than theophylline, a dramatic decrease in specificity for half the nucleobase.

every functional group upon the nucleobase. Unusually, the guanine riboswitch does not appear to use base stacking to substantially stabilize or enhance this interaction. None of the bases in the triples above or below the ligand directly stacks upon the base (fig. 1B). Stable complex formation is almost entirely dependent upon the formation of the correct hydrogen-bonding network between the ligand and the RNA (our unpublished data). This allows the riboswitch to exhibit a high degree of discrimination between guanine and related molecules such as adenine, nucleobases, nucleosides and similar metabolites such as xanthine (fig. 2A). In the complex metabolic environment of the cell where the riboswitch is confronted with a variety of closely related chemical species, the RNA is able to survey each functional group of the ligand, allowing it to achieve an extraordinary degree of selectivity.

RNA aptamers derived from in vitro selection have long been touted as being able to achieve very high degrees of discrimination. The current gold standard for this behavior is the theophylline aptamer, which exhibits over 10,000-fold selectivity for theophylline (1,3 dimethylxanthine) over caffeine (1,3,7 trimethylxanthine), rivaling that of antibodies [16]. However, this degree of selectivity is only for modifications to the five-membered ring; the aptamer shows very little ability to discriminate between xanthine species methylated at the 1 or 3 positions, as demonstrated biochemically and structurally (fig. 2B). This feature might prevent it from acting as an effective riboswitch in a cellular environment (for instance as a regulator of caffeine biosynthesis, where methylated xanthine species are present).

The theophylline aptamer is clearly a case of you get what you select for. The selection used a ligand coupled to a solid support through a carbon linker attached to theophylline via the N1 position, eliminating the ability of the RNA to monitor this position through hydrogen-bonding interactions. Furthermore, the negative selection step in the experiment solely used caffeine, which differs from theophylline by a methyl group at the N7 position. Thus, in terms of specificity, the aptamer was only asked to monitor for the presence or absence of a methyl group, yielding an RNA that only monitors the five-membered ring of the purine ligand. On the other hand, the guanine riboswitch evolved in an environment where negative selection would involve purine biosynthetic intermediates, pyrimidines, nucleosides and nucleotides, a strong selection pressure for monitoring the entire ligand. RNAs with complex ligand-binding sites like the guanine riboswitch may be achieved using selection methods that allow for complete ligand recognition, such as allosteric selection [22], and incorporate negative-selection steps that employ a comprehensive set of related compounds.

Evolving sophisticated aptamers in vitro is complicated by the fact that the initial library of variants typically contains 10^{12}–10^{14} individuals. Thus, obtaining an RNA with even a minimal amount of tertiary structure at the end of the selection would be a rare event. Larger RNAs on the order of the lysine or vitamin B_{12} riboswitches would likely be outside the range of what is obtainable. As our knowledge of the three-dimensional architecture of the natural aptamers improves, we may be supplied with a ready-made set of scaffolds that could serve as the basis for the selection of aptamers with novel binding functions. For example, selections based upon the guanine riboswitch can be envisioned in which the global fold of the RNA is kept constant by retaining the three helices and the loop-loop interaction and randomizing the sequence in the starting pool around the three-way junction of the binding pocket. This selection may have the further

advantage of allowing the majority of the RNAs in the initial screen to be folded to a significant degree, preventing their tendency to multimerize and aggregate at higher concentrations. In vitro selection experiments randomizing the binding pocket of the ATP aptamer demonstrated that RNAs with new ligand-binding specificities can be rapidly evolved [23], indicating the practicality of this approach. Using the natural aptamers as a guide may enable in vitro selection to access new functional RNAs that can cope with complex chemical environments, bringing the vast promise of SELEX closer to practical realization.

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