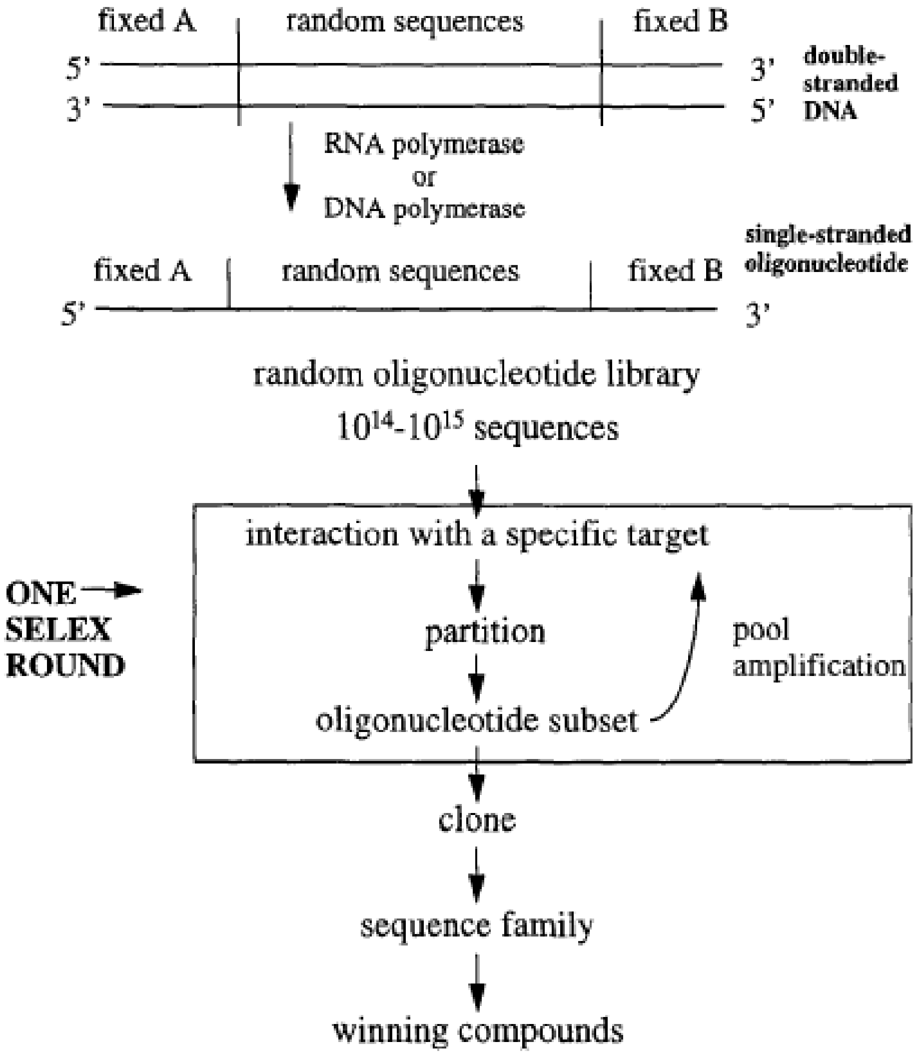
Lecture 3. 2023
RNA Binds Ligands

Targeting RNA structures with small molecules
Childs-Disney JL, Yand X, Gibaut QMR, Tong Y, Batey RT, **Disney MD**. 2022.
Nat Rev Drug Discov. 21(10):736-762.

Frameworks for targeting RNA with small molecules
Juru AU, **Hargrove AE**, 2021.
J Biol Chem 296:100191.

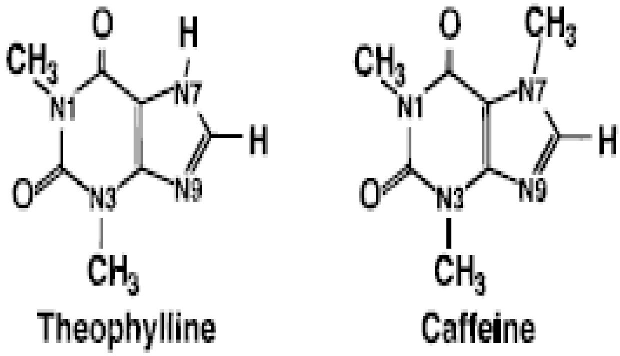
SELEX

Selection of ligands by exponential enrichment



We’ll examine a model system for identification of an RNA that binds a small molecule. In this case, the small molecule is theophylline, and the rationale for using it as a target for RNA binding was that it is a naturally occurring alkaloid that is used as a bronchodilator. Its structure is very close to that of caffeine, and monoclonal antibodies directed against theophylline show caffeine cross-reactivities of 0.2-0.3% of the theophylline signal. Can RNA also discriminate between these closely related compounds?

A



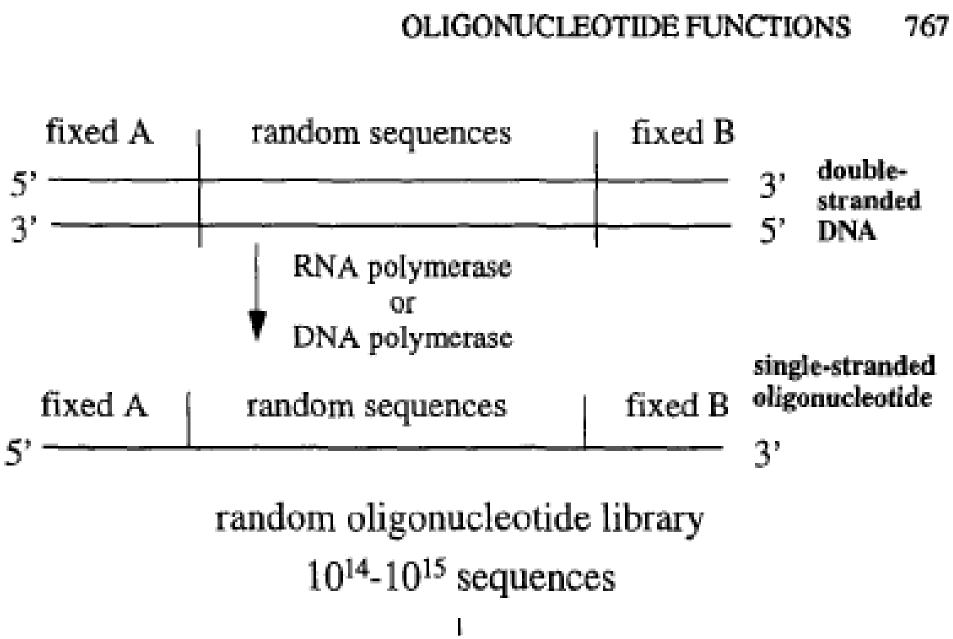
So that was the rationale. There are now many papers in the literature describing SELEX experiments to identify an RNA that binds to small molecules, proteins, drugs, etc et. A logical question to ask, and you should ask it, is “why bother?” “Why should I care about another clever RNA molecule that binds to a dumb ligand?” After a couple of years of reading these papers, most intelligent people were pretty bored with these selection tricks.

But then Ron Breaker made an amazing discovery, and that’s why we’re talking about SELEX.

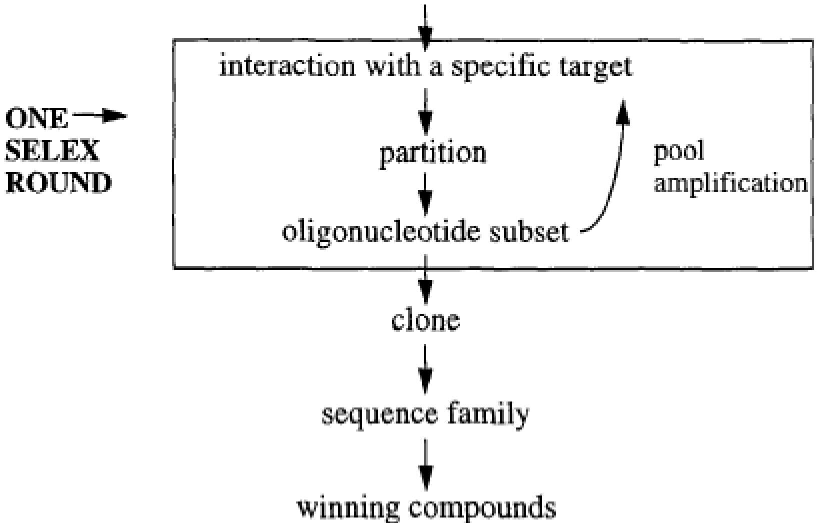
And, because now people know about the awesome power of RNA, it’s become big business.

So back to theophylline. (Jenison et al., 1994. Science 263: 1425-1428.)

The RNA pool was made of about 10^{13} molecules containing a 40-nucleotide random region. [Does this represent complete sampling of sequence space for a 40 nt random sequence? Do the arithmetic: $4^{40} = 10^{24}$ molecules]



The protocol was to immobilize theophylline on a solid matrix. The RNA pool was applied to the column, and allowed to mix for some time, at some temperature. This corresponds to the ‘interaction step’ of the process.

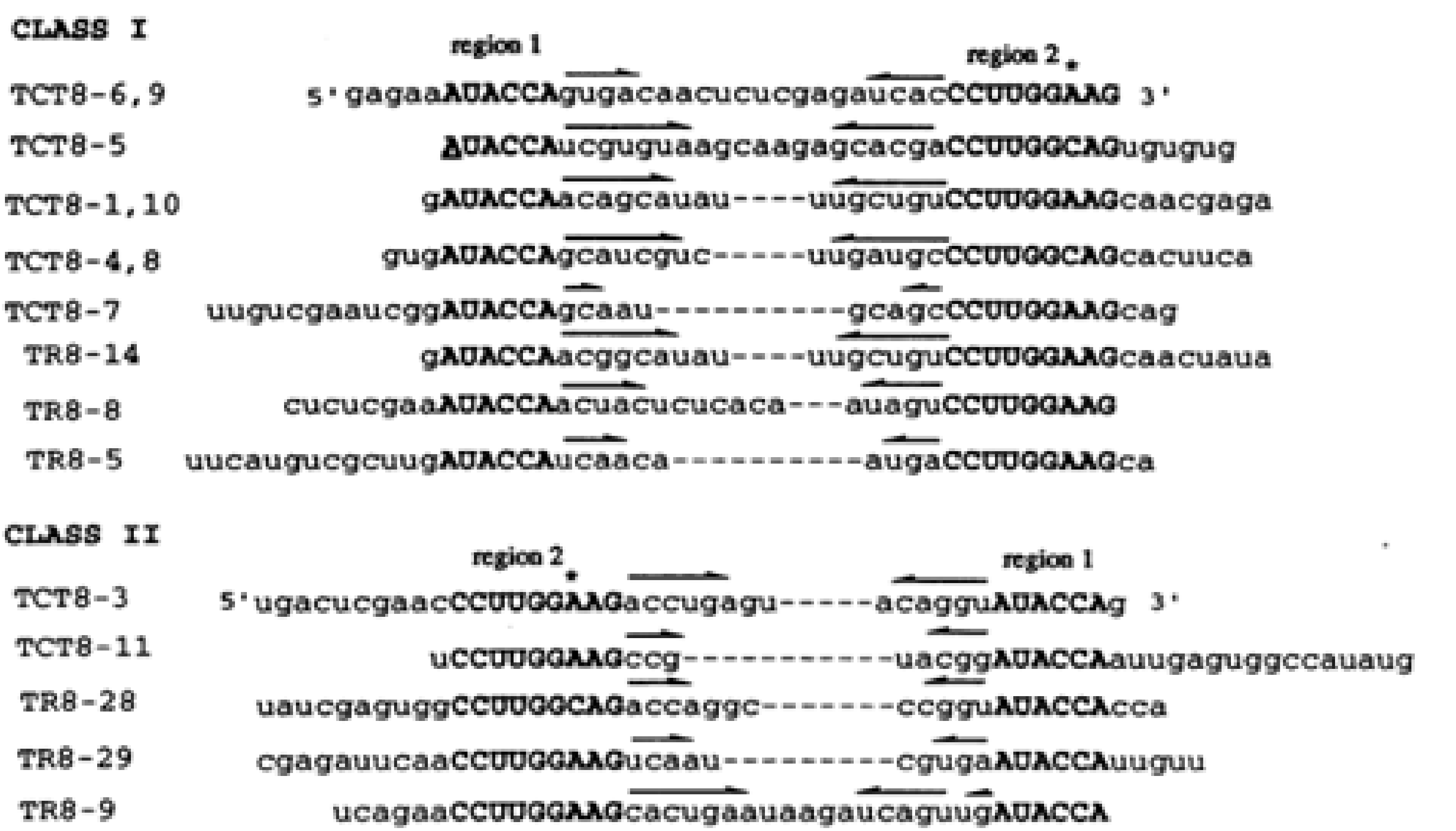


To remove the RNA, the column was washed, first with buffer to remove unbound RNA. Then to remove bound RNA, the column buffer containing free theophylline was applied, to competitively bind to the RNAs and elute them as a complex. This is the ‘partfitioning’ phase of the process.

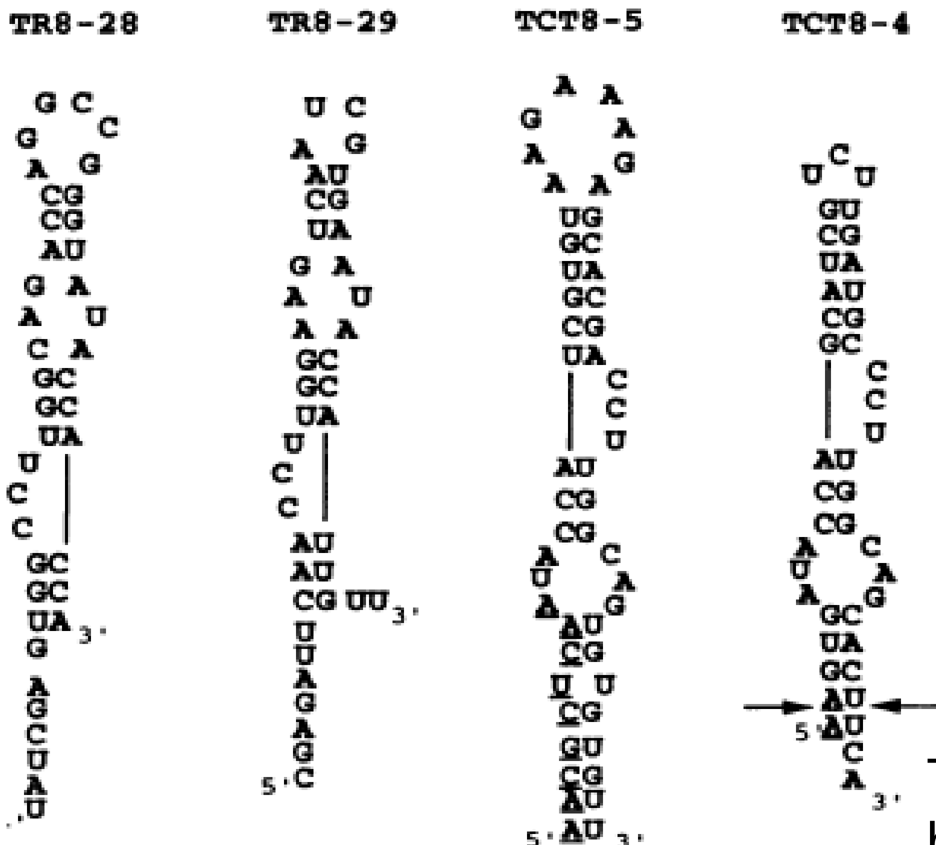
To increase specificity, a counter-selection was used. Buffer containing caffeine was applied to the column, prior to addition of theophylline-buffer. Any RNA that bound to caffeine was thus removed from the bound pool.

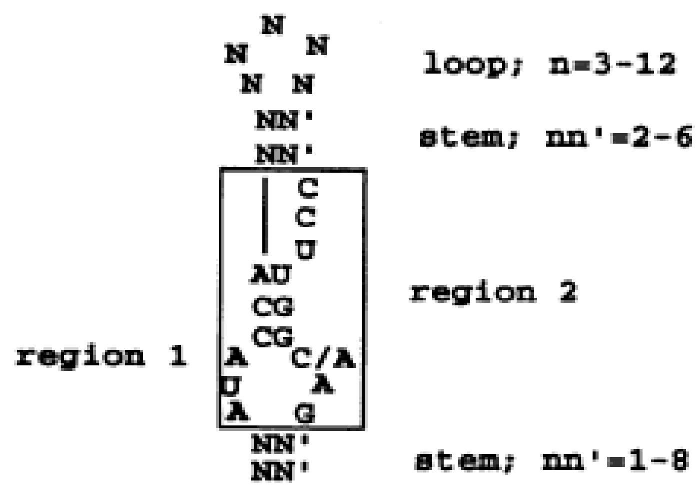
After eight rounds, most of the molecules in the pool bound to the column. They were collected and sequenced. The family of RNAs contained two sequences of six and nine nucleotides, separated by a variable span.

A

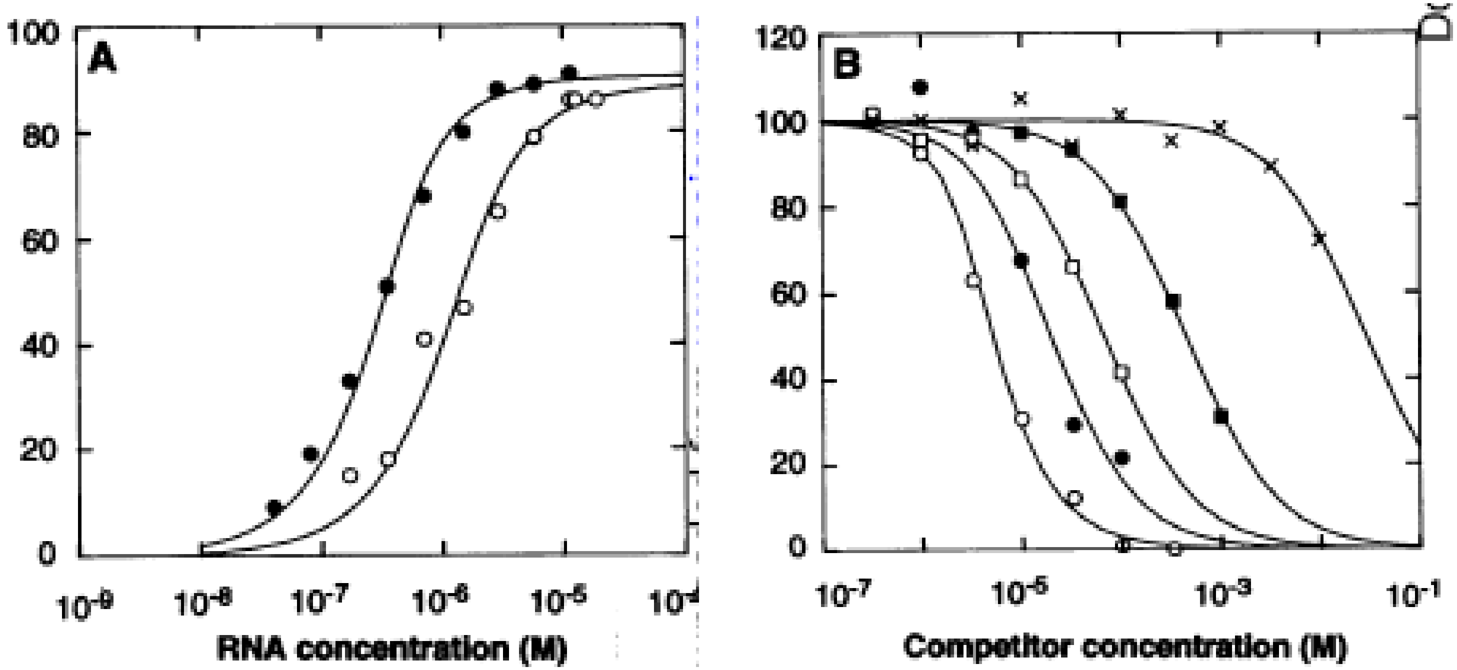


They could all be folded into a similar structure





The little minimal one contained the binding site, and it was used for structural and binding studies.

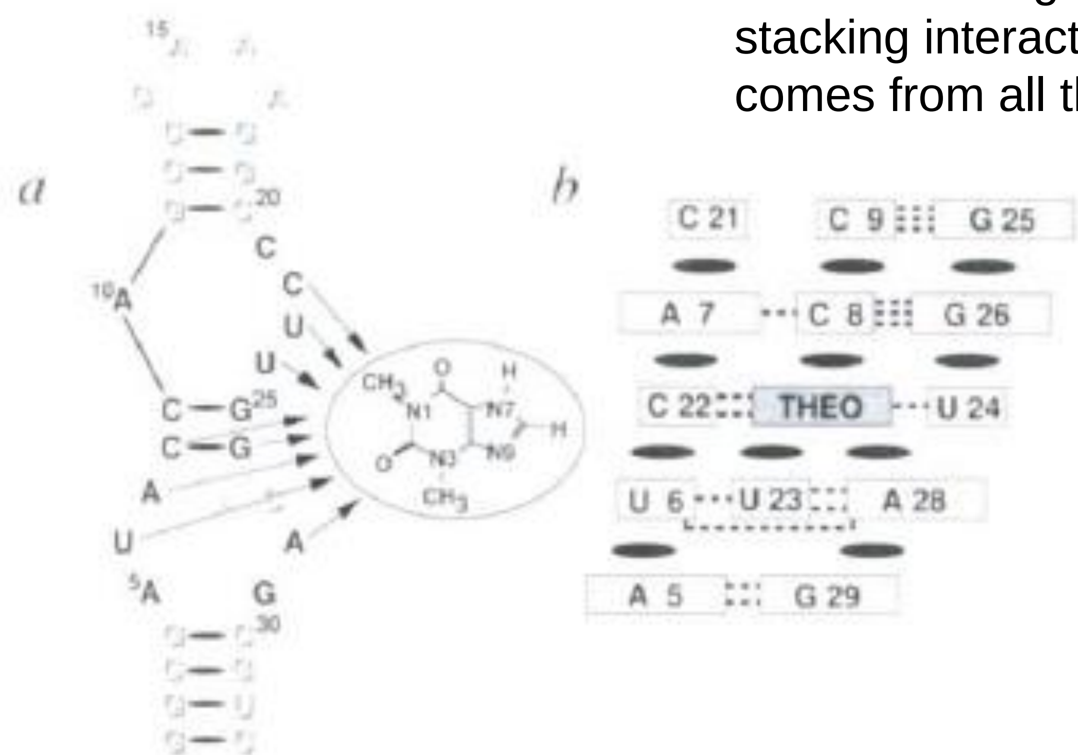


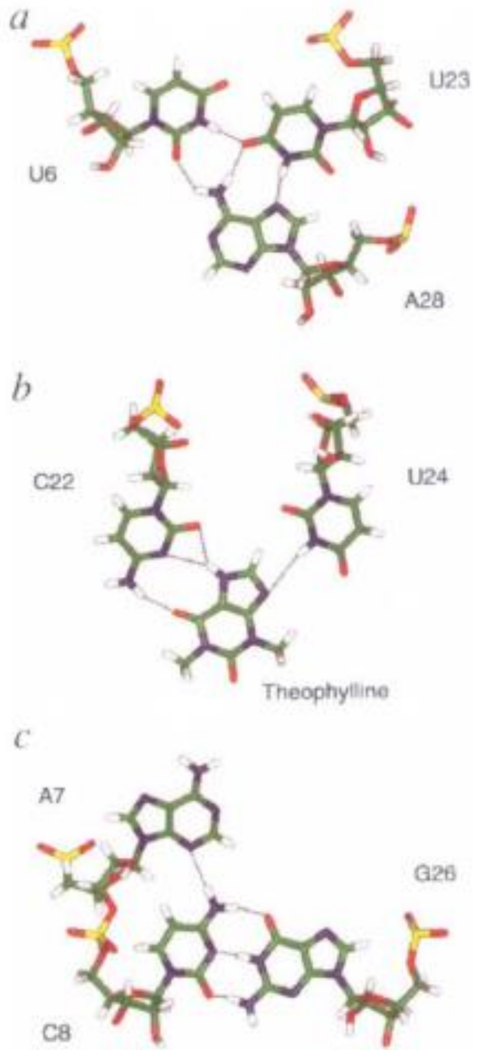
Fraction bound theophylline vs total RNA concentration.
A. ● mTCT8-4 RNA. ○ TCT8-4 RNA. The affinity of mTCT8-4 for theophylline is 0.32 ± 0.13 μM.
B. Bound 14C-theophylline was displaced by competitors: ● 3-methylxanthine, ○ theophylline, □ xanthine, ▪ hypoxanthine, x caffeine.

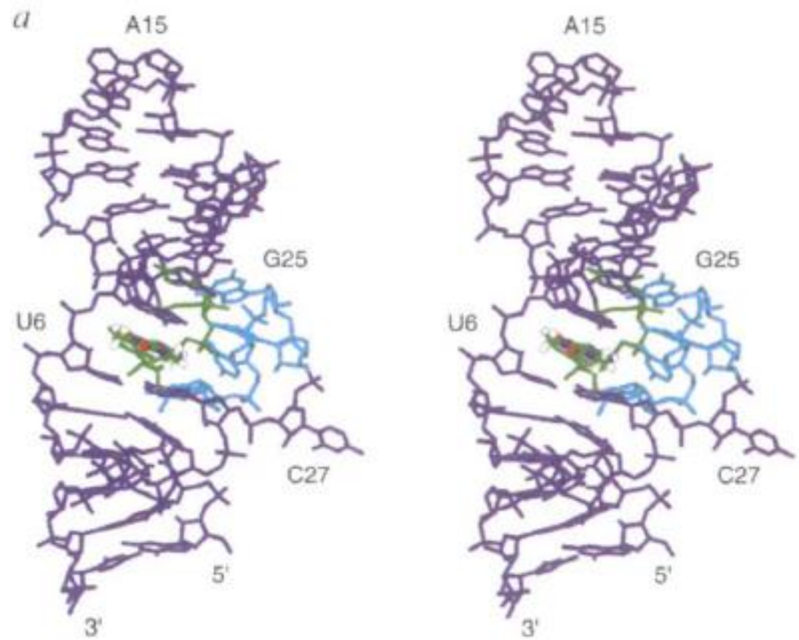
Specificity of the RNA for theophylline exceeded that of antibodies.

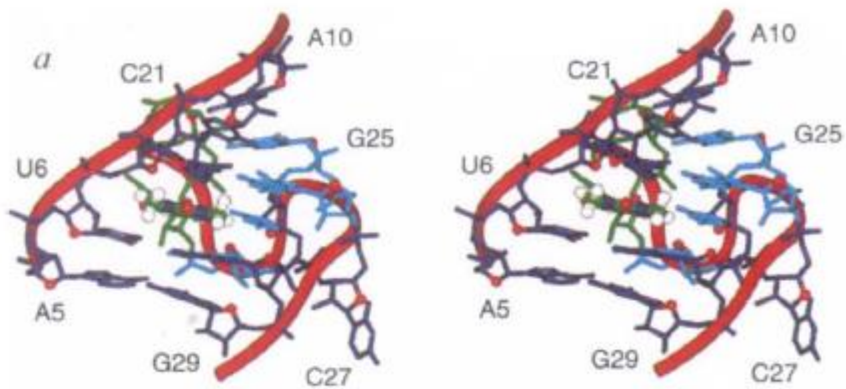
The structure of the RNA bound to theo was solved by NMR (Zimmerman et al., 1997. Nat Str Biol 4:644-649)

Note the base triples, and how the theo mimics their geometry. Packing of the theo takes advantage of the energetics of stacking interactions, but the specificity comes from all the hydrogen bonding.



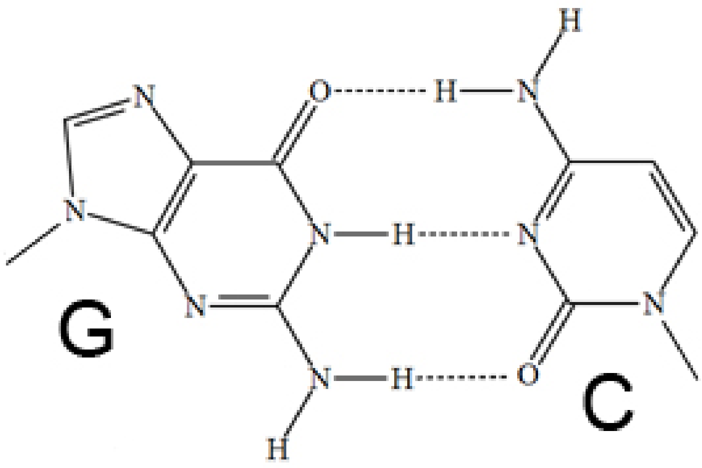

a
U23
U6
A28
b
C22
U24
Theophylline
c
A7
G26
C8

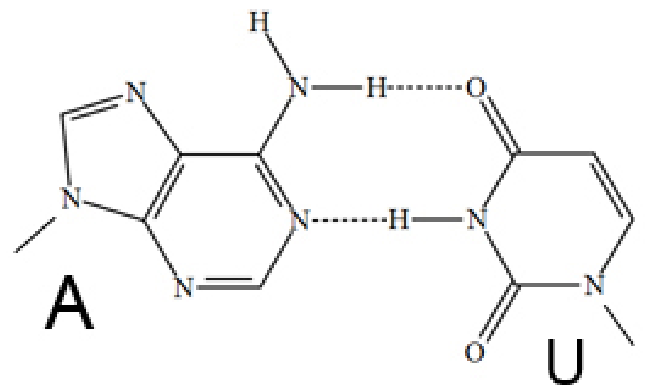

a
A15
A15
G25
G25
U6
U6
C27
C27
5'
5'
3'
3'


a
A10
A10
C21
C21
G25
G25
U6
U6
A5
A5
G29
G29
C27
C27

A (relatively) simple way to determine if the theo was bound to the RNA was to look at the imino proton spectrum.

Review of a base pair: find the imino proton.





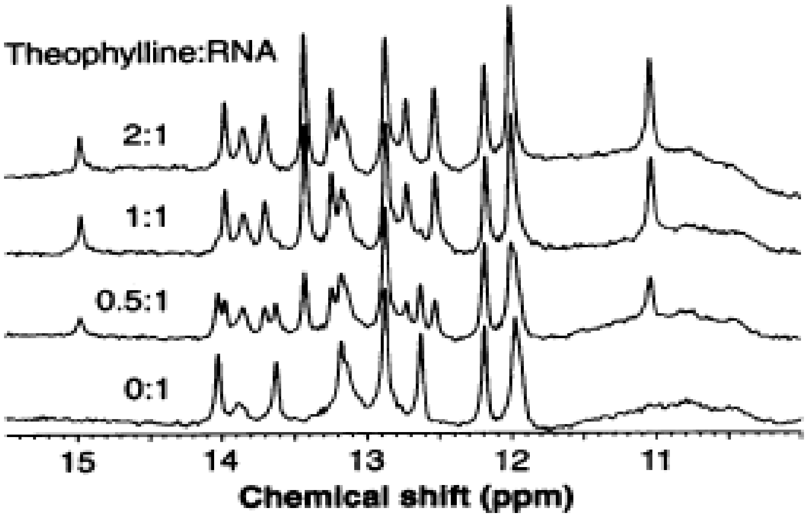


Fig. 4. Imino proton NMR spectra of 65 μM mTCT8-4 RNA in a mixture of H_2O and D_2O (9:1), 15 mM potassium phosphate, 10 mM NaCl, and 5 mM $MgCl_2$ (pH 6.8) at 20°C. Spectra were acquired at the indicated theophylline to RNA molar ratios. Each spectrum

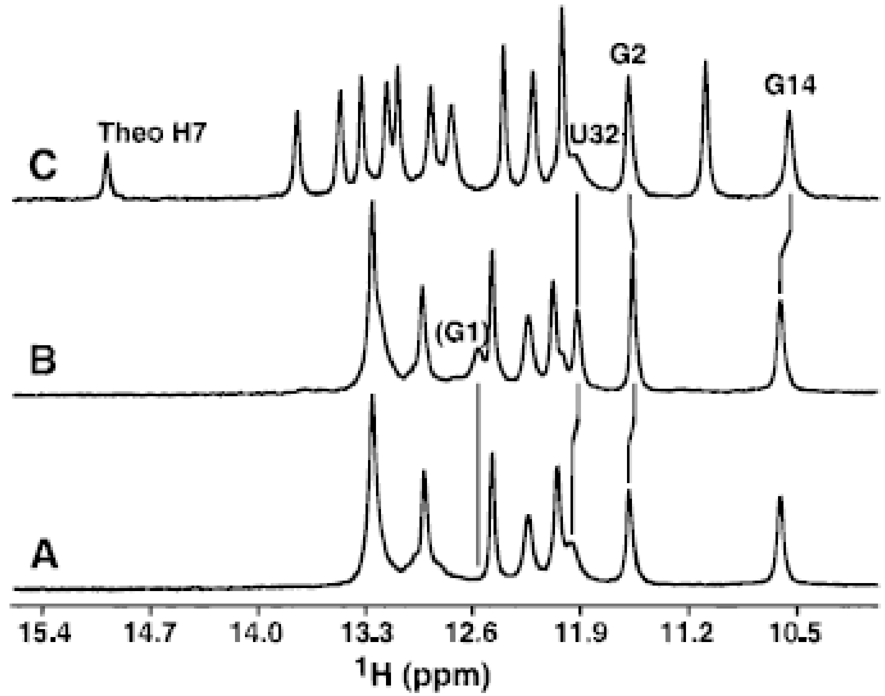


FIGURE 4. One-dimensional ^{1}H-NMR spectra of the theophylline-binding RNA (Δ-33) at 25 °C showing the effect of various ligands on the imino proton region of the spectra: **A**: no ligand; **B**: 10 equivalents of caffeine; C: one equivalent of theophylline.

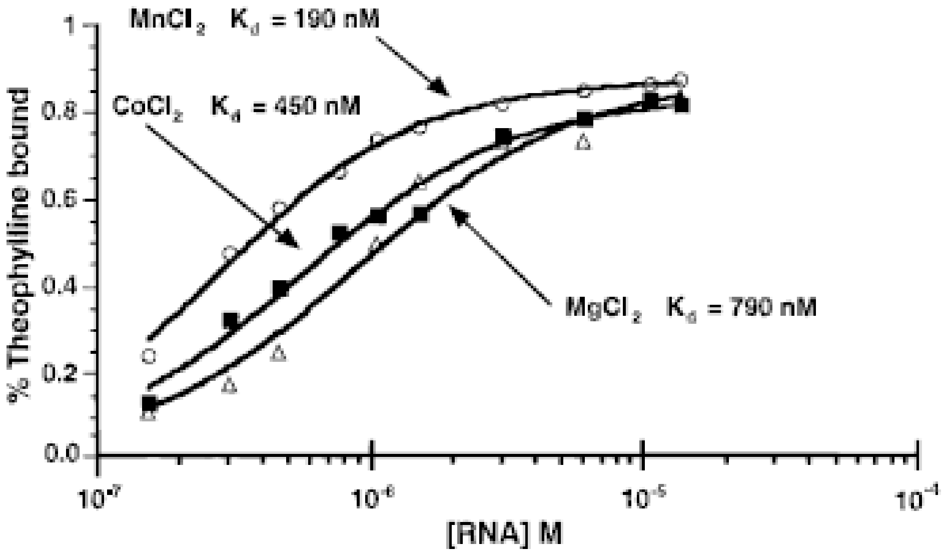


FIGURE 2. Binding curves for the RNA–theophylline complex in the presence of different divalent metals at pH 6.8, 25°C (see text). Binding data for the Δ-33 RNA (Fig. 1B) in 5 mM Mg^{2+}, Mn^{2+}, or Co^{2+} are shown as the open triangles, open circles, and filled squares, respectively. The solid lines represent the best fit to a 1:1 complex, and the calculated K_ds for theophylline binding in the presence of the different metal ions are given. Note that theophylline binding for this RNA is pH dependent with weaker binding at pH < 7 (Zimmermann et al., 1998); thus the K_d values in this figure differ slightly from those in Table 1 because of the differences in pH.

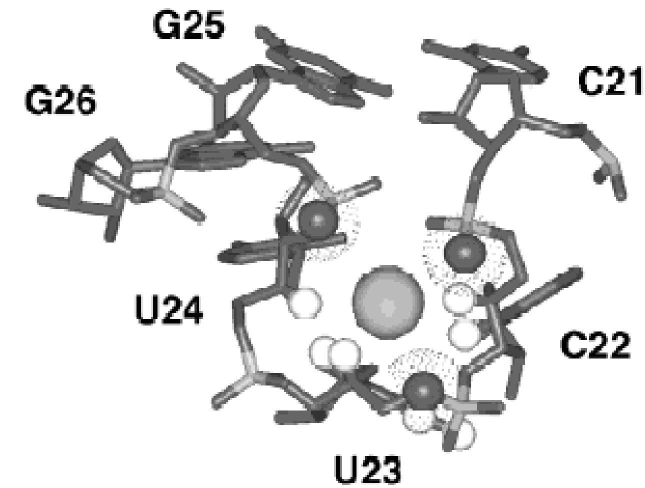
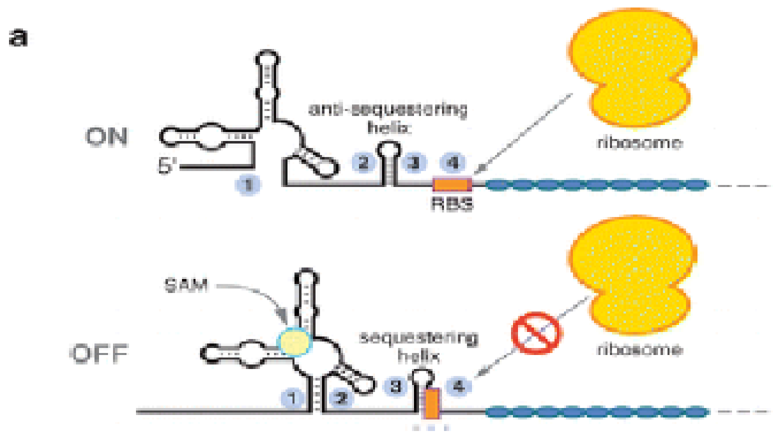


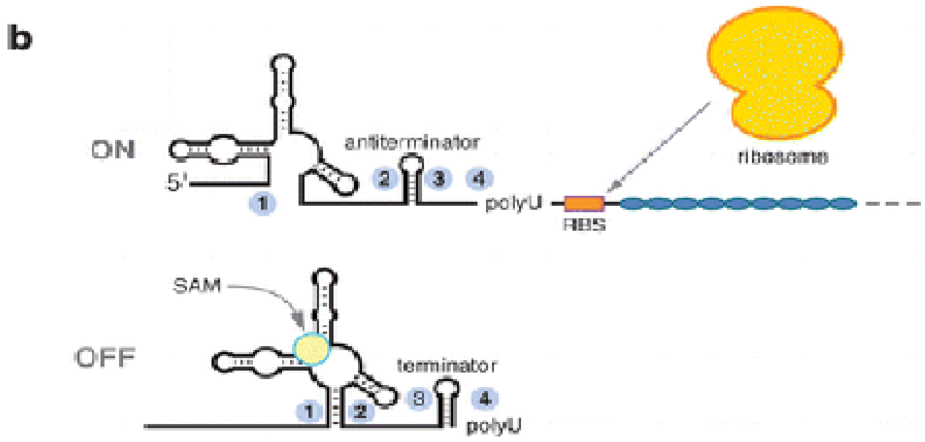
FIGURE 6. A model of the metal-binding site in the previously determined structure of the RNA–theophylline complex (Zimmermann et al., 1997). The gray sphere represents the divalent metal ion, and the white spheres indicate RNA proton resonances that are specifically broadened in the presence of 40 μM Mn^{2+} (Fig. 3). The van der Waals surface of phosphate oxygens that may coordinate the metal are stippled. The placement of the metal ion is not uniquely defined by the NMR data, and thus this picture shows one model consistent with the NMR data in the Mn^{2+}-titration (see text).

RIBOSWITCH.

What is it?
Ron Breaker (Yale) coined the name to describe the properties of an RNA sequence/structure that controlled transcription or translation through binding to a ligand and subsequently altering its conformation.



As a mechanism to regulate translation initiation, in the ON conformation, the riboswitch structure allows access to the Ribosome Binding Site (RBS). In the OFF conformation, when the metabolite (here, SAM) is bound, the RBS is part of a hairpin and not accessible to the ribosome. (Winkler & Breaker, 2005. AnnRevMicro 59:487-517).



As a transcriptional regulator, when the riboswitch is in the ON conformation, the mRNA is transcribed. In the OFF conformation, an alternative stemloop forms and with the downstream (U)n tract, forms an intrinsic (rho-independent) transcriptional terminator.

The conformational change from ON to OFF is triggered by binding of a small molecule. Typically, it's the metabolite regulated by the operon.

The Riboswitch is functionally separated into the ligand binding APTAMER and the decision-making EXPRESSION PLATFORM

Biological ligands of the known natural riboswitch classes

A

RNA-derived compounds

Coenzymes
Adenosylcobalamin
Aquacobalamin
Thiamin pyrophosphate
Flavin mononucleotide
S-Adenosylmethionine
Molybdenum cofactor
Tungsten cofactor
Tetrahydrofolate
S-Adenosylhomocysteine

Nucleotide derivatives
Guanine
Adenine
Prequeuosine$_1$
2'-Deoxyguanosine

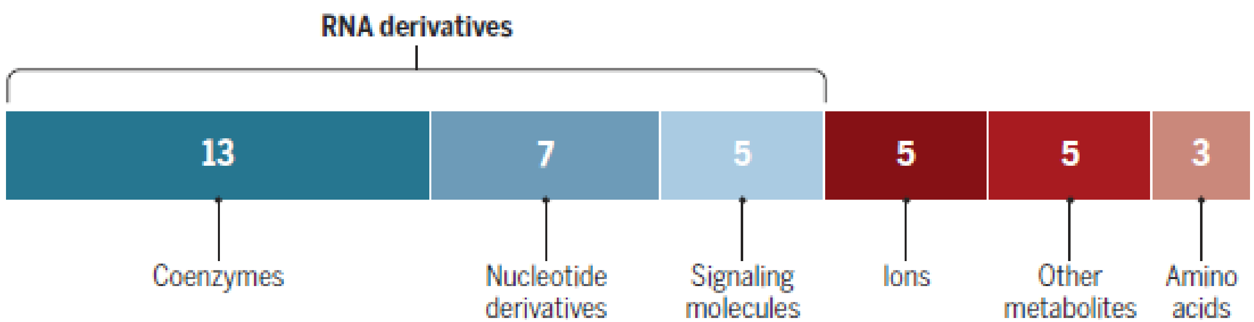
Signaling molecules
Cyclic di-GMP
Cyclic di-AMP
Cyclic AMP-GMP
ZTP

Ions
Mg^{2+}
Mn^{2+}
F^{-}
Ni^{2+}/Co^{2+}

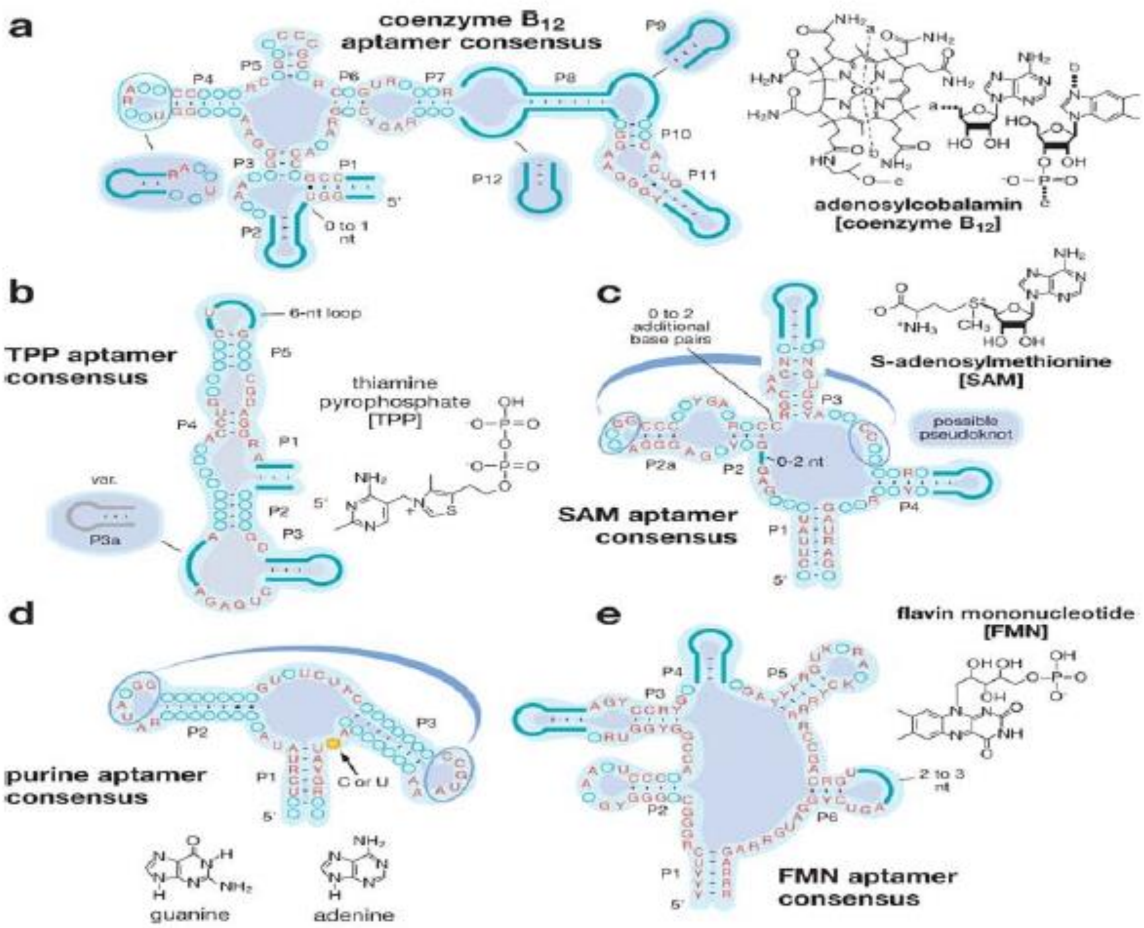
Amino acids
Lysine
Glycine
Glutamine

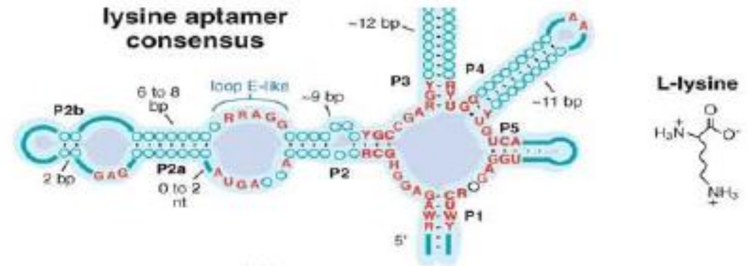
Other metabolites
Glucosamine-6-phosphate
Azaaromatics
Guanidine

B

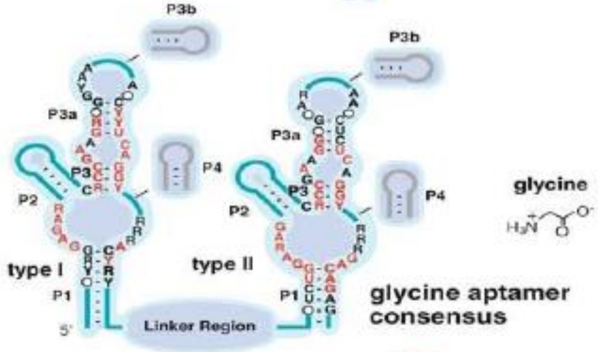


Nelson JW, Breaker RR. The lost language of the RNA World. Sci Signal. 2017. 10(483):eaam8812.

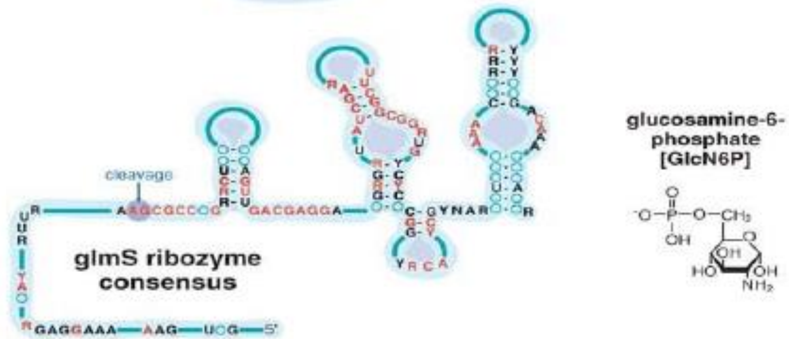

a
coenzyme B12
aptamer consensus
adenosylcobalamin
[coenzyme B12]
b
TPP aptamer
consensus
6-nt loop
thiamine
pyrophosphate
[TPP]
c
0 to 2
additional
base pairs
S-adenosylmethionine
[SAM]
possible
pseudoknot
SAM aptamer
consensus
d
purine aptamer
consensus
C or U
guanine
adenine
e
flavin mononucleotide
[FMN]
2 to 3
nt
FMN aptamer
consensus



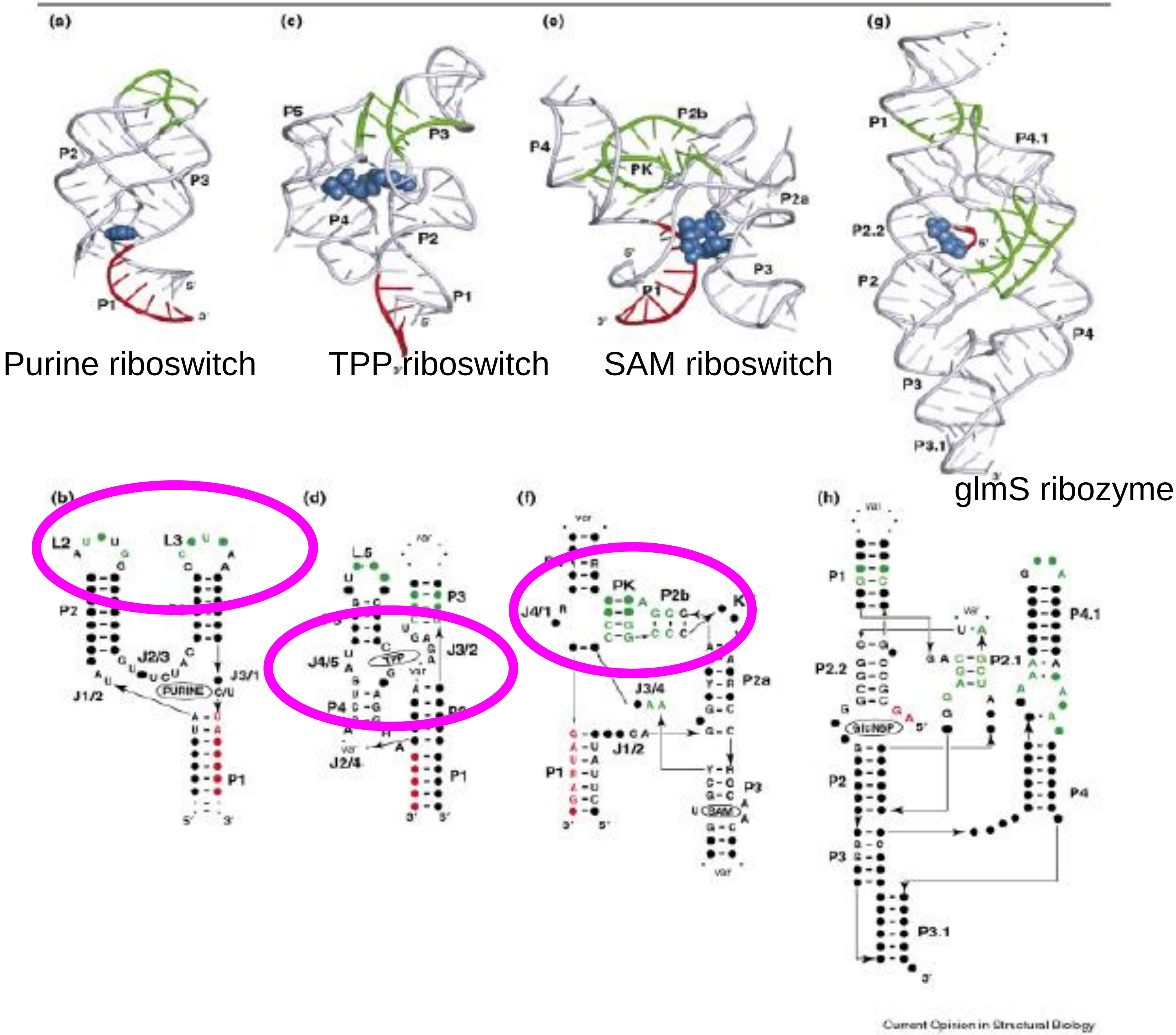
g



h



Winkler WC, Breaker RR. 2005.
Annu. Rev. Microbiol. 59:487–517

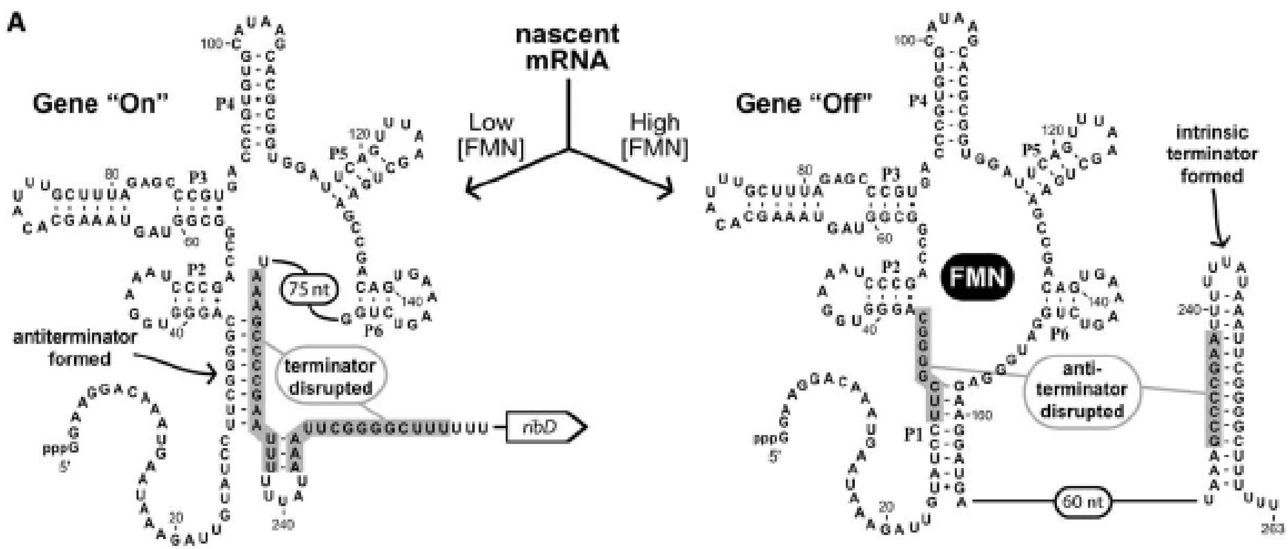


(a)
(c)
(e)
(g)
P2
P3
P1
5'
3'
Purine riboswitch
P5
P3
P4
P2
P1
TPP riboswitch
P2b
P4
PK
P2a
P3
P1
5'
3'
SAM riboswitch
P1
P4.1
P2.2
P2
P4
P3
P3.1
glmS ribozyme
(b)
L2
L3
P2
J2/3
J3/1
J1/2
PURINE
P1
5'
3'
(d)
L5
P3
J4/5
TPP
J3/2
P4
J2/4
P1
(f)
PK
P2b
J4/1
J3/4
P2a
J1/2
P1
P3
SAM
(h)
P1
P4.1
P2.2
P2.1
GlcN6P
P2
P4
P3
P3.1
Current Opinion in Structural Biology

An example of aptamer binding a ligand

An mRNA structure that controls gene expression by binding FMN.

Winkler, Cohen-Chalamish, Breaker. 2002. PNAS 99:15908-15913



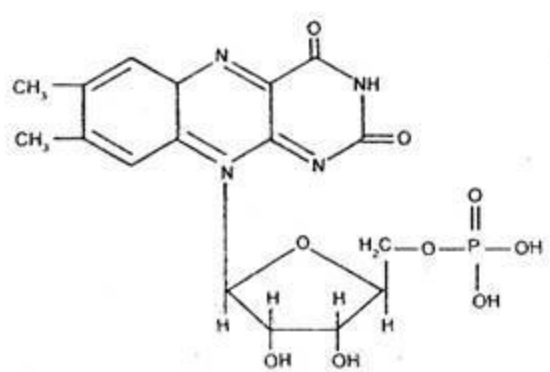
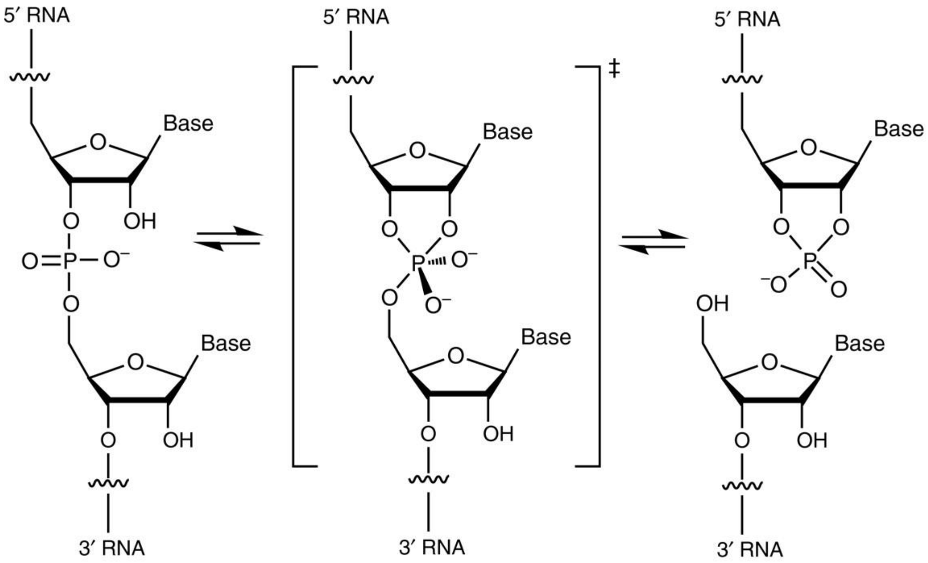


Fig. 1.37 Structure of Flavin mononucleotide (FMN).

How did the Breaker lab show that an RNA could specifically bind a ligand?

In-line probing

The principle: spontaneous cleavage of the phosphodiester backbone in the presence of Mg^{+2}.



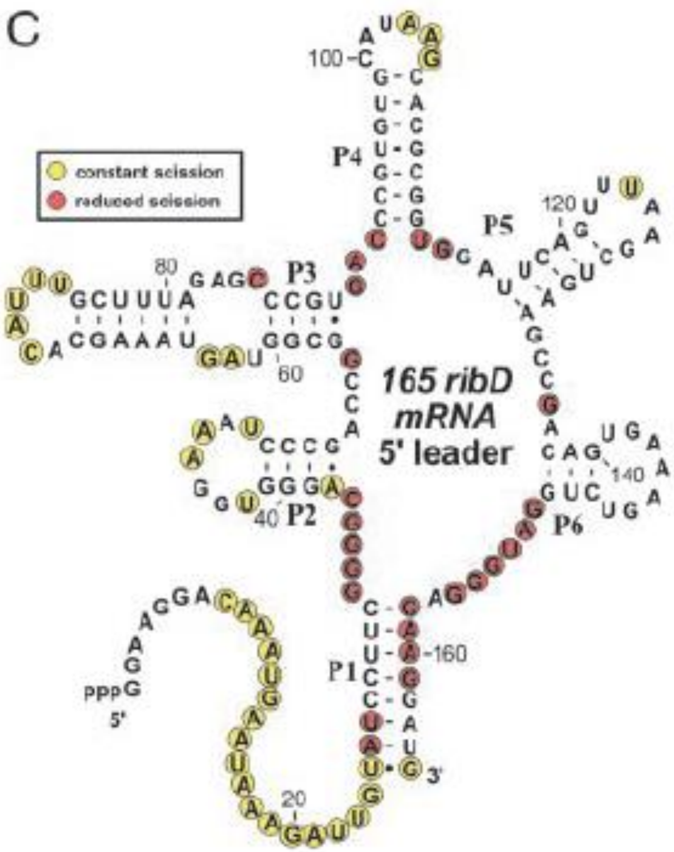
These are internal transesterification reactions that involve the 2' OH and Mg^{2+}.

This reaction requires a specific geometry (SN_2-inline displacement reaction) that does not correspond to typical conformations in structured regions of an RNA (especially duplex regions).
However, flexible regions of the RNA can access this geometry, at least transiently with some probability.
To enhance the efficiency of the transesterification reaction and thus backbone cleavage, the reactions contain 20 mM Mg^{2+} and are run at room temperature for 40 hours.

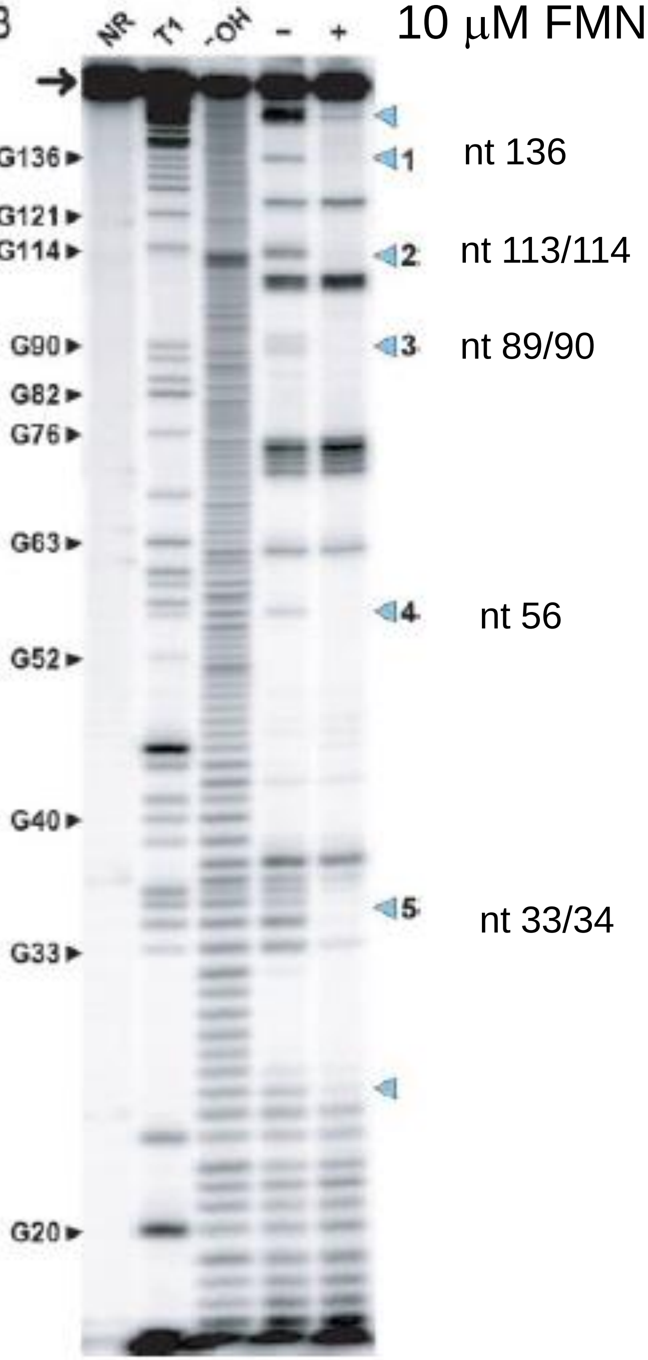
Why room temperature for 40 hours?

≈1 nM 5′ ^{32}P-labeled RNA was incubated for ≈40 h at 25°C in 20 mM $MgCl_2$/50 mM Tris·HCl (pH 8.3 at 25°C)/100 mM KCl in the presence or absence of added ligand (FMN, FAD, or riboflavin) at concentrations that are indicated for each experiment.

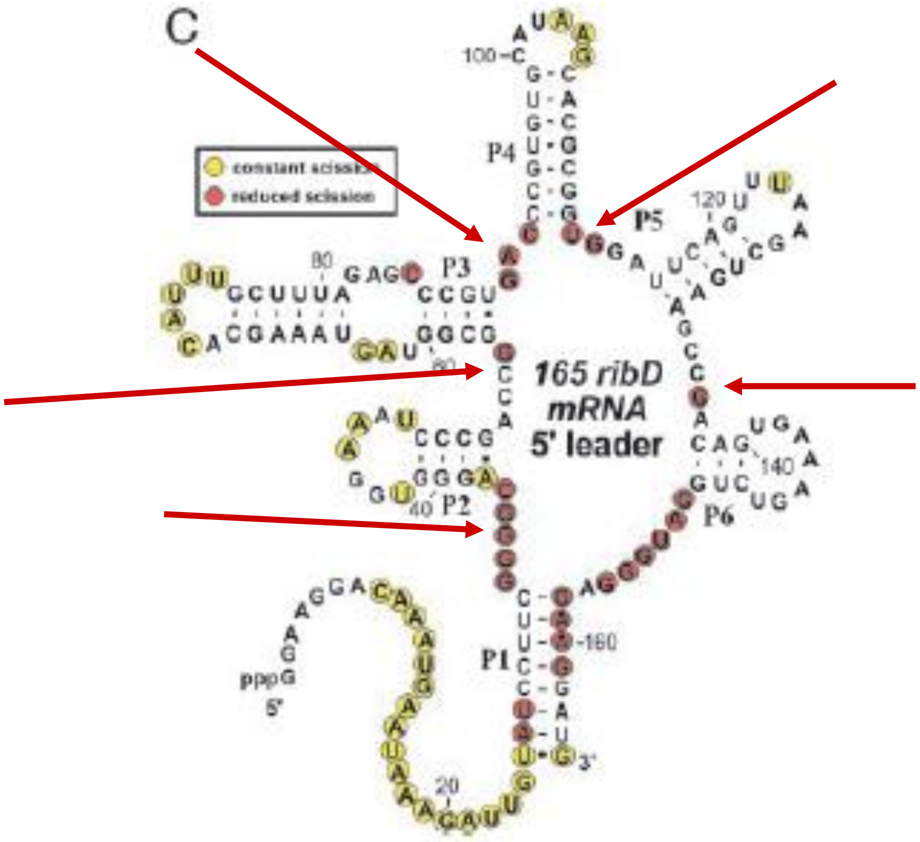
C

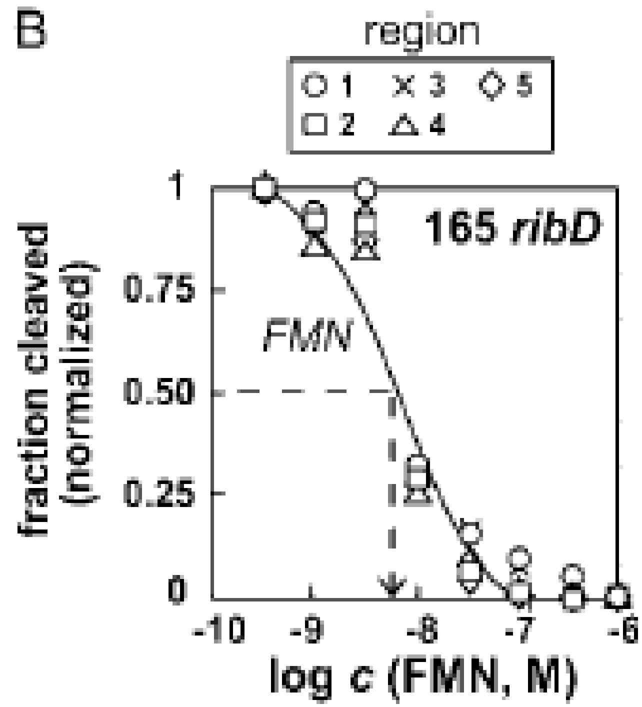


B



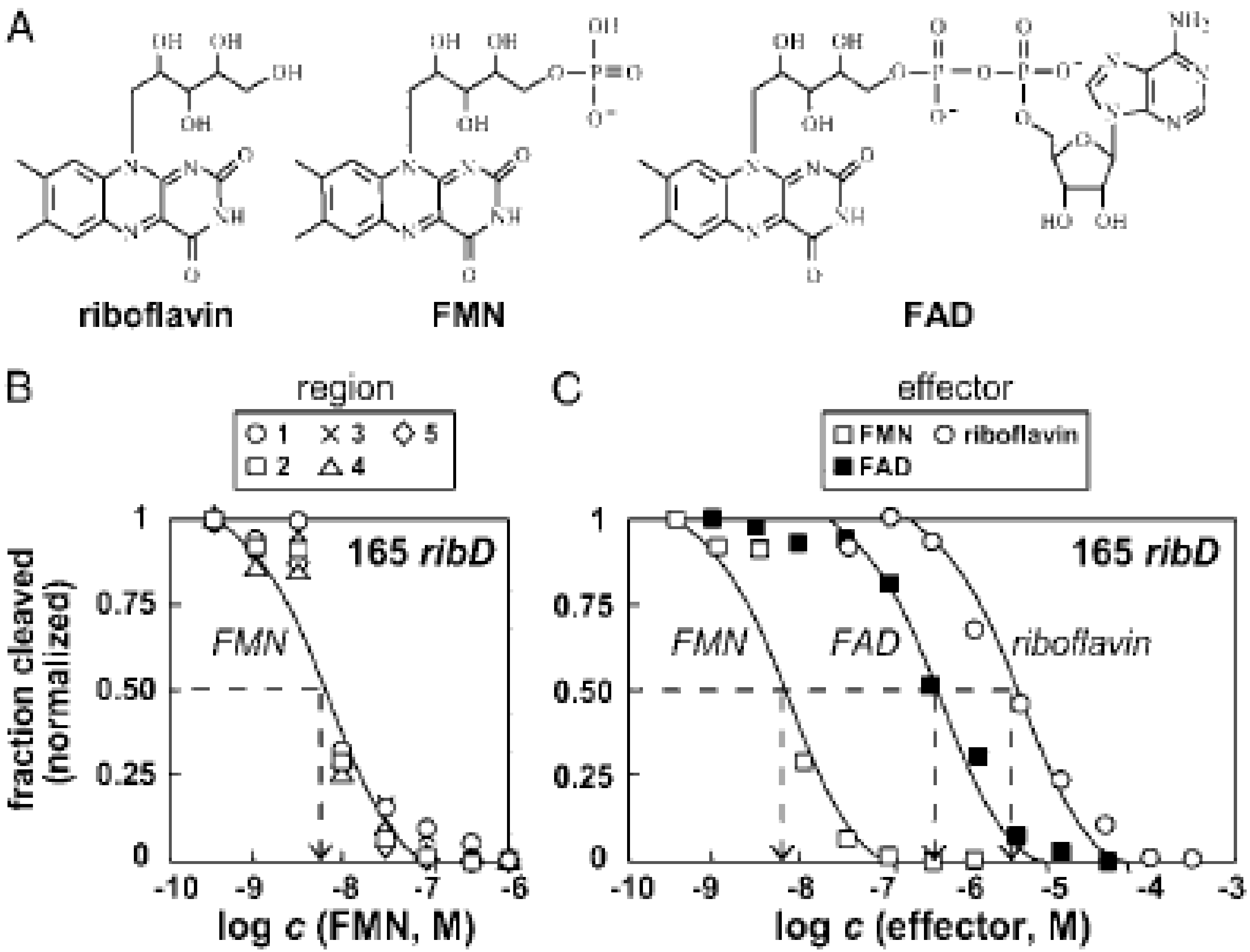
Nucleotides that become less reactive when FMN is bound.





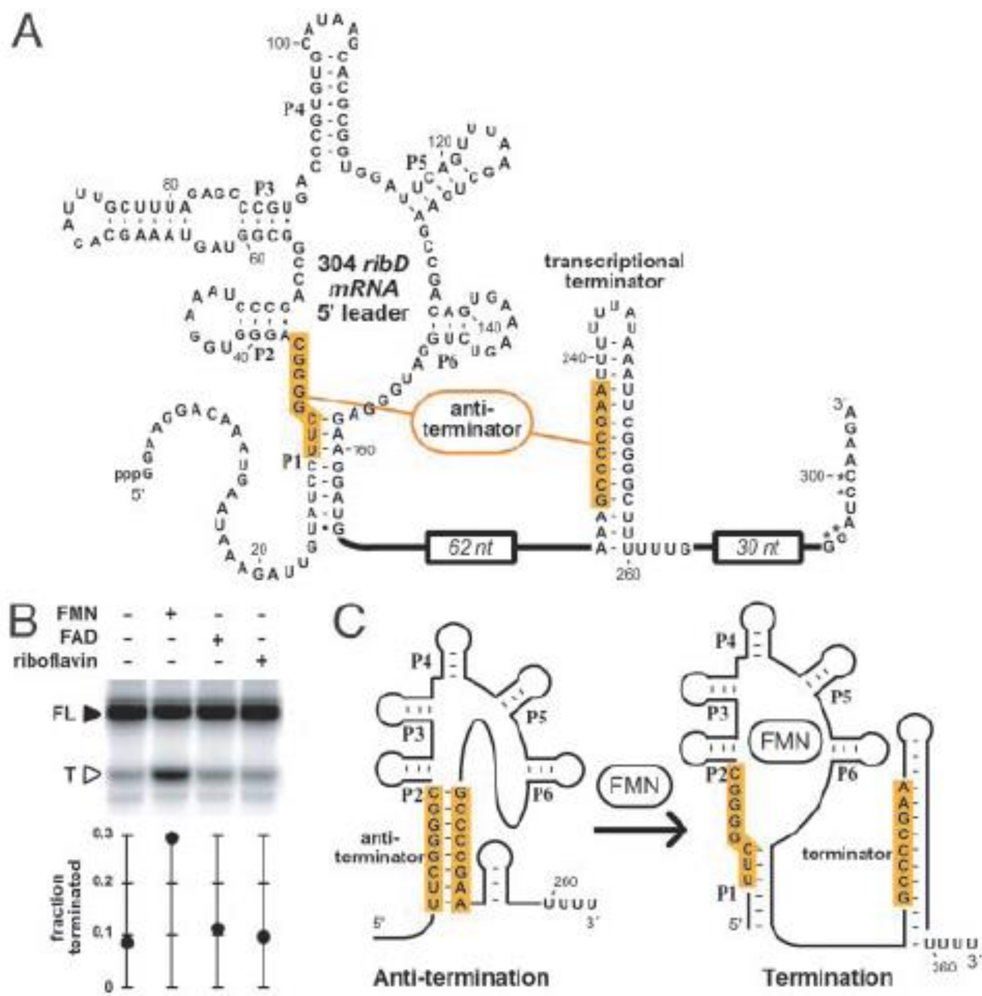
All regions show the same FMN concentration-dependence. Therefore, the conformational change is global and cooperative.

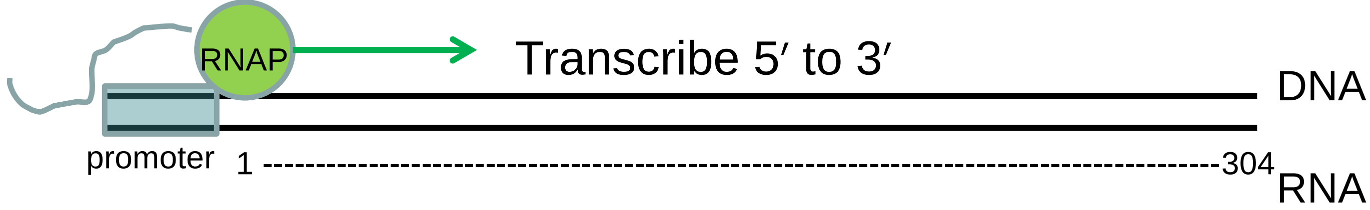
Binding is specific to FMN.

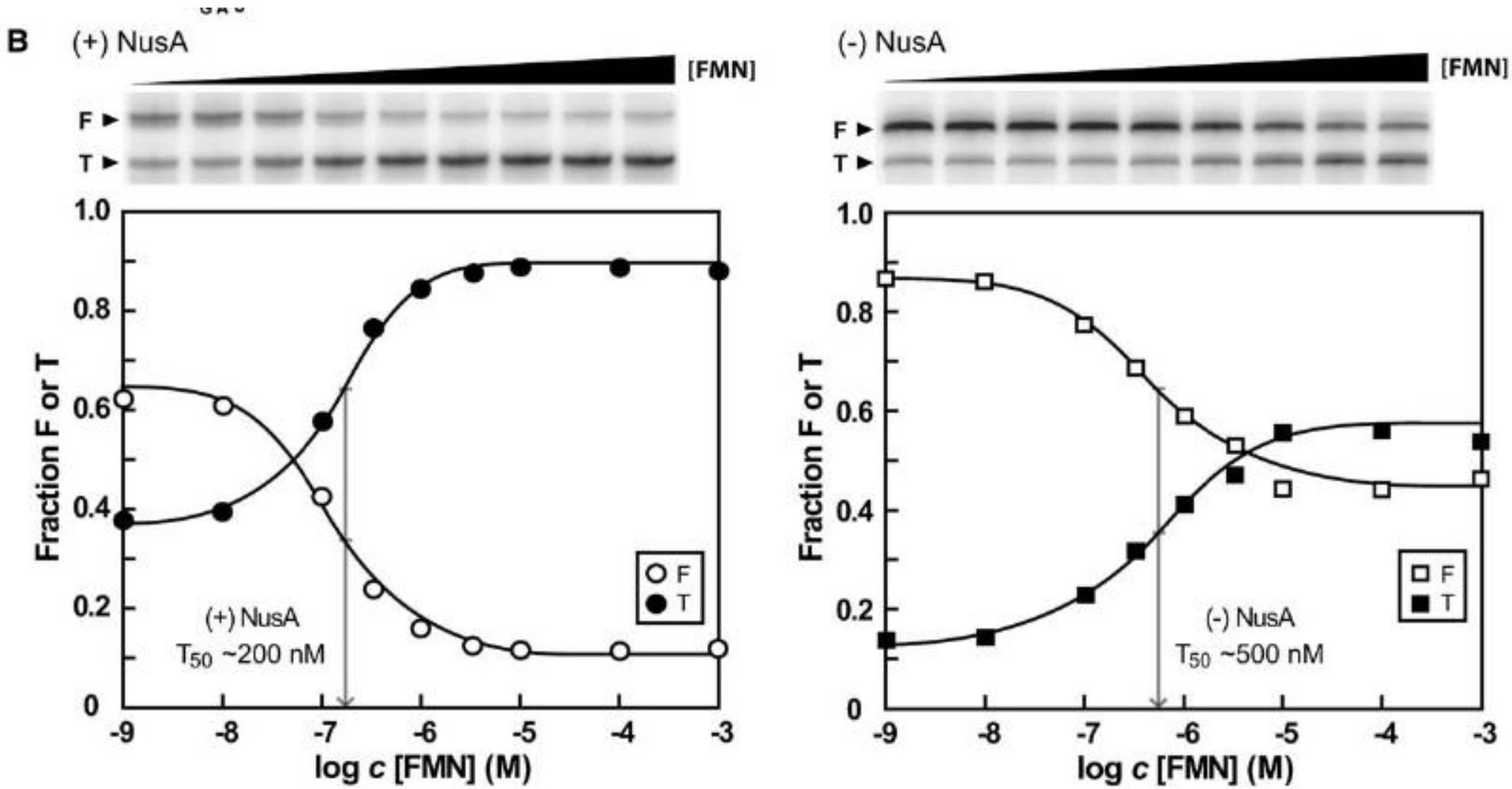


The Speed of RNA Transcription and Metabolite Binding Kinetics Operate an FMN Riboswitch

Wickiser, Winkler, Breaker, Crothers. 2005. Mol Cell. 18:490-60.



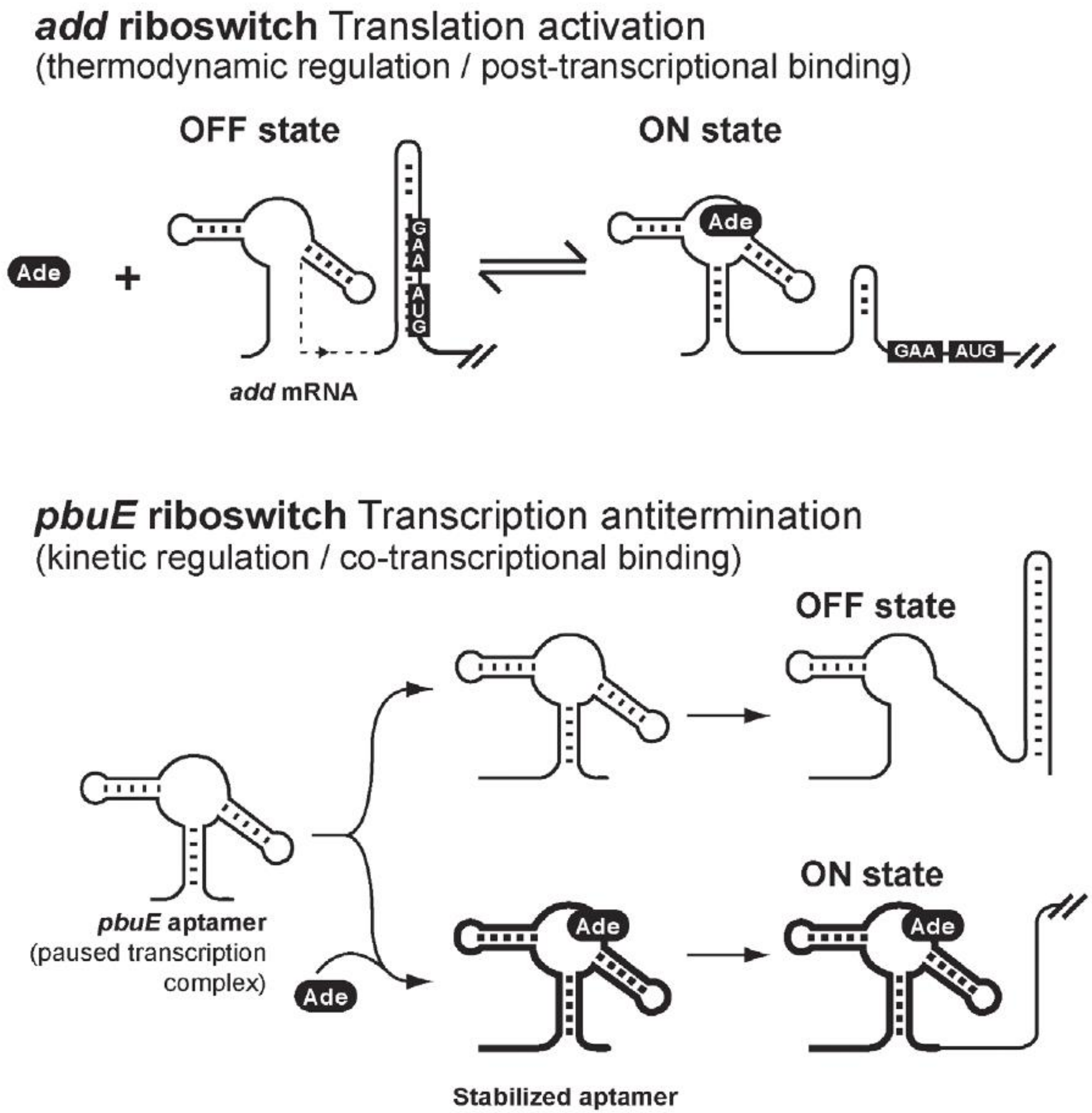




NusA is a protein that binds to bacterial RNAP and slows transcription.

This riboswitch is not at thermodynamic equilibrium at the time the choice is made to transcribe or terminate. Therefore, this decision is kinetically driven.

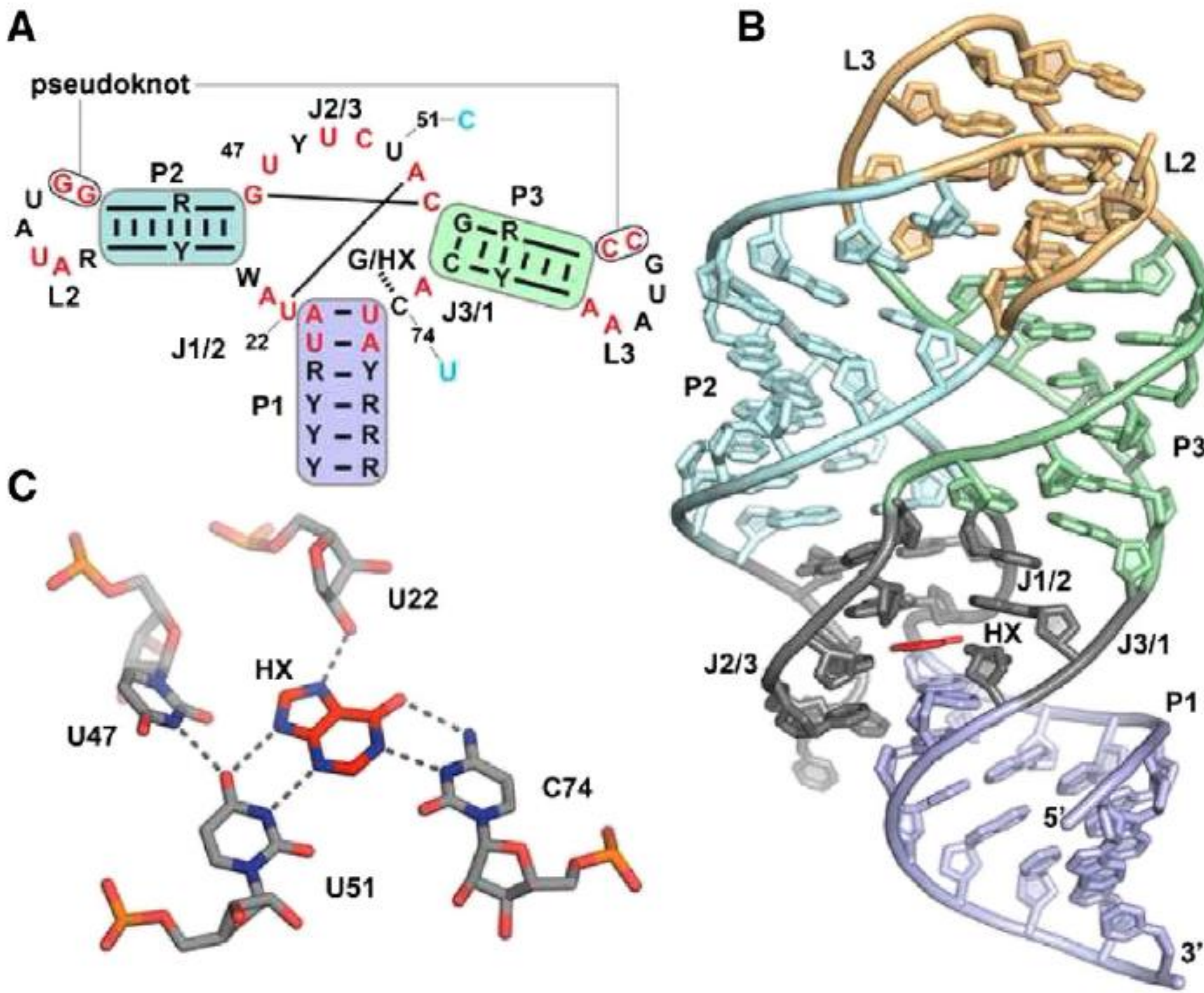
The **Purine riboswitch** operates in two modes.



Shine-Delgarno (GAA) and AUG start are paired in a stem. Adenine binding shifts the equilibrium.

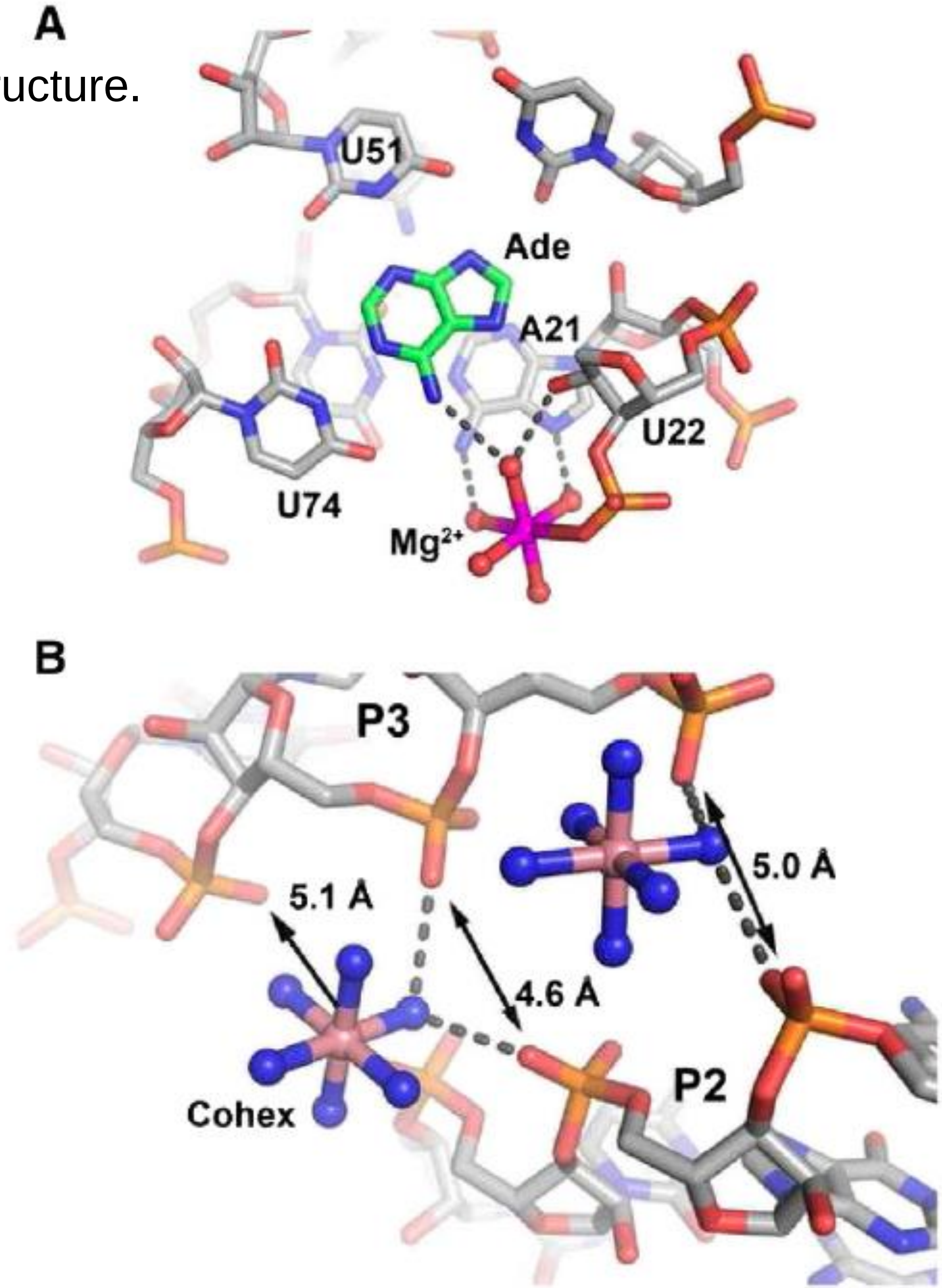
Low Adenine concentration leads to OFF state. High concentration can co-transcriptionally bind leading to the ON state.

The purine riboswitch



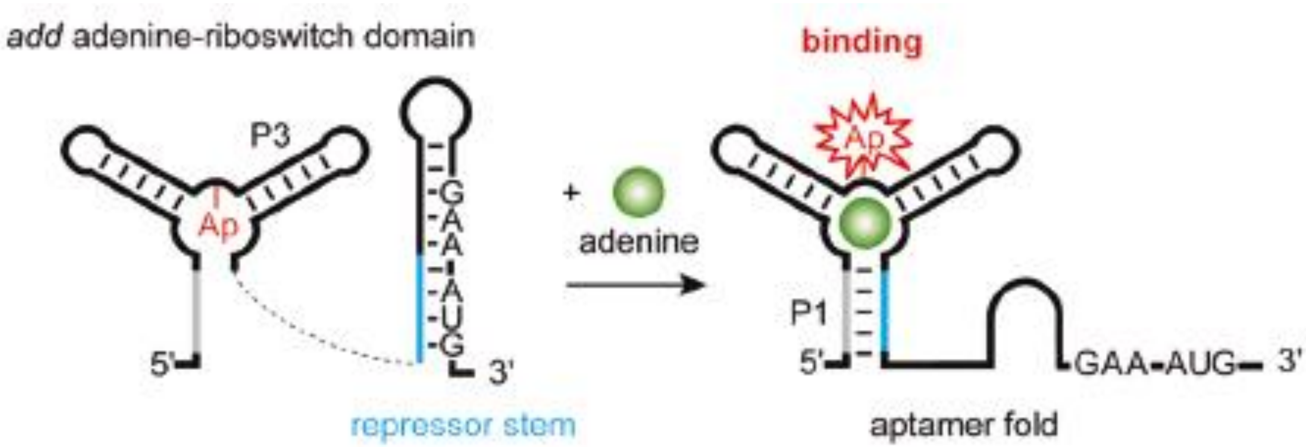
Porter et al., 2014. Biochem Biophys Acta 1839:919-930.

Ions in the crystal structure.
What do they do?



A spectroscopic method to study the ligand binding process

Examine adenine binding by the purine riboswitch. Replace a single nucleotide with the fluorescent nucleotide 2-aminopurine (Ap). When the riboswitch undergoes a conformational change, Ap fluorescence could increase or decrease (or not change), reporting on the timescale of the binding and also the folding pathway.



Haller, A, Souliere MF, Micura R (2011) The Dynamic Nature of RNA as Key to understanding riboswitch mechanisms. Accts Chem Res. 44:1339-1348.

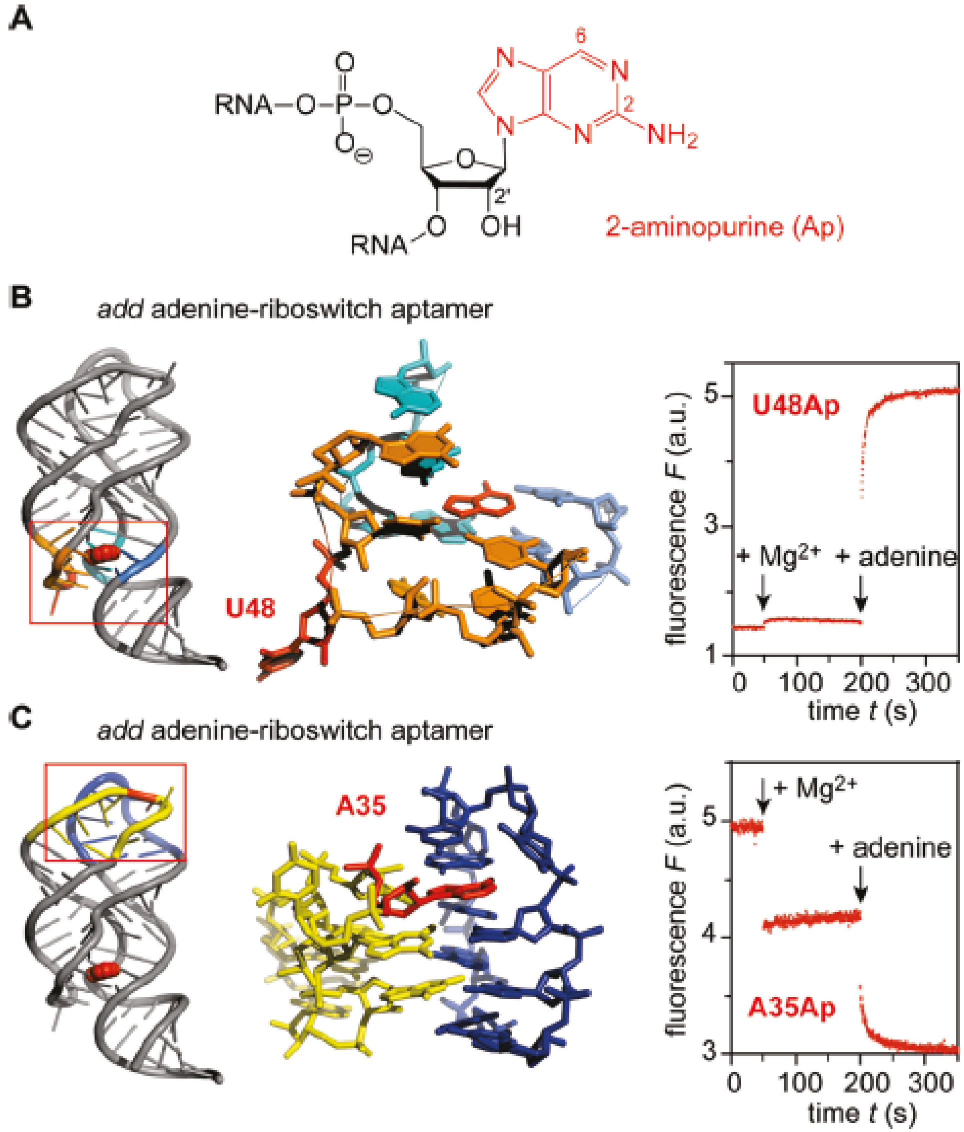


FIGURE 1. 2-Aminopurine (2Ap) labeling to study Mg^{2+}- and ligand-induced RNA folding (2ApFold approach). (A) Chemical structure of 2Ap. (B,C) Structure-based selection of 2Ap nucleoside replacement exemplified for U48 (B) and A35 (C) of the adenine riboswitch, and corresponding fluorescence response.

What do you think you would observe if adenine were added first, then Mg^{2+}?

The lock and key paradigm of substrate binding clearly fails for these riboswitches.

Instead, there is a coupled binding/folding that needs a new formalism to describe it. There are two popular models that you'll find in the literature. Induced Fit, and Conformational Selection. Are they mutually exclusive mechanisms, or are they the same mechanism with different names, or can both be present in the same system?

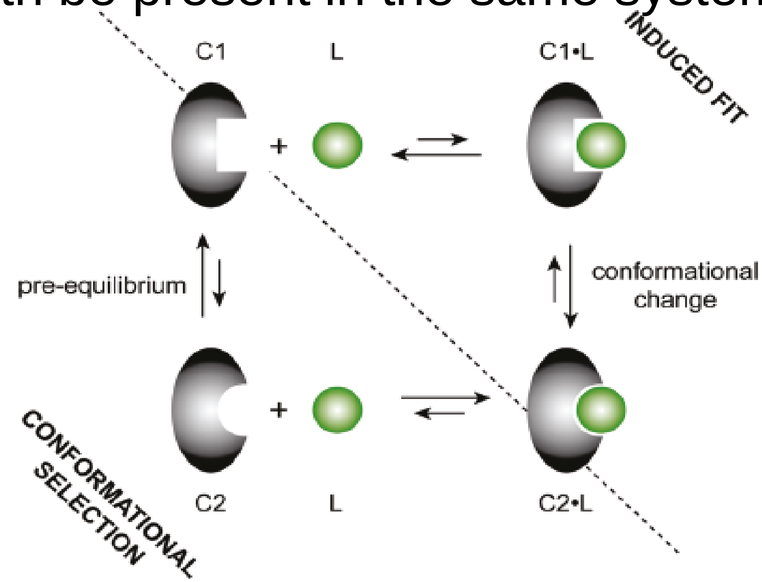


FIGURE 5. Two models for molecular recognition: induced fit and conformational selection.[63] In conformational selection, the binding-competent conformation (C2) is pre-existing in solution before the addition of ligand (L). In induced fit, initial binding contacts (C1•L) between ligand and receptor induce conformational rearrangements to achieve the conformation C2•L of the complex.

Vogt & Di Cera . Biochemistry. 2012 51(30):5894-902.