Module Overview

Day	Lecture	Lab
1	Introduction	DNA library synthesis (PCR)
2	SELEX I: Building a Library	DNA library purification (agarose gel electrophoresis)
3	SELEX II: Selecting RNA with target functionality	RNA library synthesis (*In vitro* transcription = IVT)
4	SELEX III: Technical advances & problem-solving	RNA purification and heme affinity selection
5	Characterizing aptamers	RNA to DNA by RT-PCR
6	Introduction to porphyrins: chemistry & biology	Post-selection IVT Journal Club 1
7	Aptamer applications in biology & technology	Aptamer binding assay
8	Aptamers as therapeutics	Journal Club 2

Aptamer Structure Characterization

20.109 Lecture 5

17 February, 2011

Today's objectives

- Aptamer characterization
  - Structure (what do we want to know and how do we analyze?)
    - Primary
    - Secondary
    - Tertiary
  - Examine some methods for characterizing aptamer (RNA) structure
    - DNA sequencing
    - RNA footprinting
    - High resolution structural methods

Aptamer primary structure

Definition:

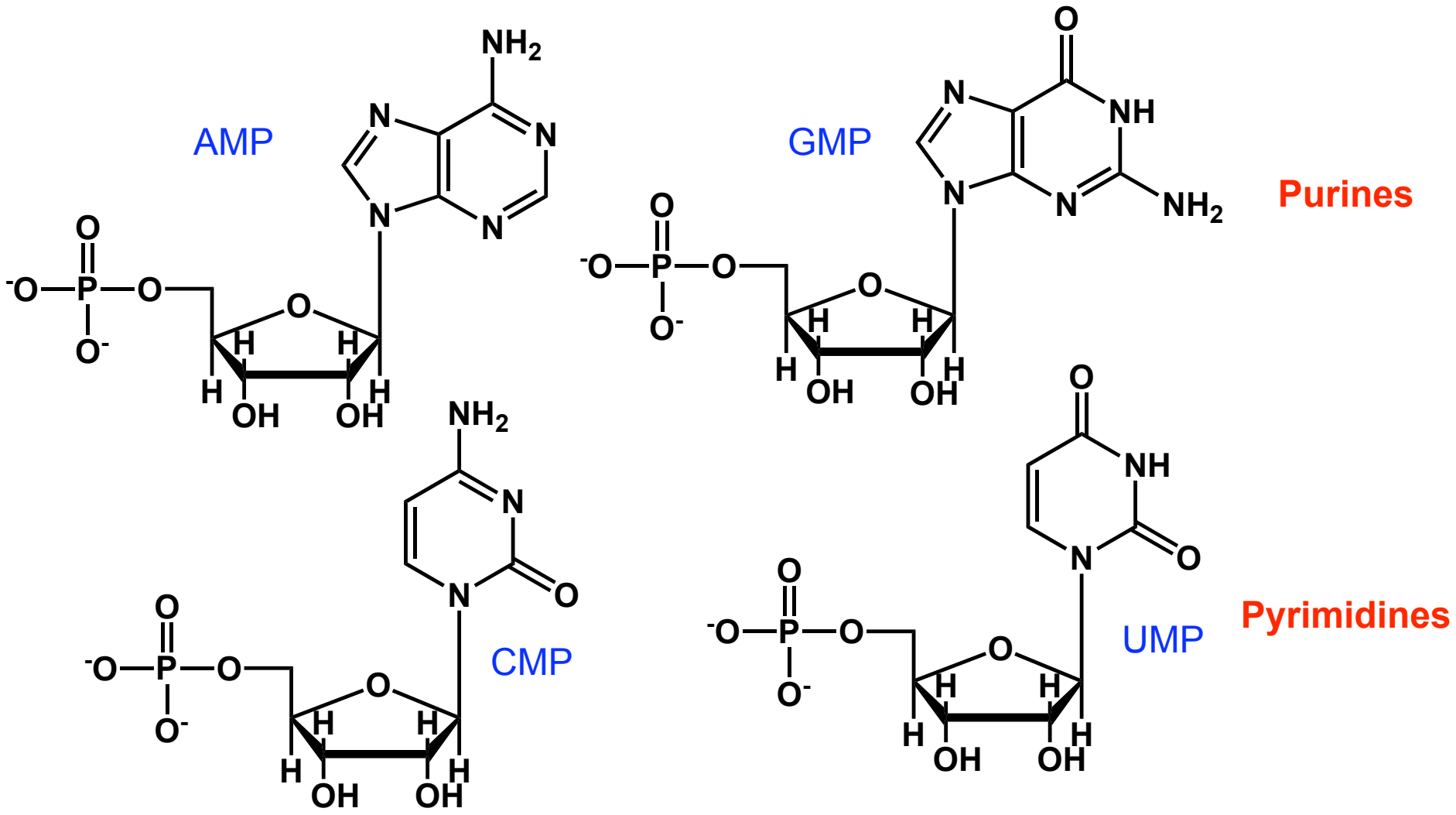
- Sequence of nucleotide building blocks making up the aptamer

- Four nucleotide building blocks: G, A, C, U

  – Can you identify them by structure?

Aptamer primary structure

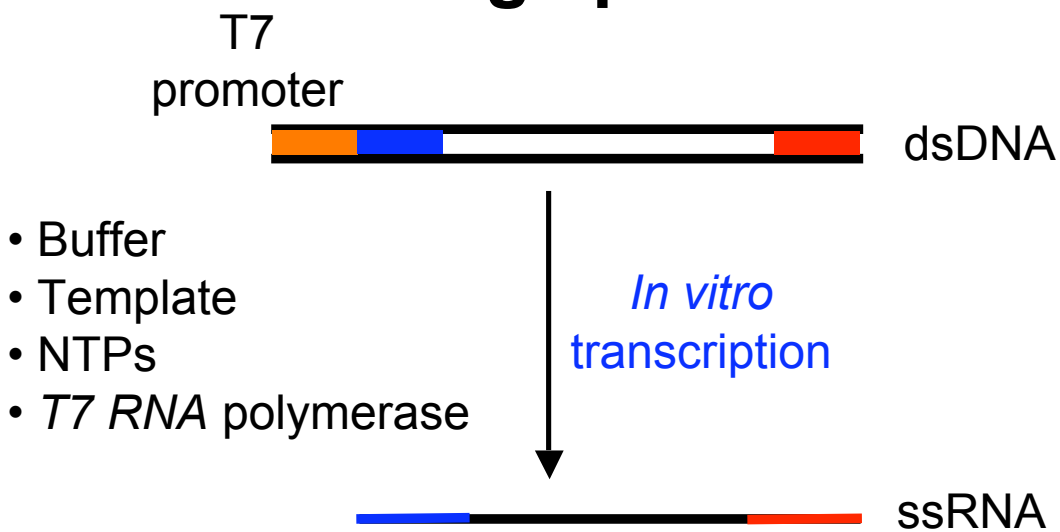
- **The nucleotide building blocks**



Aptamer sequencing

- How do we determine the sequence of an isolated aptamer?
  - *Directly sequence RNA*
    - Possible
    - More difficult than sequencing DNA
    - Less robust than sequencing DNA
  - *Sequence the DNA encoding the RNA*
    - Routine
    - Use simple rules to convert DNA into RNA sequence

Converting aptamer DNA into RNA sequence



T7 promoter

TAATACGACTCACTATAGGGTACTT…

DNA sense strand

transcribe

GGGUACUU…

(ssRNA)

Rules

- RNA begins at the G residue immediately downstream of the T7 promoter
- RNA has identical sequence to the DNA *sense* strand
- RNA contains uridine in place of thymidine

How do you sequence DNA?

- **Sanger method is used most routinely**
  - Uses primer extension/PCR
  - Induced stochastic termination during chain extension
    - Generate fragments of various lengths
    - Each fragment terminates in base encoded at that position
  - High resolution method required to resolve these fragments
    - Require single base resolution
  - Must be able to uniquely identify the base terminating a given fragment

http://www.mwit.ac.th/~deardean/link/All%20Course/pic/secuencia.swf

Analyzing primary structure (sequence) data

- *What are we trying to learn?*
  - The identity of selected aptamers
  - The frequency at which any given aptamer occurs
    - Reflects degree of convergence relative to original library
  - Insights into conserved sequence elements that may be related to function
    - Direct binding?
    - Required structural feature, but no direct binding?
  - Generate hypotheses for further testing

Aptamer RNA secondary structure

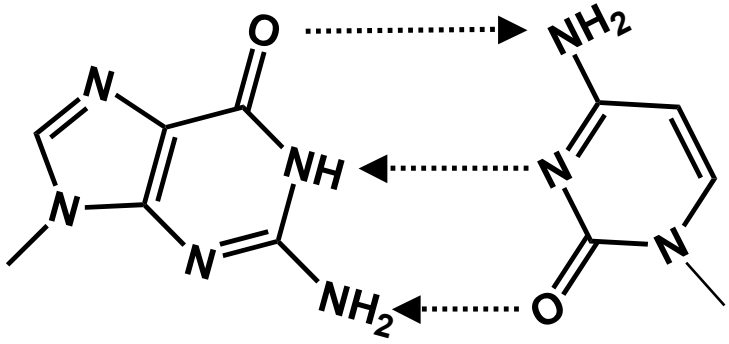
Definition:

- The base pairing interactions occurring within an RNA molecule

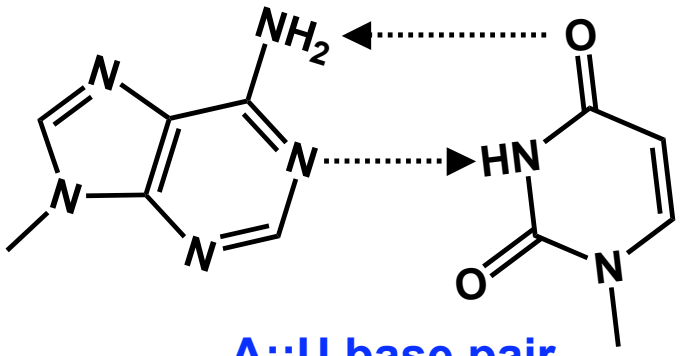
  - *What are the possible base pairing interactions contributing to RNA secondary structure?*

Aptamer RNA secondary structure

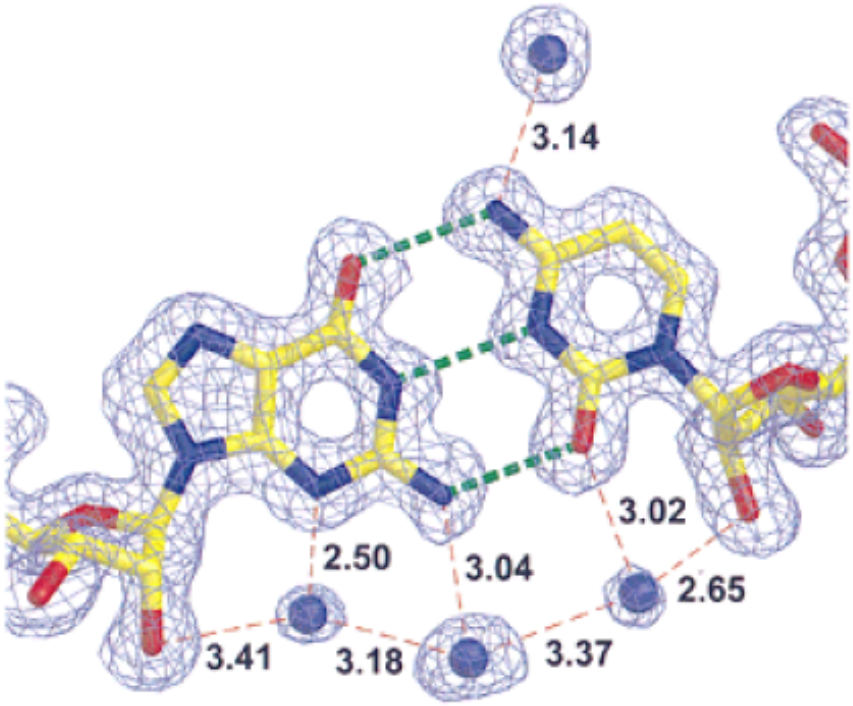
RNA base pairs contributing to its secondary structure



G:::C base pair



A::U base pair

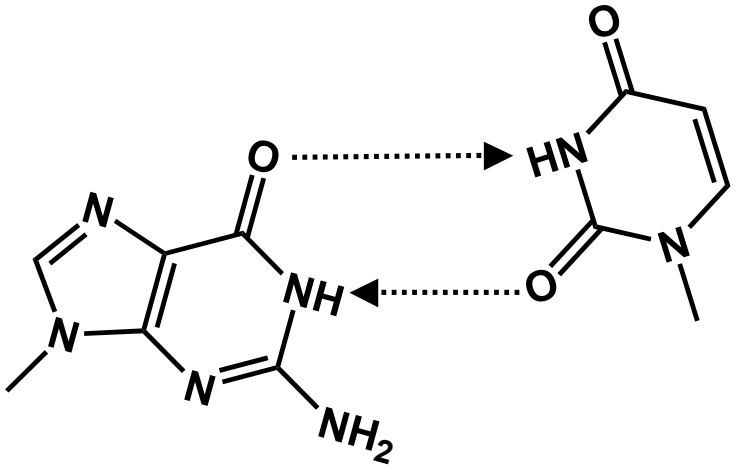


Watson-Crick base pairs

Mueller *et al*, *RNA*, 5:670-677 (1999)

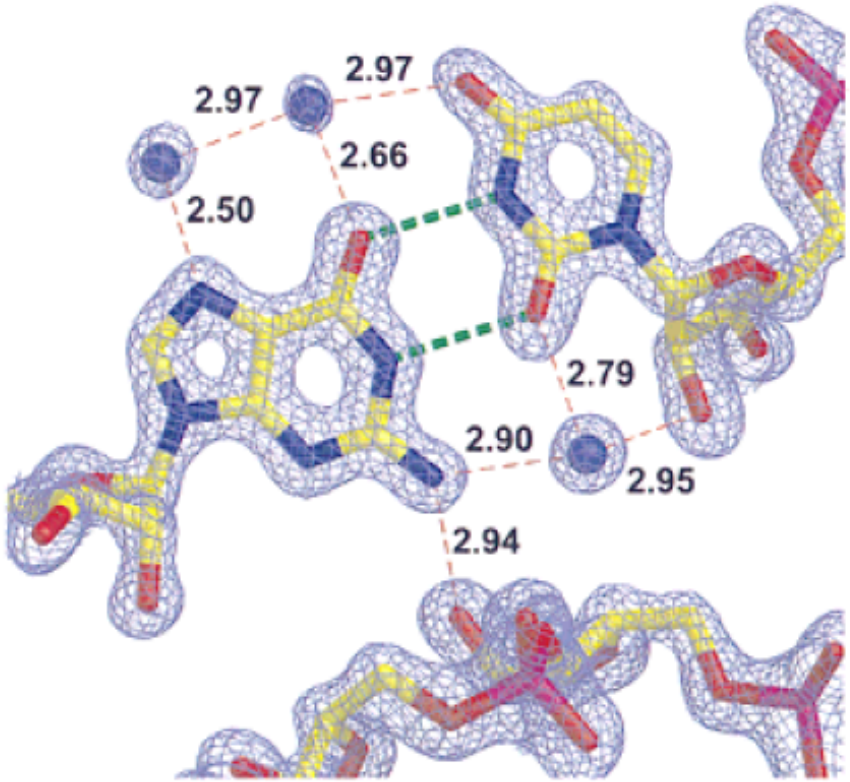
Aptamer RNA secondary structure

RNA base pairs contributing to its secondary structure



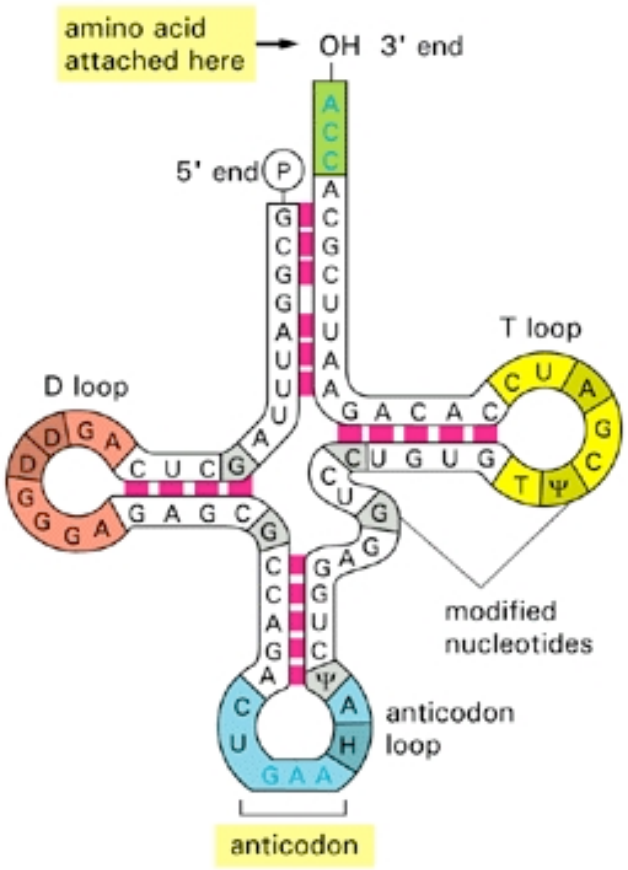
G::U base pair

“Wobble” base pair

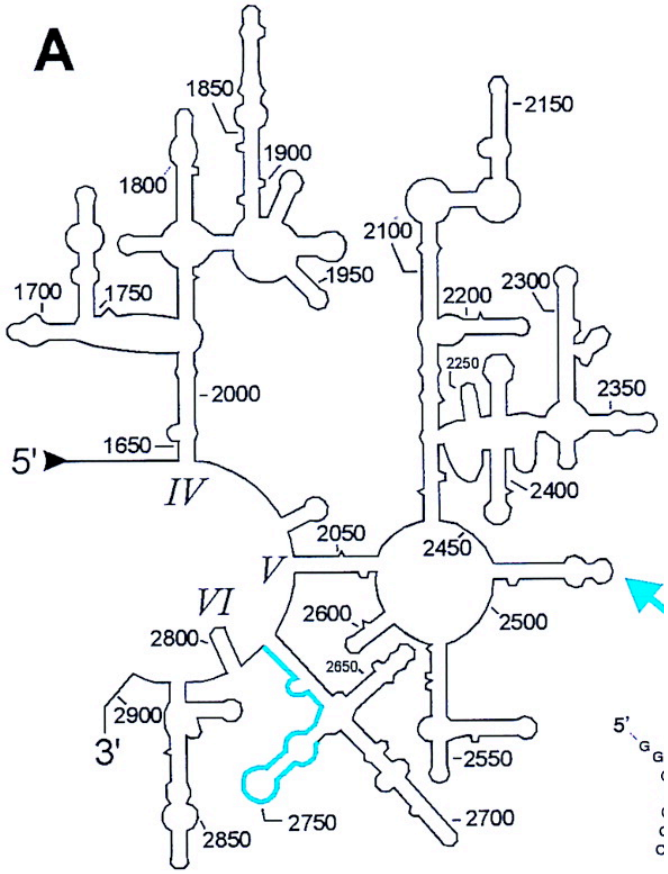


Mueller *et al*, *RNA*, 5:670-677 (1999)

Aptamer RNA secondary structure



tRNA



23S rRNA (partial)

Bruce Alberts *et al*, Molecular Biology of the Cell (NCBI Bookshelf)

Stelzl U. *et al*. *PNAS* 97:4597-4602 (2000)

Determining RNA secondary structure

- ***In silico* methods** (e.g. mfold)
  - Energy-minimization algorithm
  - Nearest-neighbor energy rules

- Advantages
  - Easy and fast
  - Can be fairly accurate
  - Rapid hypothesis generation and testing

- Disadvantages
  - Not necessarily accurate

Determining RNA secondary structure

- **Experimental methods**

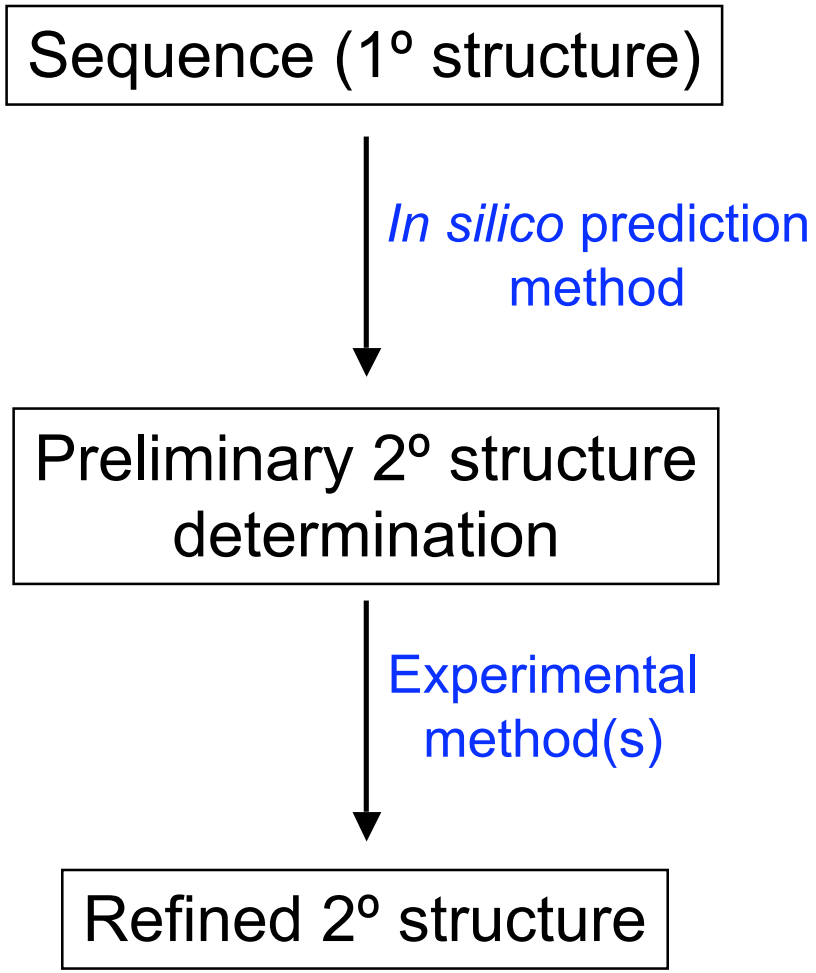
- Advantages
  - More likely to reflect *actual* RNA 2º structure

- Disadvantages
  - Laborious!

  - Technical details important to be sure that 2º (and not 3º) structure is being probed

Determining RNA secondary structure

Approach to determining RNA 2º structure



Experimentally determining RNA secondary structure

- **General principles:**
  - RNA 2º structure directly impacts its reactivity wtih
    - Chemicals
    - Enzymes (nucleases)
  - These reagents cause RNA fragmentation
    - Directly or indirectly
  - The RNA fragments are separable with high resolution
    - Single base resolution required
    - 2º structure inferred from fragmentation pattern

Experimentally determining RNA secondary structure

- **2º structure dependent fragmentation**

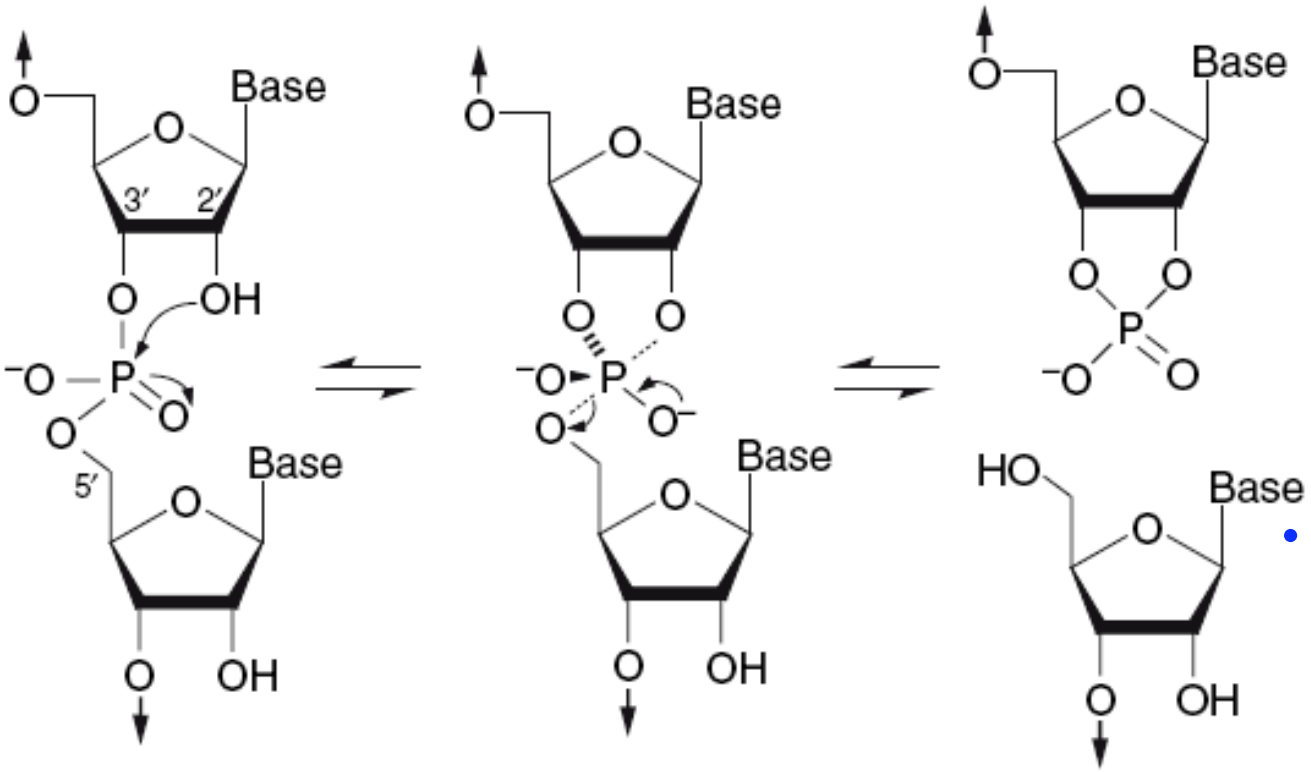
  - Chemical methods

    - Spontaneous RNA hydrolysis (In-line probing)

    - Metal ion-induced hydrolysis (e.g. Pb^{2+})

Experimentally determining RNA secondary structure

- **In-line probing**



- Sufficient flexibility in local structure required to attain an “in-line” configuration
  - Greater flexibility increases probability of sampling this configuration
- Spontaneous cleavage reaction proceeds once favorable configuration occurs

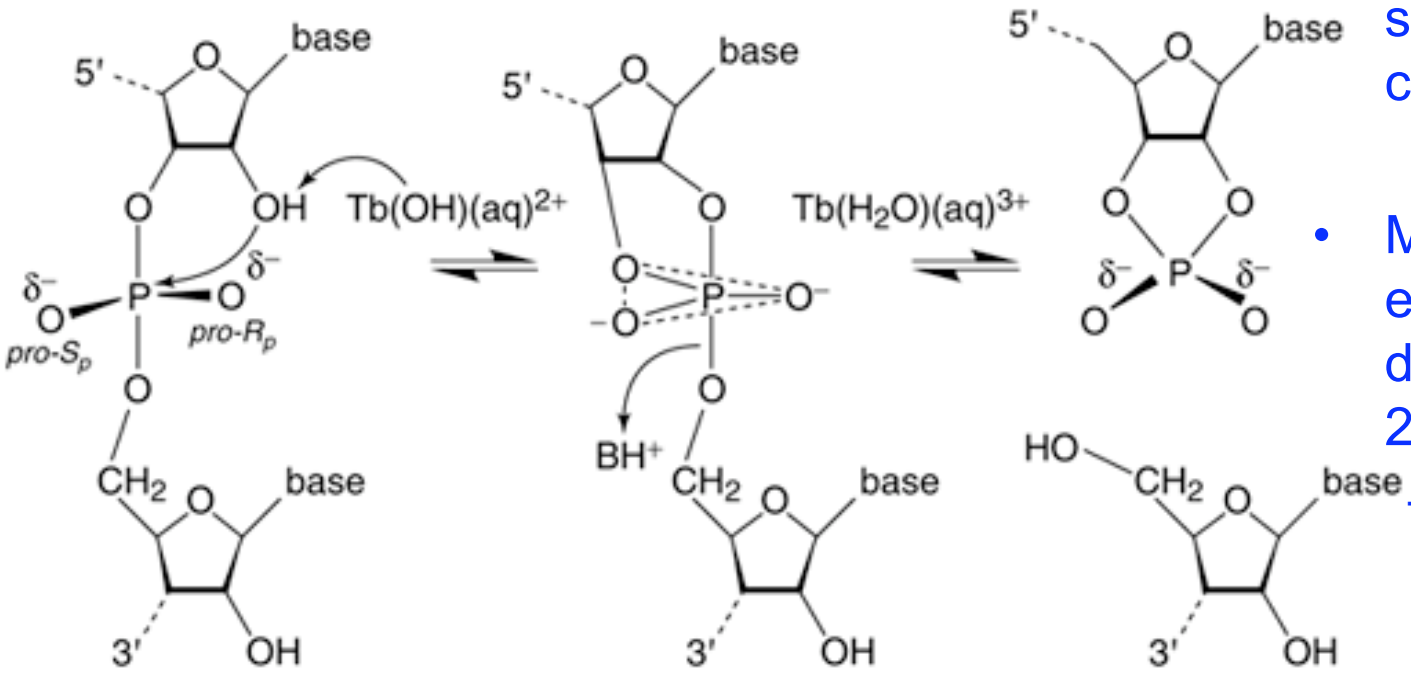
Regulski, E.E. and Breaker R.R., *Methods in Molecular Biology*, 419:53-67 (2008)

Experimentally determining RNA secondary structure

- **Metal ion-dependent cleavage**
  - Metal ions can directly bind RNA
    - Phosphate groups
    - Nucleobase (e.g. N7 guanine)
  - Metal ion concentration can impact cleavage specificity
    - High affinity versus low affinity sites
    - Inner versus outer sphere chemistry

Experimentally determining RNA secondary structure

Metal ion-dependent cleavage chemistry



- Same basic chemistry as during spontaneous cleavage
- Metal ion hydrate enhances deprotonation of the 2'-OH group
  - Significant enhancement in reaction rate

Harris D.A. and Walter N.G., *Current Protocols in Nucleic Acid Chemistry*, Unit 6.8 (2003)

Experimentally determining RNA secondary structure

- **2º structure dependent fragmentation**

  - Enzymatic cleavage methods

    - Use RNA nucleases (RNases) to selectively cleave RNA

    - Cleavage “rules”:
      - RNase A
        - » Cleaves single stranded RNA after C/U residues
      - RNase V1:
        - » Cleaves based-paired nucleotides (double stranded RNA)
      - RNase T1
        - » Cleaves single stranded RNA after G residue

Experimentally determining RNA secondary structure

Test RNA

5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

- Decide to probe secondary structure using enzymes

- **First question**:
  - How will we resolve the various fragments generated?
  - High resolution PAGE (Polyacrylamide Gel Electrophoresis)
  - Capillary Electrophoresis (CE) also an option

Experimentally determining RNA secondary structure

Test RNA

5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

- Decide to probe secondary structure using enzymes

- **Second question**:
  - How will we detect the various fragments generated?

  - PAGE (denaturing)
    - Radioactivity (^{32}P)
    - Fluorescent label

  - Capillary Electrophoresis
    - Fluorescent label

Experimentally determining RNA secondary structure

Test RNA

5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

- Decide to use **PAGE** with ^{32}**P** labeling

- **Question**:
  - How will we label the various fragments generated?

  Options:

  1. Label the fragments once generated

  2. Label the precursor RNA

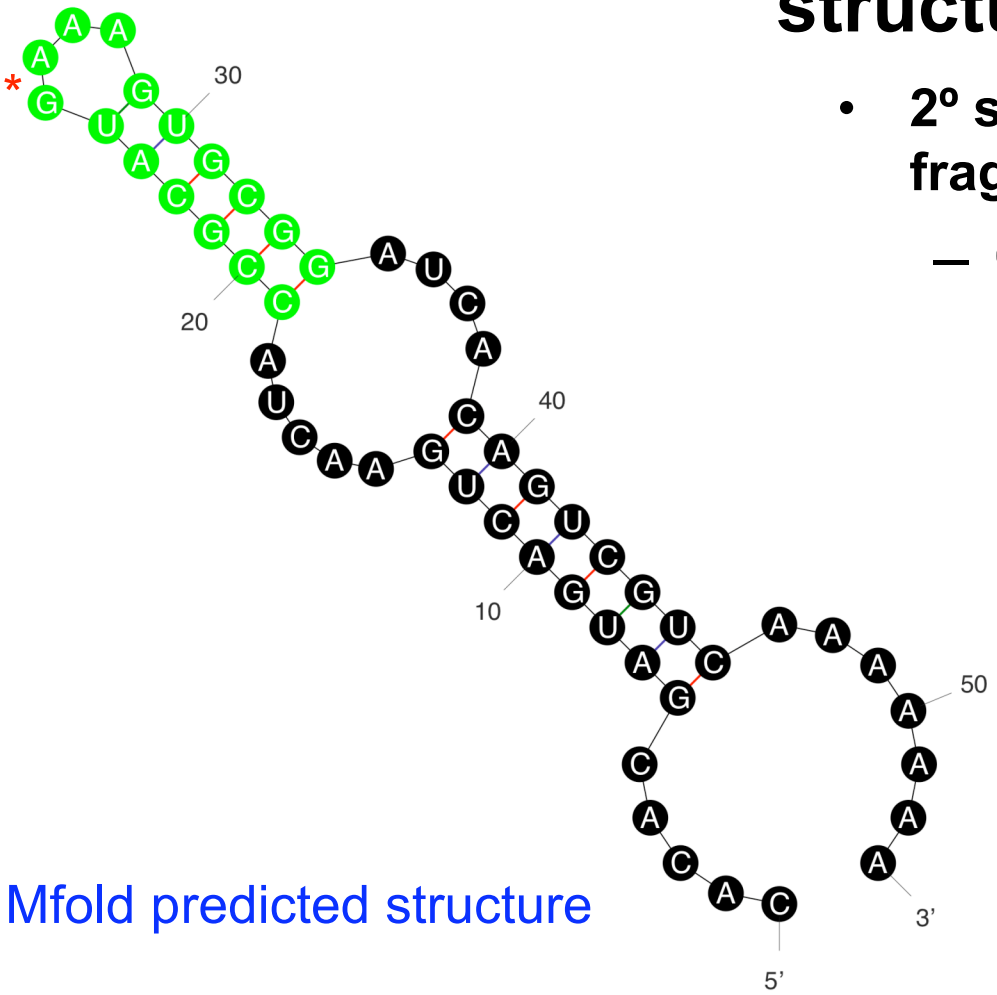
Experimentally determining RNA secondary structure

Test RNA

5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

- There are convenient enzymatic options for ^{32}P labeling RNA
  - 5’-end: e.g. T4 polynucleotide kinase
  - 3’-end: e.g. RNA ligase
- Typically, label one end (e.g. 5’- terminus)

Experimentally determining RNA secondary structure

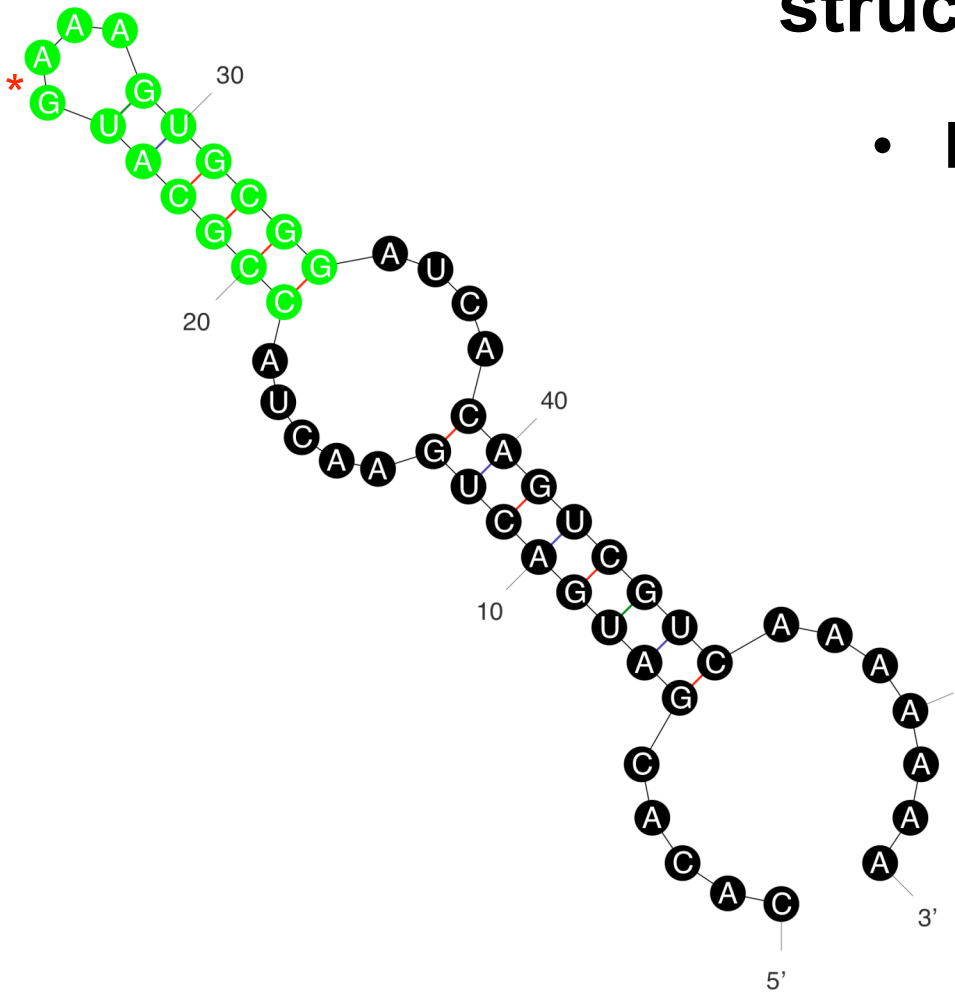


Mfold predicted structure

- **2º structure dependent fragmentation**
  - Cleavage “rules”:
    - RNase A
      - Cleaves single stranded RNA after C/U residues
    - RNase V1:
      - Cleaves based-paired nucleotides (d.s. RNA)
    - RNase T1
      - Cleaves single stranded RNA after G residue

5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

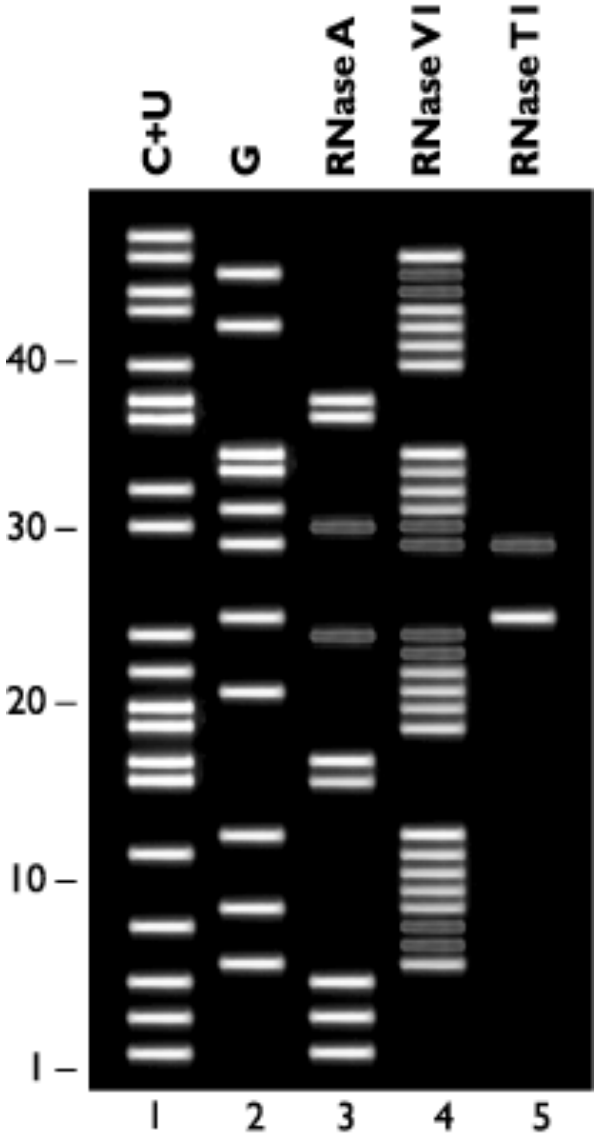
Experimentally determining RNA secondary structure



- **RNase T1 cleavage**
  - Single site predicted
  - Expect 2 fragments
    - 25 bases long (5'-fragment)
    - 29 bases long (3'-fragment)
  - Only 5'-end is labeled
    - Expect to detect the 25 base fragment

5'-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

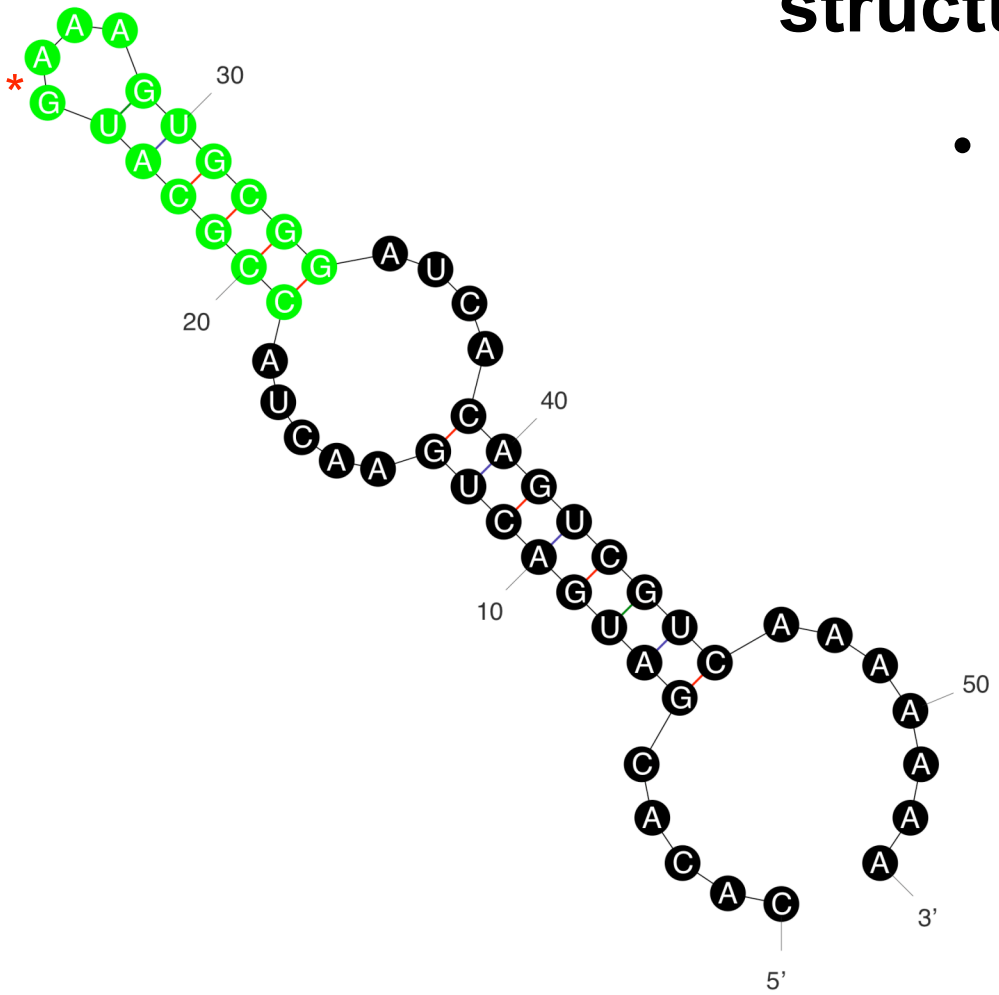
Experimentally determining RNA secondary structure



- **RNase T1 cleavage**
  - Expect to see 25-base fragment
  - Also detect a 29-base fragment!
    - What's going on?

www.ambion.com

Experimentally determining RNA secondary structure



- **Interpretation**
  - G29 is actually in a single stranded loop
  - Experiment refines the secondary structure prediction

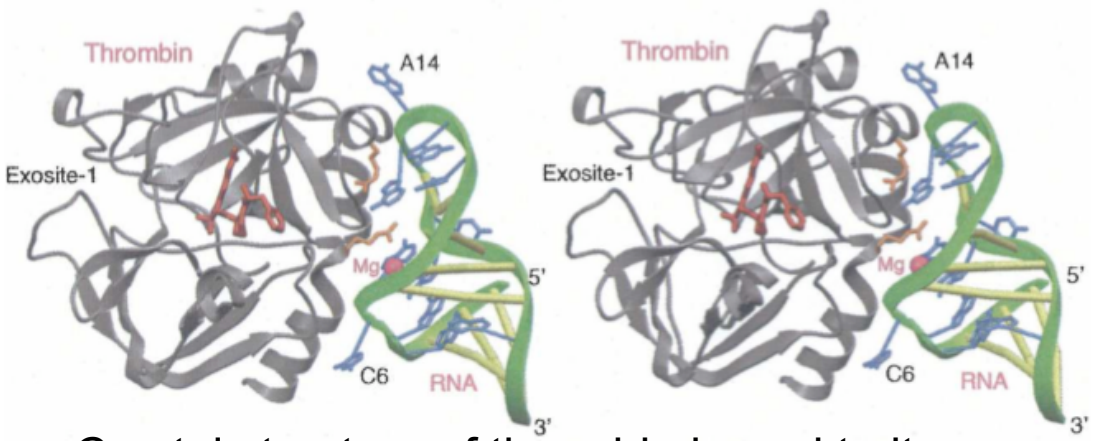
5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

Experimentally determining RNA tertiary structure

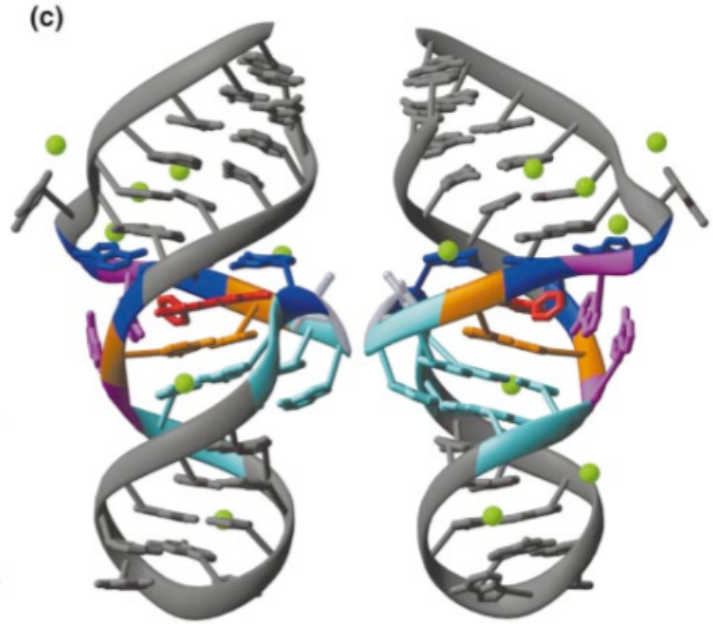
- **3º structure by fragmentation methods**
  - Chemical methods
    - Hydroxyl radical (•OH)
    - Metal-dependent hydrolysis (e.g. Pb^{2+}, Tb^{3+})
  - Tertiary structure differentially limits access of chemical reagent to potential cleavage site
    - **Cannot be used to precisely determine the 3D folded state of the RNA**

Experimentally determining RNA tertiary structure

- High resolution structural methods
  - NMR
  - X-ray crystallography



Crystal structure of thrombin bound to its aptamer (Long *et al*, *RNA*, 14(12):2504-12 (2008)



Crystal structure of TMR bound to its aptamer (Baugh *et al*, J. Mol. Biol, 301(1): 117-128 (2000)

Experimentally determining RNA tertiary structure

- **Significant challenges**
  - RNA quality significantly impacts success
    - Heterogeneity (e.g. length)
  - RNA is inherently flexible
    - Large uncertainties in data possible
    - Difficulty crystallizing
  - EXTREMELY laborious (with no guarantee of success!)
    - NMR requires isotope enrichment studies (e.g. ^{13}C, ^{15}N)
    - Relatively large amounts of material
    - Size limitation
    - Crystallography requires screening large numbers of conditions to achieve a diffraction quality crystals

Summary

- We have defined broadly RNA structure: 1º, 2º and 3º

- Explored various methods (*in silico* and experimental) for investigating RNA structure
  - Frequently combine these methods to efficiently evaluate RNA structure

  - Recognize that obtaining more refined RNA structural information becomes increasingly difficult

- High resolution structural methods (e.g. NMR and crystallography) are gold standard methods
  - All (1º, 2º and 3º) structural information can in theory be derived from these methods
  - However, it can be difficult to obtain these data for many RNA targets