# 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<br>(*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<br>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

23 February, 2010

# 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**:

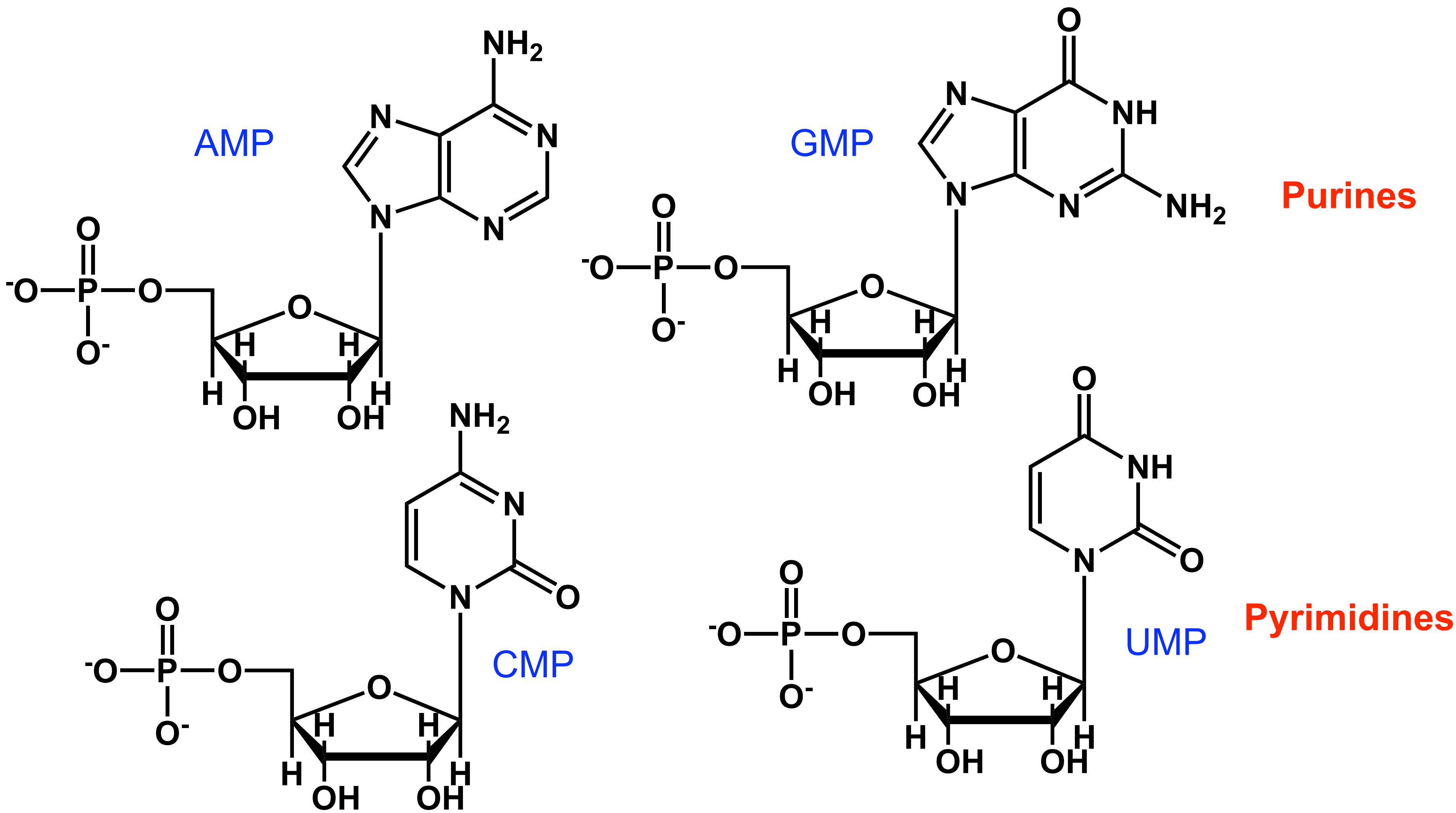
- Sequence of nucleotide building blocks making up the aptamer

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

  – Can you identify them by structure?

# Aptamer primary structure

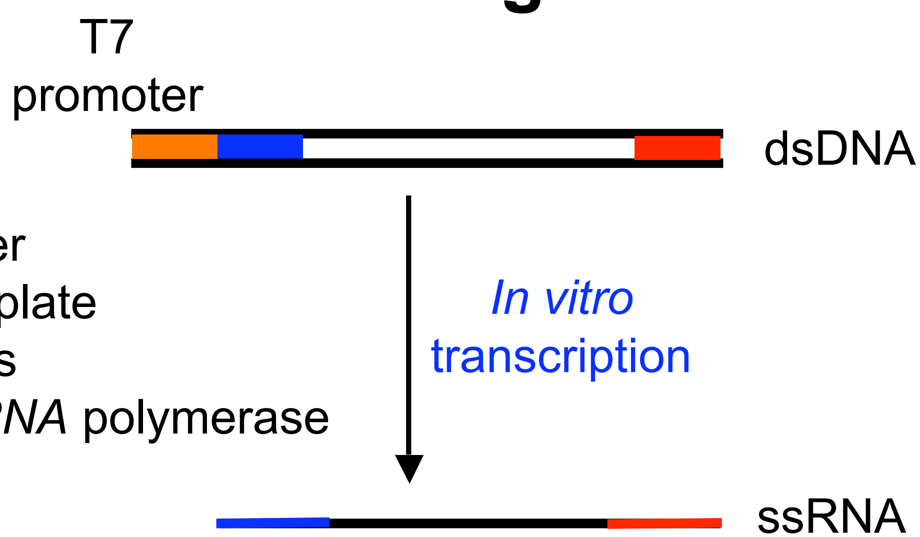
- **The nucleotide building blocks**



# 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 DNA into RNA sequence




- Buffer
- Template
- NTPs
- *T7 RNA* polymerase

<u>T7 promoter</u>

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 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

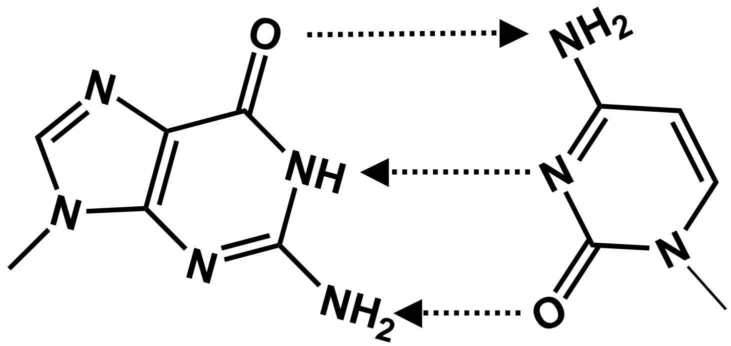
**Definition**:

- The base pairing interactions occurring within an RNA molecule

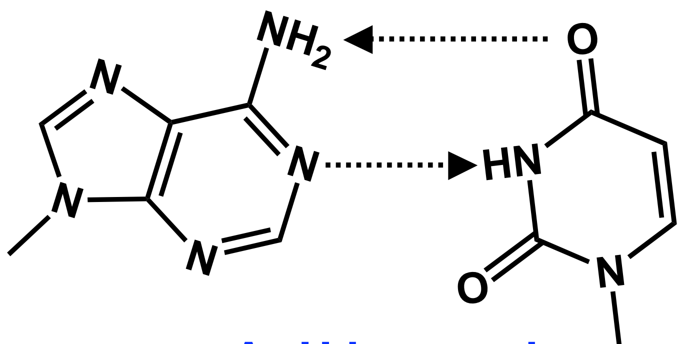
  - *What are the possible base pairing interactions contributing to RNA secondary structure?*

# Aptamer RNA secondary structure

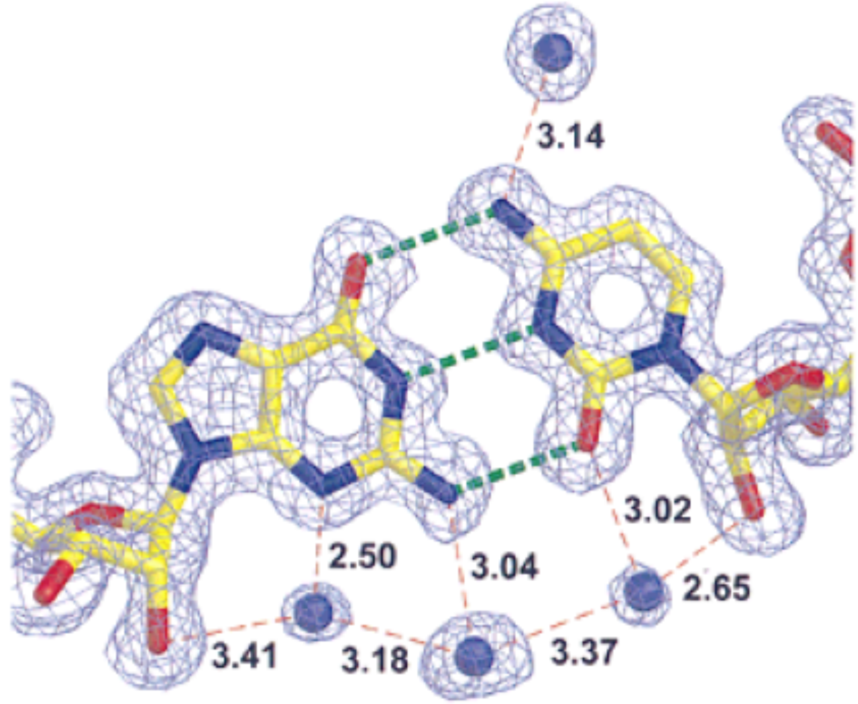
## RNA base pairs contributing to its secondary structure



G:::C base pair



A::U base pair

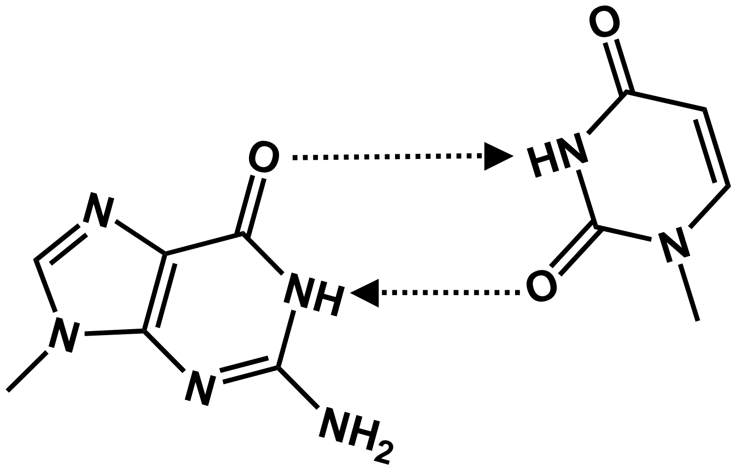


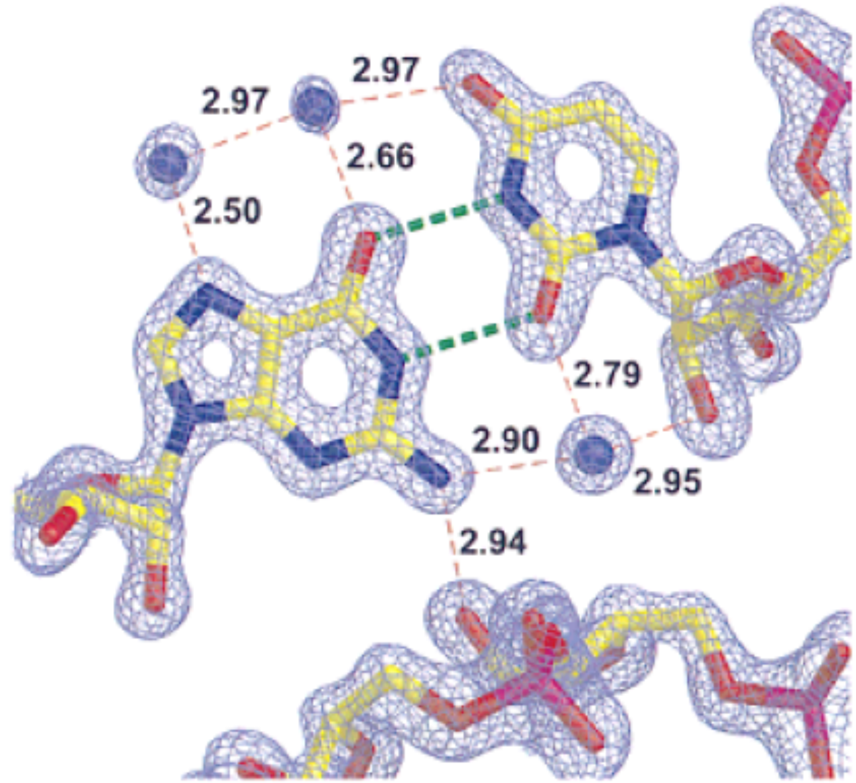

**Watson-Crick base pairs**

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

# Aptamer RNA secondary structure

## RNA base pairs contributing to its secondary structure



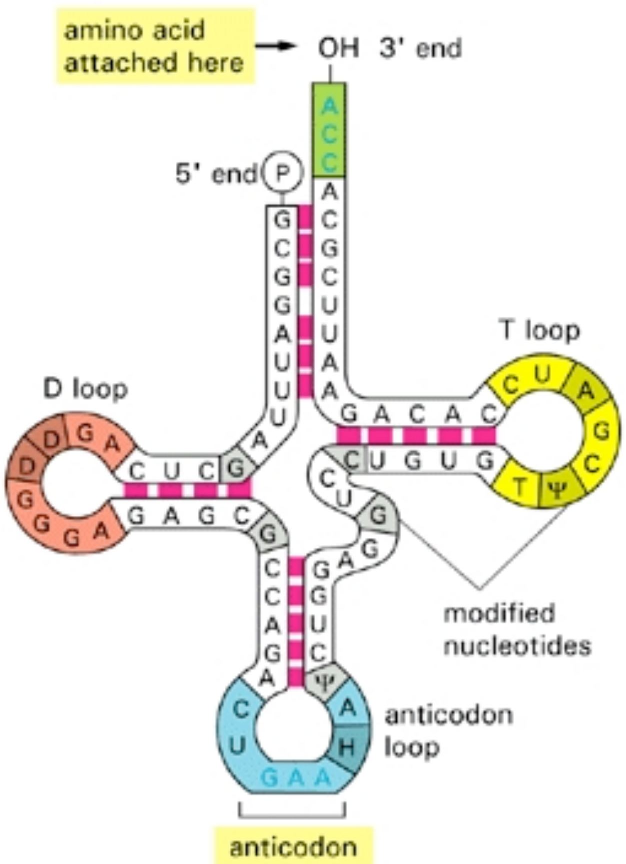




**G::U base pair**

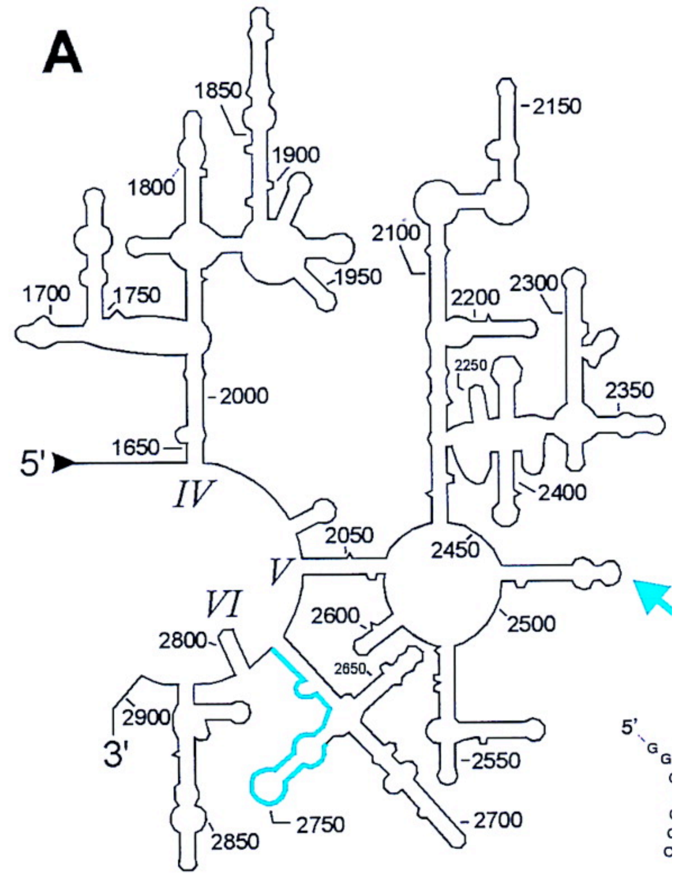
**“Wobble” base pair**

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**

Sequence (1º structure)

↓ *In silico* prediction method

Preliminary 2º structure determination

↓ Experimental method(s)

Refined 2º structure

# Experimentally determining RNA secondary structure

- **General principles:**
  - RNA 2º structure directly impacts its reactivity wtih
    - Chemical reagents
    - Enzymatic reagents
  - 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**

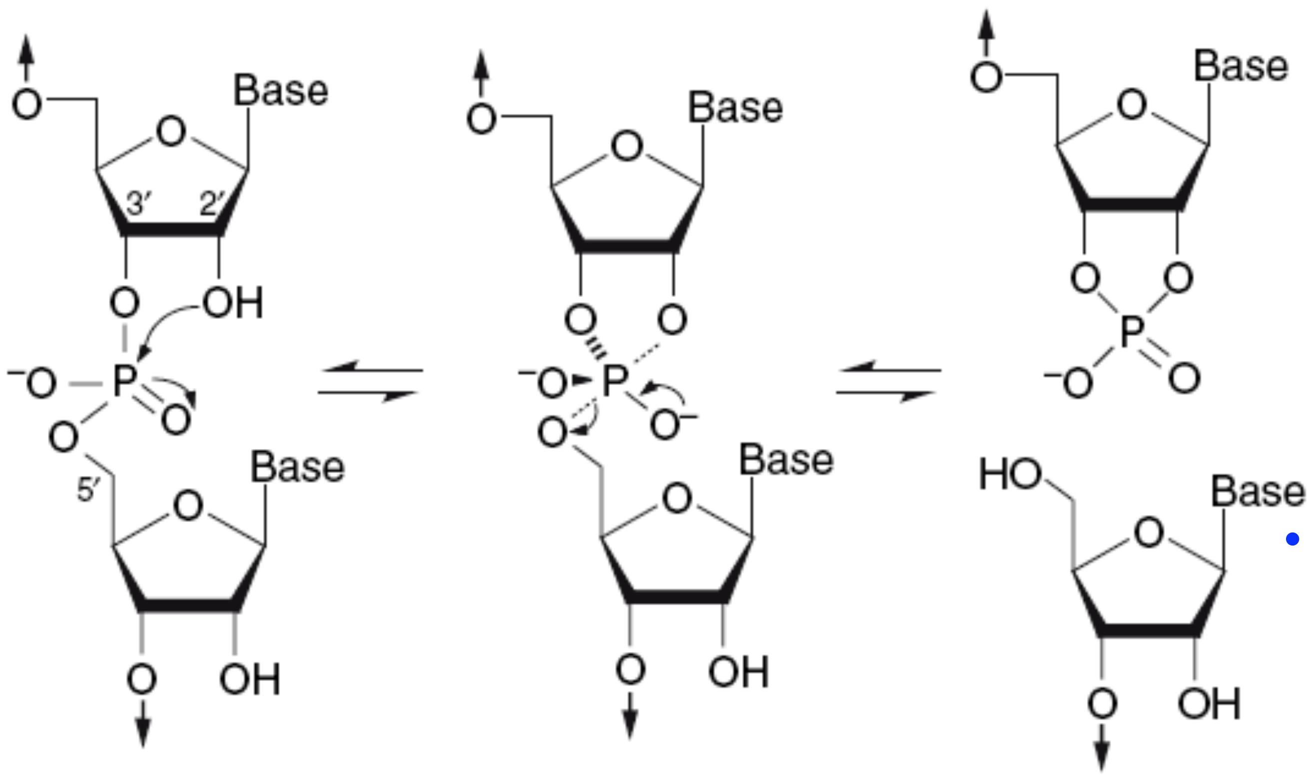
  - Chemical methods

    - 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

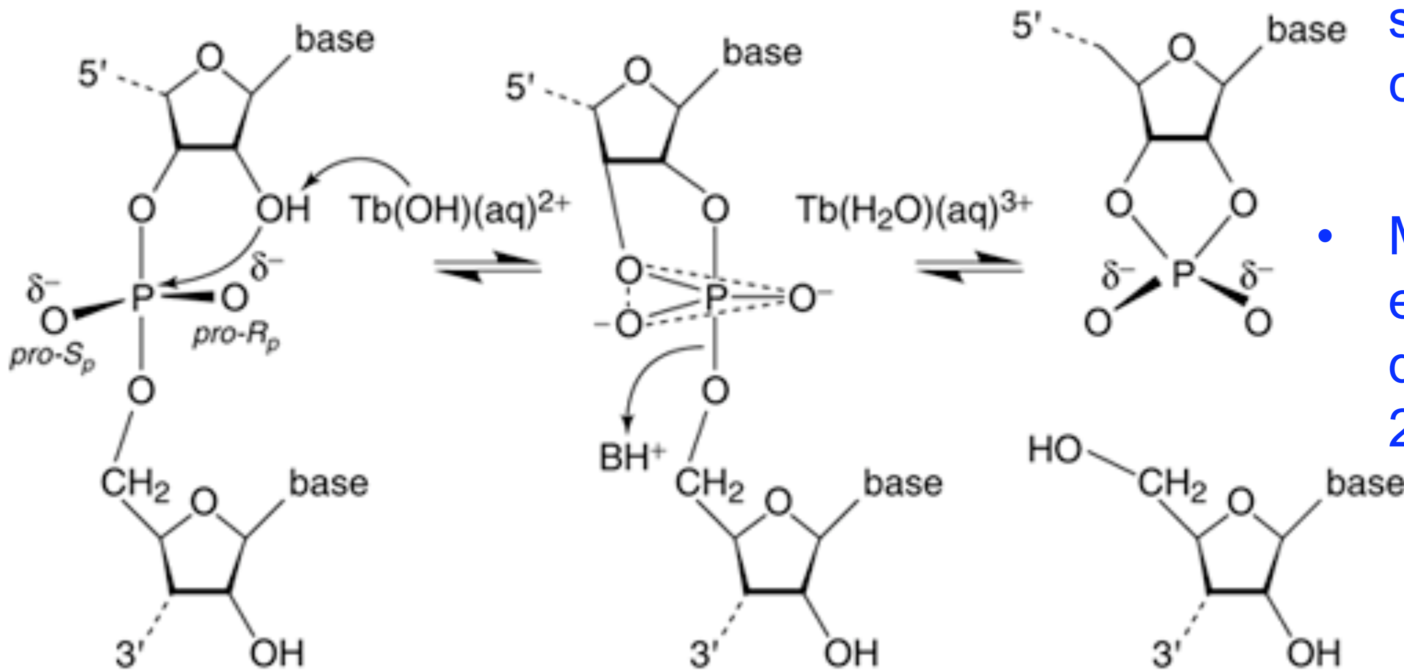
Regulski, E.E. and Breaker R.R., *Methods in Molecular Biology*, 419:53-67 (2008)

# Experimentally determining RNA secondary structure

- **Metal ion-dependent cleavage**
  - Metal ion can directly bind RNA
    - Phosphate groups
    - Nucleobase (e.g. N7 guanine)
  - Metal ion concentration can impact cleavage specificity
    - High affinity v. low affinity sites
    - Inner v. 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 (ds RNA)
      – RNase T1
        » Cleaves single stranded RNA after G residue

# Experimentally determining RNA secondary structure

**Test RNA**

5'-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAA

- 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'-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAA

- 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'-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAA

- 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

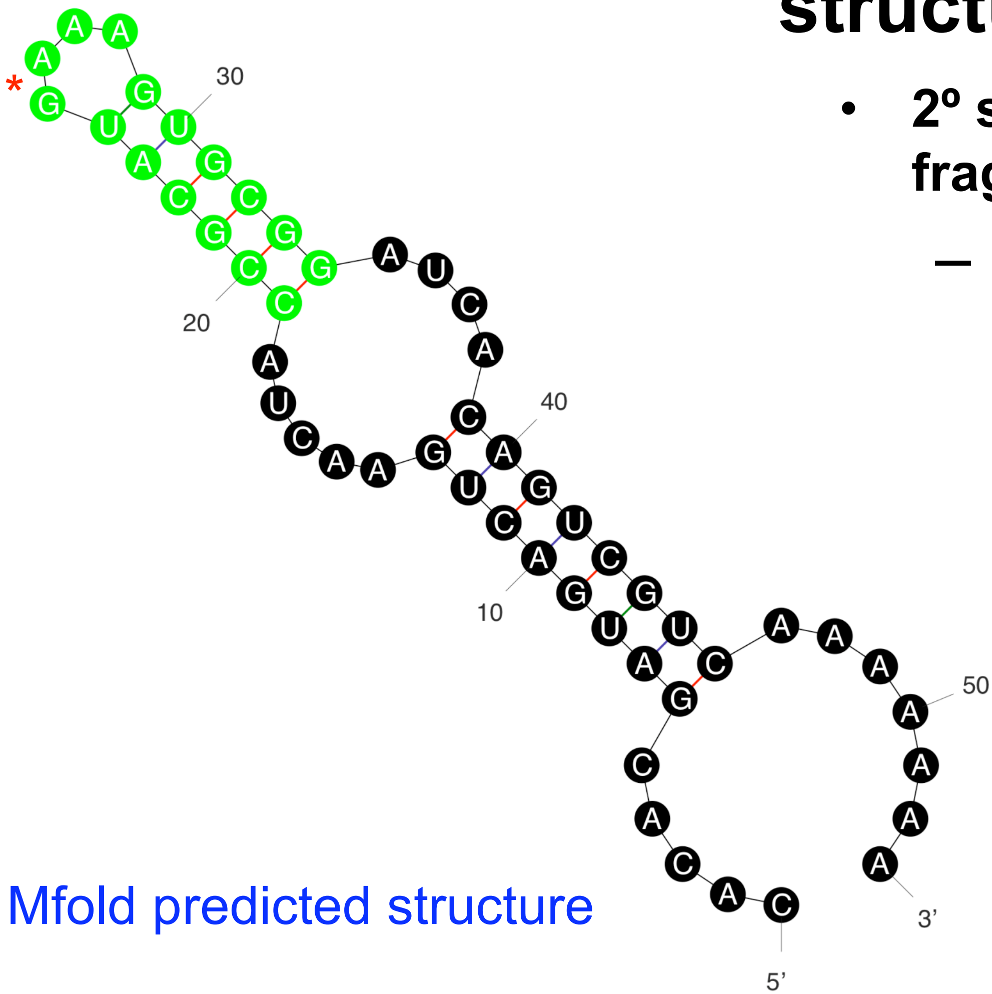
# Experimentally determining RNA secondary structure

**Test RNA**

5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAAA

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

# Experimentally determining RNA secondary structure

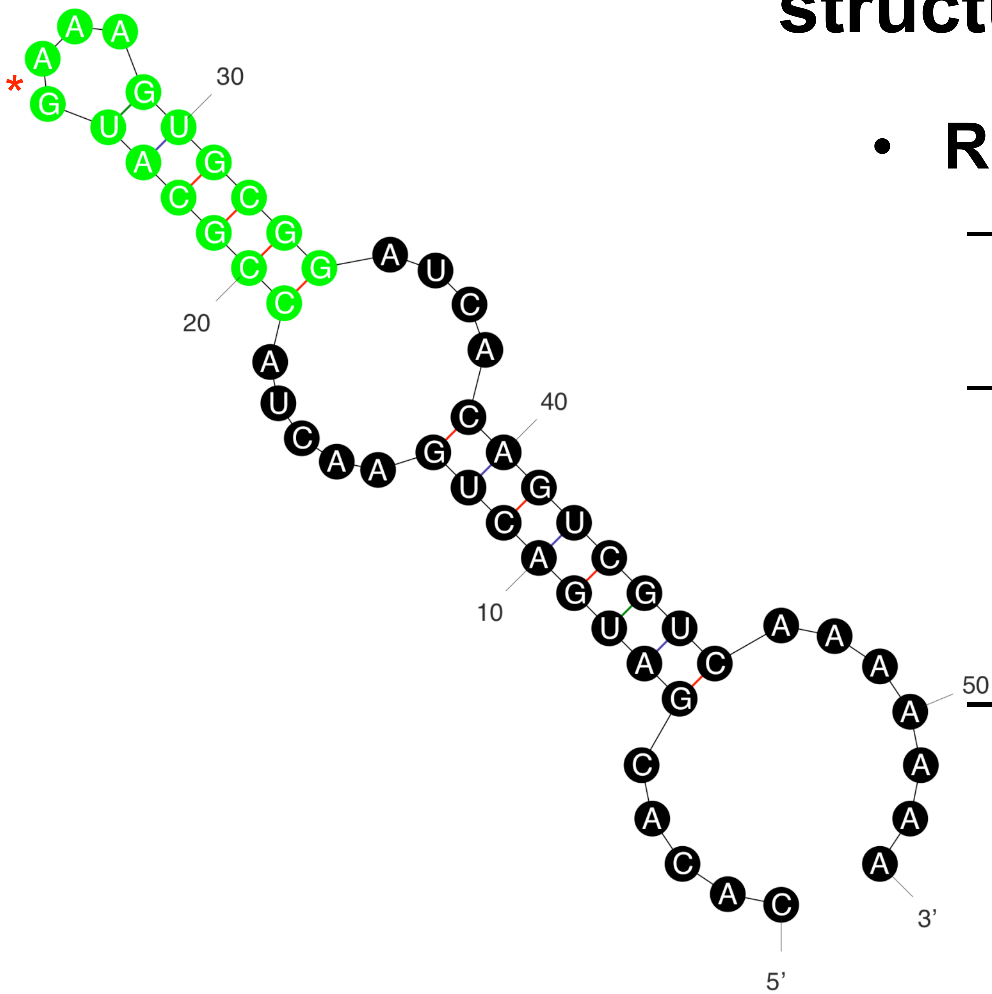



- **2º structure dependent fragmentation**
  - Cleavage “rules”:
    - RNase A
      - Cleaves single stranded RNA after C/U residues
    - RNase V1:
      - Cleaves based-paired nucleotides (ds RNA)
    - RNase T1
      - Cleaves single stranded RNA after G residue

Mfold predicted structure

5’-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAA

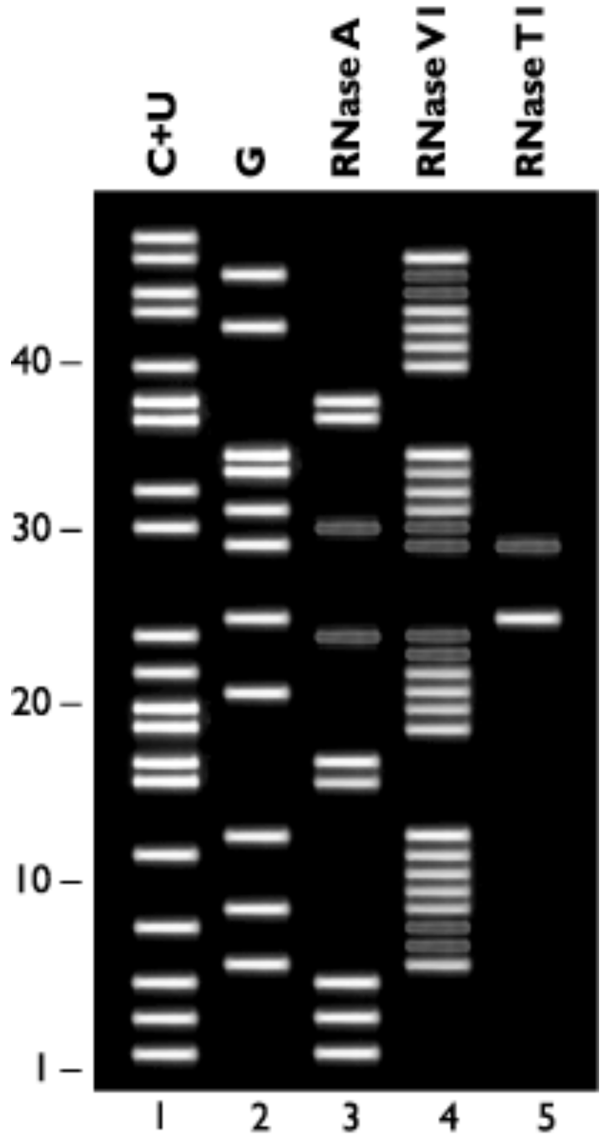
# Experimentally determining RNA secondary structure




- **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'-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAA

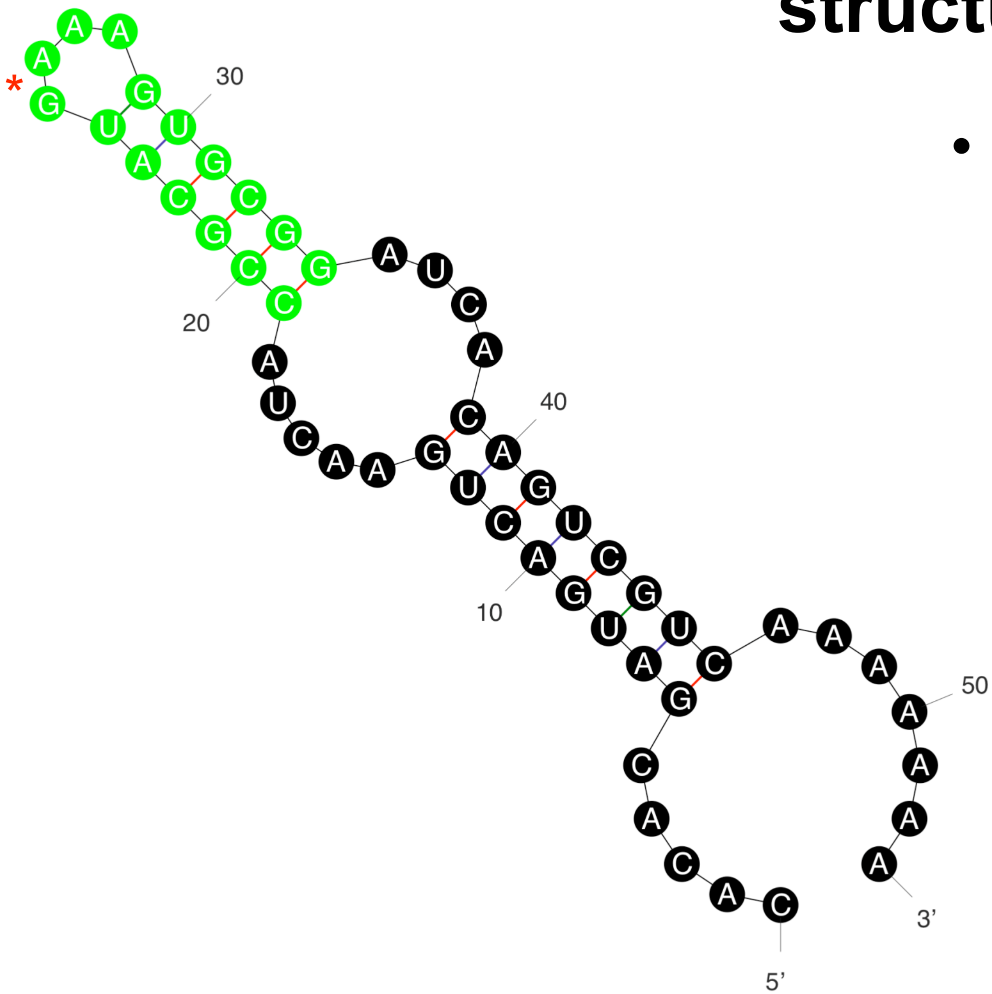
# Experimentally determining RNA secondary structure




www.ambion.com

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

# Experimentally determining RNA secondary structure




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

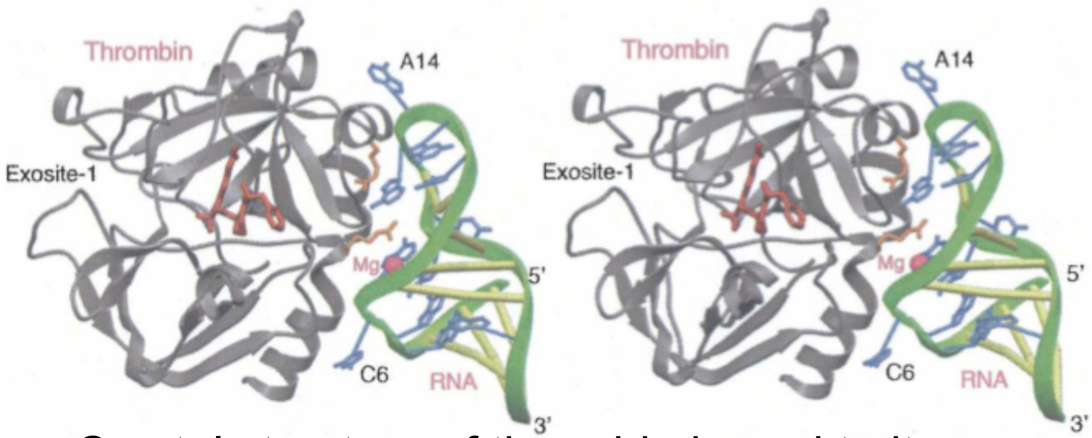
5'-CACACGAUGACUGAACUACCGCAUGAAAGUGCGGAUCACAGUCGUCAAAAAA

# Experimentally determining RNA tertiary structure

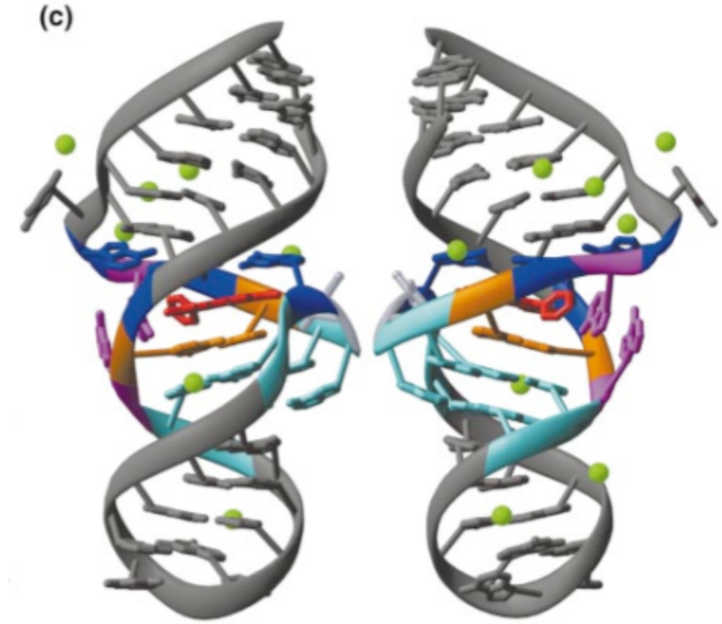
- **3º structure by fragmentation methods**
  - Chemical methods
    - Hydroxyl radical (•OH)
    - Metal-dependent hydrolysis (e.g. $Pb^{2+}$, $Tb^{3+}$)
  - Tertiary structure differentially limits access of chemical reagent to potential cleavage site
    - **Cannot be used to precisely determine the 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
  - RNA is inherently flexible
    - Large uncertainties in data possible
    - Difficulty crystallizing
  - EXTREMELY laborious (with no guarantee of success)
    - NMR requires isotope enrichment studies ($^{13}C$, $^{15}N$)
    - Relatively large amounts of material
    - Size limitation
    - Crystallography requires screening large numbers of conditions to achieve a diffraction quality crystal

# 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 is difficult to obtain these data for many RNA targets