

Lecture #19

Lecture 19 3/17/04

Exam tonight 7:30-9:30pm

References for CryoEM (not required reading)

Frank, J. *Am Rev Biophys Biomol Structure* (2002) **31**, 303-19 (details about the technique)

Biopolymers (2003) **68**, 223-33 (CryoEM of ribosome)

Last time: We learned how to site-specifically attach a probe to RNA and to protein. We discussed hydroxyl radical (HO•) footprinting with BABE (uses Fe²⁺ and H₂O₂ to generate diffusible hydroxyl radical HO•)

Noller experiment: *Biochemistry* (1999) **38**, 945-951

Can we use the hydroxyl radical footprinting system to learn about tertiary interactions in the 16S RNA of the ribosome?

->Had to find a place to put the probe where you can still reconstitute the system to a large enough extent to execute experiments.

16S RNA nts: 1-1542

Noller placed the thiophosphate at nt 361, He generated 1-360 and 361 to 1542. He did not ligate the two pieces of RNA in this set of experiments he just reconstituted the 30S ribosomal particle with 1-360 and 361(babe)-1542 (both 5' to 3')

Also, carries out a number of controls described below.

Reconstitutes 30S subunit, isolates 50S subunit, then reconstitutes 70S and purifies it using sucrose density centrifugation

See Table 1 in Noller paper for results of reconstitutions and control reconstitutions

-> low % yield of 70S

Actual experiments

1-360	361(BABE)-1542	No Fe ²⁺	Control
1-360	361(no label, uses phosphate rather than the thiophosphate primer in the transcription)-1542)	add BABE and Fe ²⁺ separately	Control
1-360	361(BABE)-1542 (ascorbate keeps the Fe ²⁺ , reduced)	add Fe ²⁺ , H ₂ O ₂ , ascorbate	Experiment

- 1) All experiments must be carried out under **Single hit** conditions (single hit means each molecule of RNA is only modified once) if you generate too much hydroxyl radical (HO•), you would completely destroy all the RNA

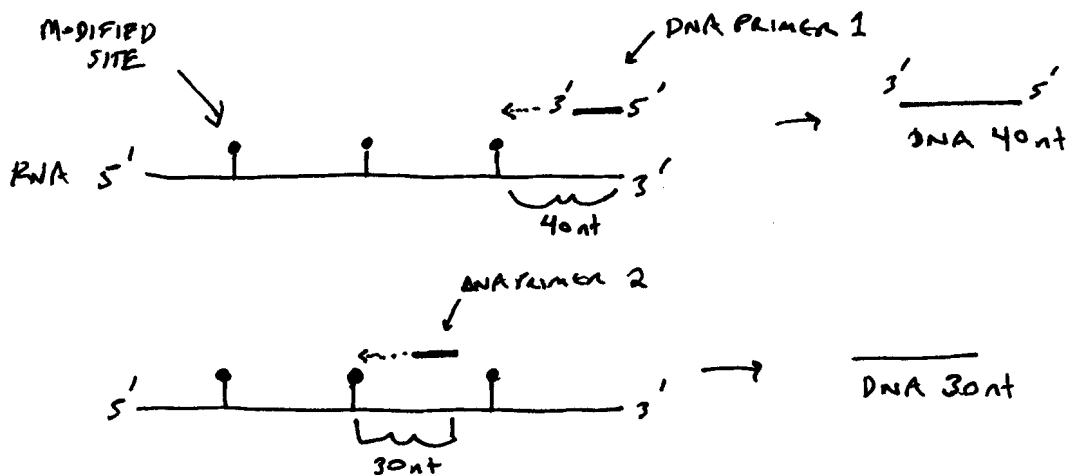
Use reverse transcriptase and primer extension (Methods in enzymology, (1988) 164, 481-9. Available in reference section of the library)

How is the nucleic acid modified by the reaction with hydroxide radical? Is this alteration recognized by the reverse transcriptase (RT)? The assay requires that the RT recognizes a lesion and stalls in putting in the dNTP, relative to the unmodified RNA. Thus the RT can dissociate, leaving a piece of RNA whose length is from the primer to the dissociation site.

Strategy:

Make DNA primers complimentary to the RNA approximately every 150 nts. Either end label the primers with ^{32}P or extend the primer with $[\alpha\text{-}^{32}\text{P}]\text{-dNTP}$. Both labeling methods allow detection by phosphorimaging.

Use processive polymerase (reverse transcriptase) that stalls when it hits a modified site \rightarrow makes a piece of DNA that indicates the location of the modification
> the intensity (amount of this length of DNA made) correlates to the distance from the site of generation of hydroxyl radical



Drawing of modified RNA and strategy to find location of these modifications

Problem: Secondary structure could also cause the enzyme to stall

This is why the control experiments are important- they indicate where the enzyme stalls due to secondary structure.

Look at the primary data in the Noller paper- lanes 1&2 are controls and lane 3 is the real experiment

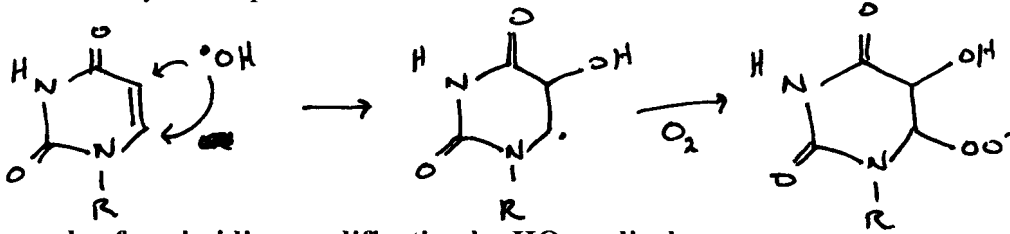
You need to compare the differences between control and experiment to see what sites correspond to $\text{HO}\cdot$ modification of the RNA

(If it stops in all 3 lanes, indicates it is stalled by secondary structure of RNA)

You can now map the intensity and location of sites onto a flat picture (16S secondary structure) to learn about tertiary interactions

*What is the damage caused by the hydroxide radical, and is it recognized by the reverse transcriptase?

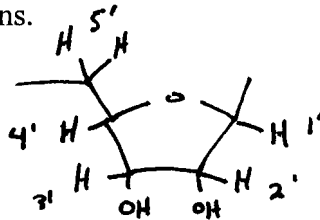
HO• can react with the nucleic acid base (80% of the time) or the sugar (20%)
 Can cause complete cleavage of the RNA (takes about 5 steps for this to occur)
 This chemistry is complex!



Example of pyrimidine modification by HO• radical

Changes sp² to sp³ -> structural change in the ring structure would stall enzyme
 Remember, a key question is whether the enzyme will be stalled by a modification or simply read through the modification

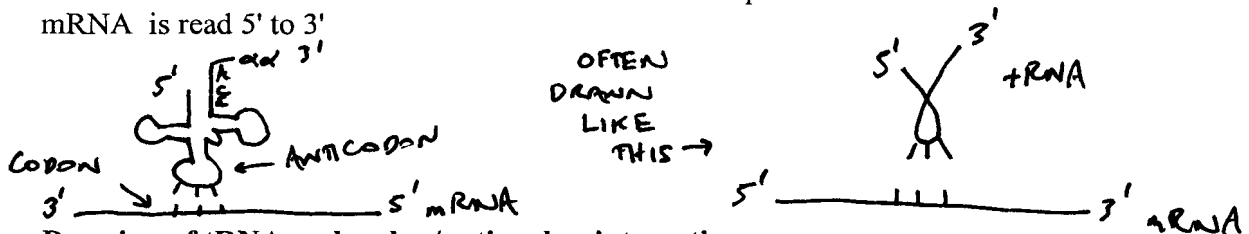
With a sugar, the HO• can remove a hydrogen atom from any position (1, 2', 3', 4', 5'.5'') of the ribose. The resulting radical can be trapped with oxygen and then undergo a variety of additional reactions.



Drawing of sugar and all the hydrogens that can be abstracted by hydroxide radical

tRNA synthases (RS) and Fidelity

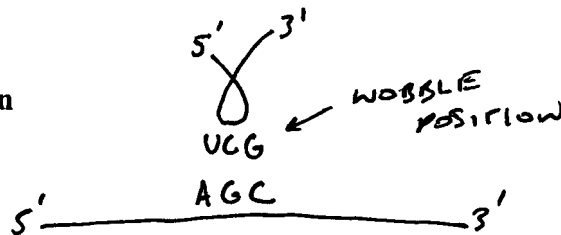
remember the codon-anticodon interaction must be antiparallel!
 mRNA is read 5' to 3'



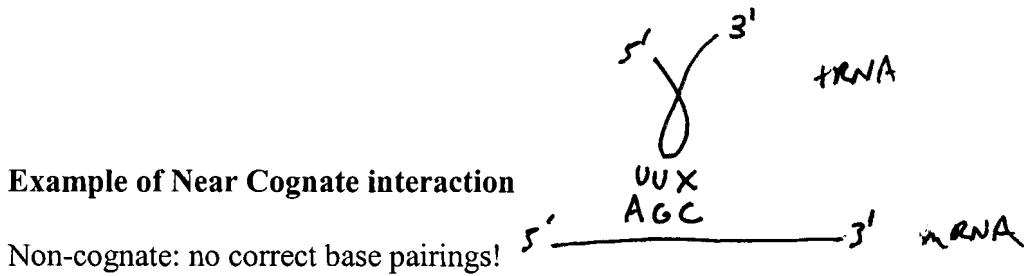
Drawing of tRNA and codon/anti-codon interaction

Cognate interaction: all 3 bases in anticodon and codon match

Example of Cognate interaction

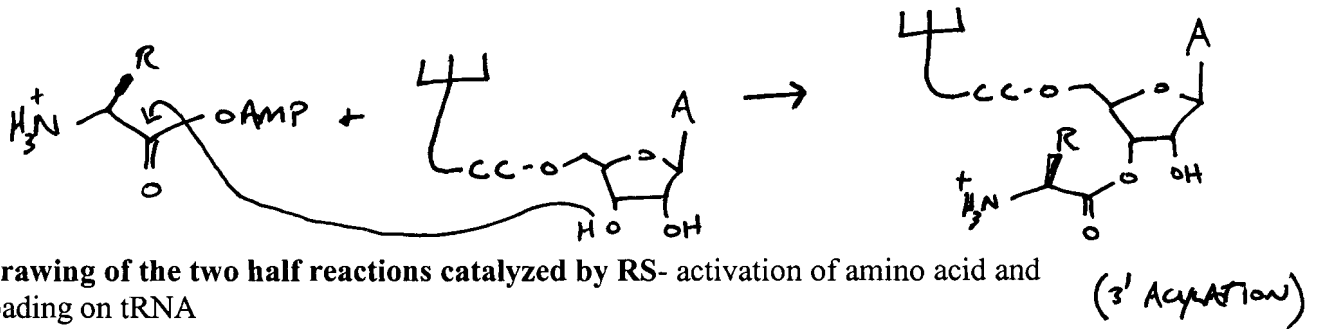
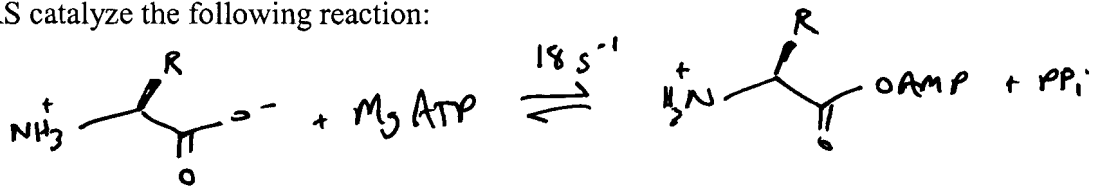


Near Cognate: first base or second base is correct (need at least one correct bp and a second bp, like GU that is not too bad)



p. 3 Handout 3b talks about the mechanism of fidelity induced fit of the tRNA in the 30S subunit
 3 nucleotides A1492, A1493 and G530 are key players- located near the A-site

All RS catalyze the following reaction:



You can assay the loading onto tRNA through radiolabeling PPi and looking for incorporation of radioactive PPi into ATP.

Either the 2' or 3' position is acylated depending on the class of the RS

Class 1-> acylates at 2'

Class 2-> acylates at 3'

The second half reaction can also be monitored using a radiolabeled amino acid. The amino acid can be separated from the charged tRNA.