TD 6: Structure of the 16S rRNA in the ribosome
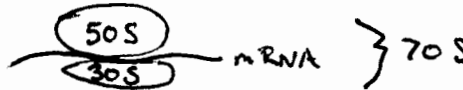
Reference: Noller, Biochemistry (1999) **38**, 945-951

Techniques:
-RNA labeling
-ribosome reconstruction
-Hydroxyl radical ($\bullet$OH) footprinting

1. Goal of the study: to obtain a set of pairwise RNA-RNA distance constraints -> use these to generate a model of 16S rRNA structure <u>within the intact ribosome</u> (biologically relevant)

2. Background:

The 50S subunit: peptide bond formation
The 30S subunit: decoding site
70S ribosome (50S+30S) S stands for sedimentation coefficient, is not additive

30S composition:
16S rRNA (1542 nucleotides (nt), one single RNA+ 21 proteins ("TP30"= total small subunit proteins)

previously observed: protein/RNA footprinting and crosslinking studies show no interactions between protein and many regions of 16S rRNA-> probably there are large RNA rich, protein free regions of 30S that can only be studied with RNA/RNA footprinting or crosslinking

3. Overall approach:
    1. prepare 16S rRNA w/ EDTA-Fe^{2+}(BABE) label at beginning or middle

    2. reconstitute full 70S ribosome w/ labeled rRNA
    3. initiate $\bullet$OH formation with H_2O_2
    4. Use RT (reverse transcriptase) and primer extension analysis to figure out where RNA modification or breakage has occurred

Ex: if EDTA label at position 1 of 16S rRNA causes strand breakage at position 601-> nt 1&601 are close in space in ribosome 3D structure

4. Technique 1- RNA labeling
-RNAs up to ~50 nt can be chemically synthesized (~20x more expensive than DNA chemical synthesis- DNA can make up to ~100deoxynt) chemistry must be specific for 2' vs 3' hydroxyl, which is not a problem for deoxynucleotides of DNA
-16S rRNA = 1542 nt -> no possibility of chemical synthesis
-can make long RNA by in vitro transcription

DNA → RNA   (with NTPs and RNA polymerase-ex. T7, T3, Sp6)

DNA template must have double-stranded promoter (the rest can be either double or single stranded; polymerase will accept either
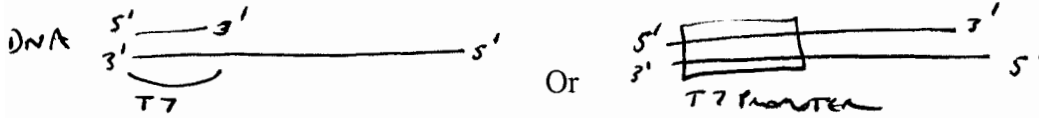
DNA 5' TAATACGACTCACTATAGG NTP
DNA 3' ATTATGCTGAGTGATATCCATGCTCATCG.......
        T7 PROMOTER
The first base that will be incorporated into the new RNA is G

In vitro transcription gives RNA product: 5' GGUACGAGUAGC…..

-you can easily make 5' end labeled RNA because the RNA polymerase is tolerant. Instead of GTP, it can use for the 1st RNA nt: GMP (monophosphate), GMPS (monothiolphosphate), or GpG (dimucleotide)
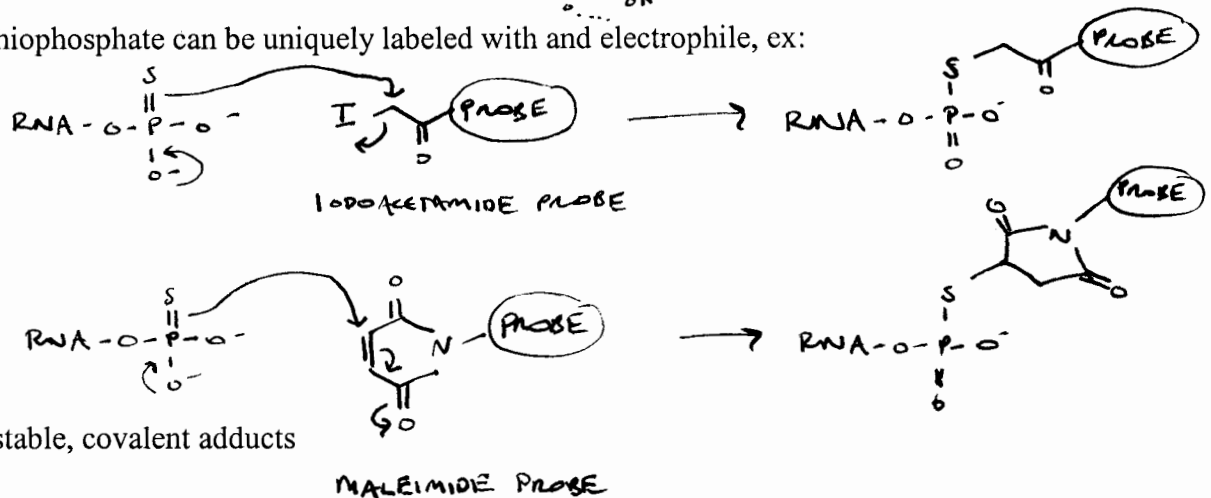Note: there is no chemical reason for the 1st nt to be in activated form
Still need GTPs for the rest of the transcription

If you use GMPS as the primer, you get:
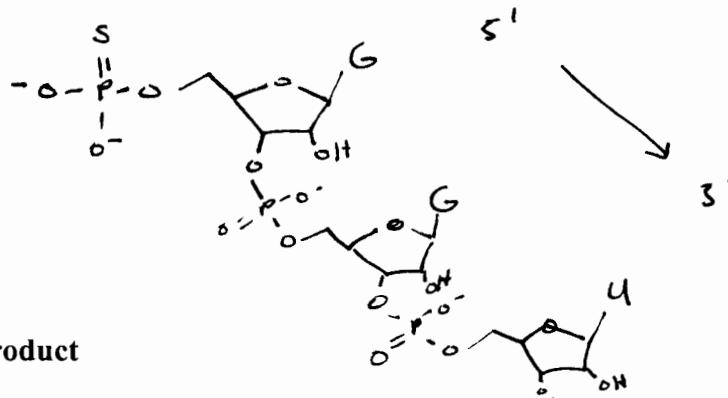
RNA product

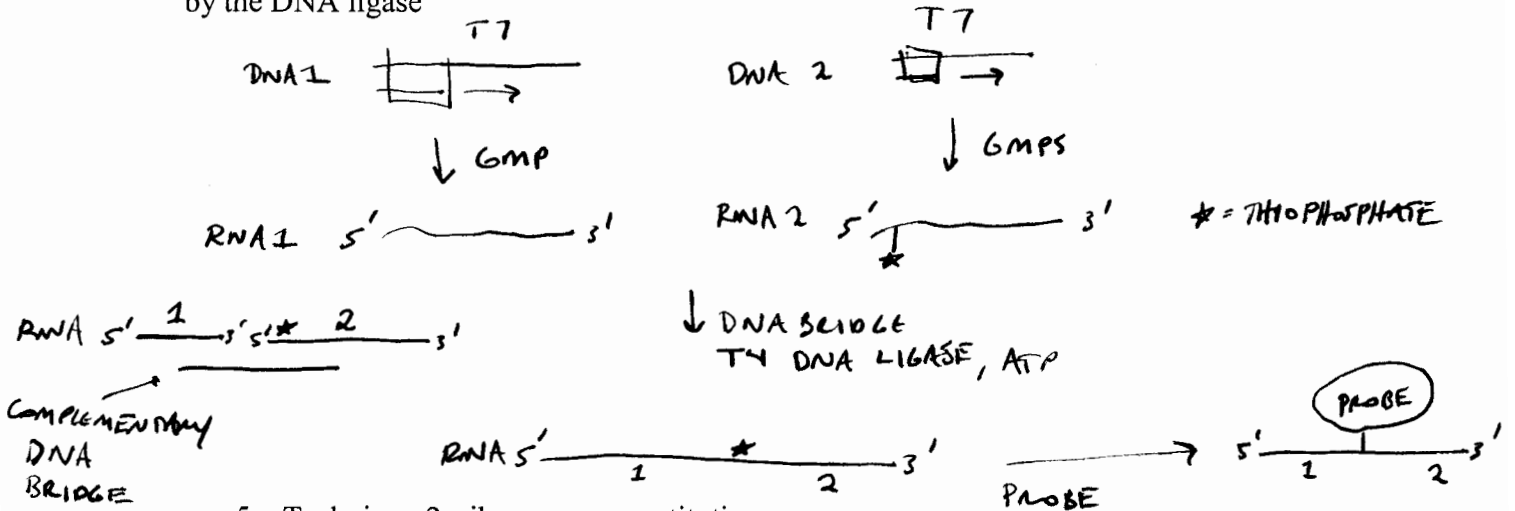The thiophosphate can be uniquely labeled with and electrophile, ex:

IODOACETAMIDE PROBE

Give stable, covalent adducts

MALEIMIDE PROBE

-Sharp and Moore developed a trick to make internally labeled RNA:

      make two pieces of RNA (with the second labeled at 5' end) and ligate them with T4 DNA ligase and a DNA bridge

      must add the probe AFTER the ligation, because the probe would not be tolerated by the DNA ligase

T7   T7

DNA 1   DNA 2

↓ GMP   ↓ GMPS

RNA 1 5' ～——— 3'   RNA 2 5'┬——— 3'   ⚹ = THIOPHOSPHATE
                                      ⚹

                                  ↓ DNA BRIDGE
RNA 5' ——1——,5' 5'⚹——2——,3'   T4 DNA LIGASE, ATP
     ——————

COMPLEMENTARY
DNA
BRIDGE   RNA 5'————⚹————3'   ————→ 5'——1—|—2——3'
                            1   2   PROBE   PROBE
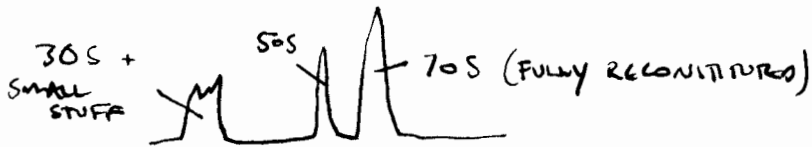
5. Technique 2: ribosome reconstitution

16S rRNA + TP30 (all 21 proteins)

↓   pH 7.5 MgCl$_2$

heat 15 min each at 40, 43, 46, 48, 50 deg C, cool quickly to 4 deg

| add 50S (purified from natural source, not reconstituted- no modifications)

↓

37 deg, 30 min
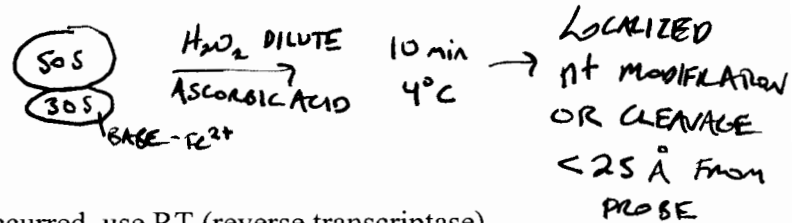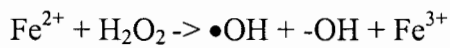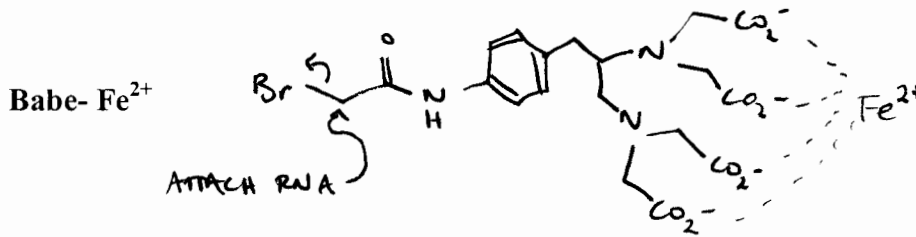
↓

run sucrose gradient ultracentrifugation to purify 70S

30S +
SMALL
STUFF   50S   70S (FULLY RECONSTITUTED)

TOP OF TUBE   BOTTOM

Check reconstituted 70S for activity using tRNA binding assay (^{32}P labeled tRNA^{Phe})

6. Technique 3: •OH footprinting

-probe attached to 16S rRNA is BABE (an EDTA ligand) + Fe^{2+}

Babe- Fe^{2+}

$Fe^{2+} + H_2O_2 \rightarrow \bullet OH + -OH + Fe^{3+}$

•OH is diffusible

-to determine where modifcation/ cleavage occurred, use RT (reverse transcriptase) primer extension analysis

DNA -> RNA transcription (w/ RNA polymerase)

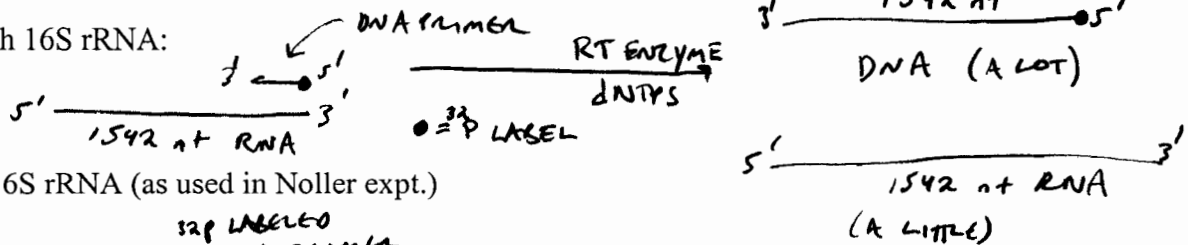RNA -> DNA   reverse transcription (w/ reverse transcriptase)
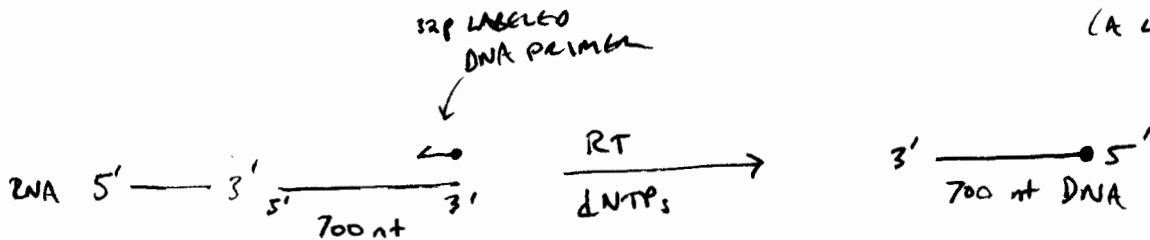
Why RT?
-introduce ^{32}P label (in primer)
-amplify amount (start with small amount of RNA, get lots of DNA product)
-allows you to see modification without complete breakage of RNA strand (stalls reverse transcriptase enzyme, but RNA is not cleaved)

For full-length 16S rRNA:

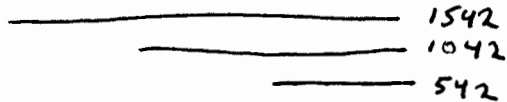For cleaved 16S rRNA (as used in Noller expt.)

Example: If •OH can modify nt's 500 and 1000 of 16S rRNA

RNA 5' —————X———————X———•————3'
                    500   1000   1542 nt

X = SITE OF MODIFICATION

DNA PRIMER w/ ^{32}P LABEL •

You get a heterogeneous population of RNAs:
RT analysis with a primer that anneals to nt 1542 will give the following DNA products:

——————————————— 1542
———————————— 1042
————— 542

The gel comparing a control (with no H_2O_2, no footprinting) to the footprinting expt would look like this:

+ H_2O_2 − H_2O_2

1542   ——   ——

1042   ——

542   —
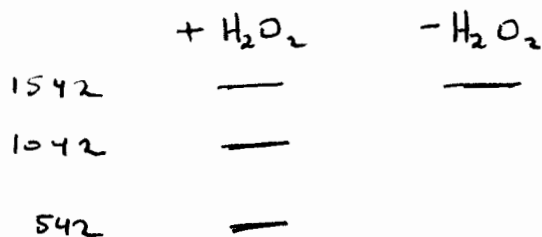
If a primer is used that anneals to nt 300, the gel would look like this:

DNA PRIMER

5'————•—X————X————3'
            500   1000

RNA

+ H_2O_2 − H_2O_2

1542

300   ——   ——
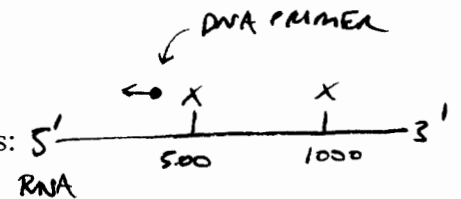
No difference between +/- H_2O_2 indicates that there is no footprinting between nt 1-300

7. Noller study
3 reconstituted ribosomes studied
   -one made from Fe(II) 16S
   -one made from 1-360 + Fe(II) 361-1542
   -one made from 1-448 + Fe(II) 449-1542

The fragments were not ligated, probably because they could not get it work

Controls:
-Does splitting up the 16S into 2 pieces mess up the ribosome?
   *reconstitution still works, giving full size 70S structure
   *can still bind tRNA and tRNA binding is polyU dependent
-Does having the Fe(II) Babe probe in the middle of the ribosome mess it up?
   *same controls as above, compare the labeled w/ unlabeled ribosome

-How efficient is the RT analysis? Can RNA secondary structure cause RT termination, giving false positives?

  *do a control (go through entire process of reconstitution and RT) with ribosome with NO Fe(II) Babe

-How localized is •OH modification? How specific is the attachment of Fe(II) Babe to just the one position in the RNA?

  *do a control w/ Fe(II) babe free in solution (unattached to rRNA and compare to experimental results

Look at the experimental data in the paper (gels)

Note: the numbers refer to nt positions, not MW markers, so large numbers actually are smaller pieces of DNA

AG= ladder standards
Lane 1: Control with No Fe(II) Babe
Lane 2: Control with Fe(II) Babe free in solution
Lane 3: Expt

RT primer binds to nt 1542 at 3' end of RNA

Look for footprinting near probe in primary structure of the RNA (if probe is at nt 1, should see footprinting at nts 2-6)

Conclusions:

  nt361 of 16S rRNA is near nt 34, 160, 497, 512, 520, 537, 552, 615, 1410, 422, 1480, 1490
  nt 449 is near 488, 42, 617
  nt 1 is near 5, 601, 615, 642

Look at the models of 16 structure based on these constraints