Lecture #18

Lecture 18
3/15/04

Review Tuesday 7-8:30 pm
Exam Wednesday 7:30-9:30 pm
TD 9am 3/19 cancelled! (try to attend one of the other sections)
12 noon moved to different building
We will have a make-up TD session if you cannot make one of these the week after break

From last time: CryoEM and atomic resolution structures, antibiotics
We discussed the cryoEm of 70S ribosome stalled with the antibiotic, kirromycin in the presence of Ef-Tu•GTP•tRNA. When compared to the x-ray structure Ef-Tu•GTP•tRNA is modeled into the electron density, it does not fit! With any rigid body motions of this ternary complex, the electron density still does not fit. If the condon interaction with the anit condon on the tRNA is made to fit, then to fit the rest of the electron density the TRNA in the ternary complex must be bent- fit the anti-codon at the tip with the cryoEM electron density, then kink the structure to fit the rest of the electron density
This experiment indicated a conformational change in the Ef-Tu•GTP•tRNA

CHEMICAL METHODS
-site specific modification of protein or RNA, gives information about the dynamics and 3-D structures of protein/protein and protein/nucleic acid interactions, nucleic acid/nucleic acid interactions

1) Preparation of protein or RNA with a single reactive group
2) Attach a specific probe (photoaffinity label, crosslinker, hydroxide radical renerator, used for footprinting, fluorescent probes)
3) Reassemble the modified protein or RNA into the ribosome

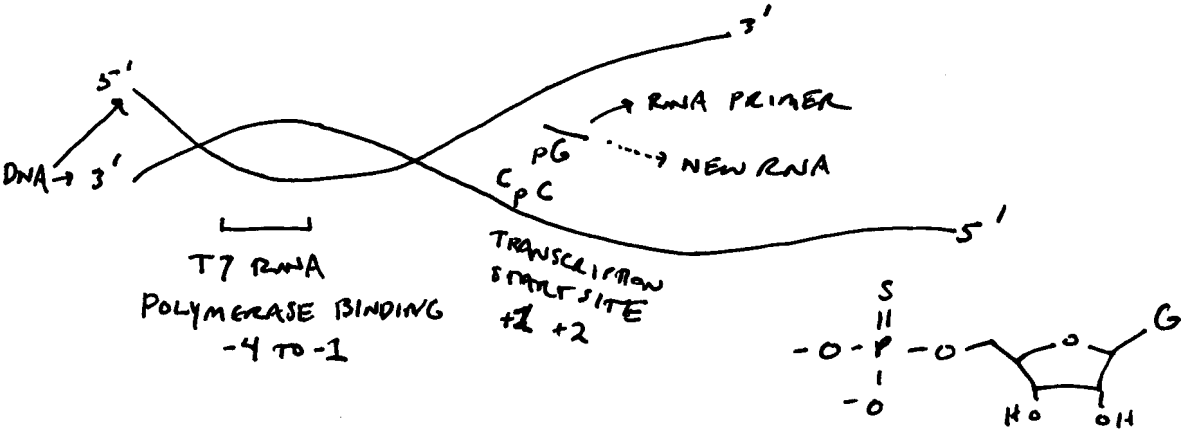
1.Preparation of protein or RNA with a single reactive group

-Built around the unique chemical reactivity of thiolates

Proteins: Use structural information to identify a site where the probe will be attached
Use site-directed mutagenesis (SDM) to insert a cysteine site-specifically at that site
Problem: What if there is more than one cysteine in the protein that is chemically accessible in the native state?
-Use SDM to mutate the other reactive cysteines to serine or alanine (you must characterize this new protein to be sure you have not drastically altered the structure)
Leaves only one reactive cysteine

RNA: Science (1992) **256**, 992 Moore and Sharp
Use Uhlenbeck's method with T7 RNA polymerase to synthesize new RNA, but use a primer with a thiolphosphate
Need recombinant DNA with the sequence you want that contains the promoter and start site for transcription

RNA polymerase binds upstream of the start site and causes a "bubble" of unwound DNA that allows the primer to bind
The primer is either ribonucleotides, pG or GpG, with a thiolphosphate



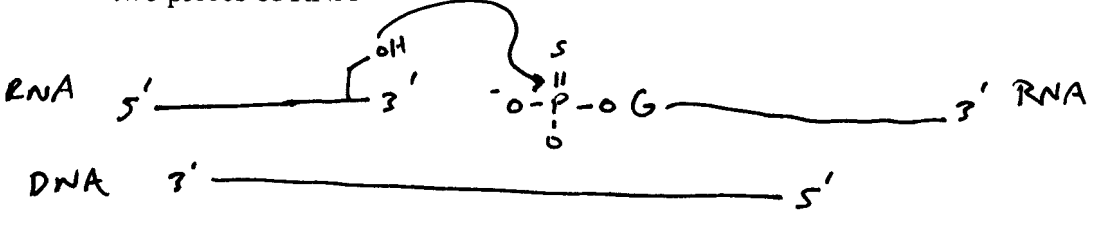
Drawing of initiation of transcription **thiolphosphate primer**

The product is a piece of RNA w/ a thiolphosphate at the 5' end

Product

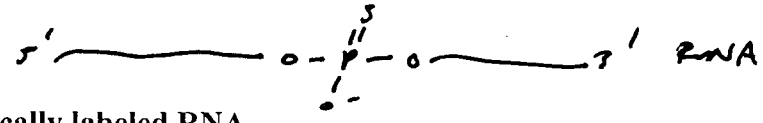
How do you put a label in the middle of a strand of RNA?

-make a second piece of RNA that corresponds to the first half of your desired product (use Uhlenbeck's method)
-Ligate the two pieces
-add a complementary piece of DNA- to allow unique ligation
make <100 nucleotides(nt) of DNA by synthesis or >100nt by molecular biology
use T4 DNA ligase and Mg^{2+} to ligate the two pieces of RNA
(remember Mg^{2+} is needed to neutralize charge of phosphates)
T4 DNA ligase activates the thiolphosphate by adenylation
Attack of the 3'-OH on the activated thiophosphate kicks off AMP and joins the two pieces of RNA



Drawing of ligation

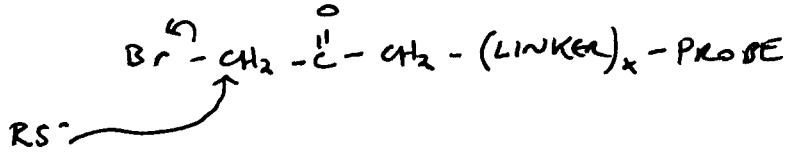
This gives an RNA/DNA duplex w/ a thiol specifically incorporated in the RNA
-Destroy the DNA, left with specifically labeled RNA



specifically labeled RNA

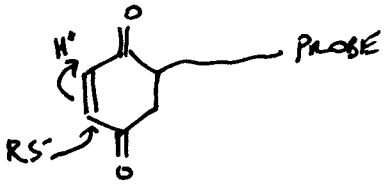
2. Attach the specific probe (photoaffinity label, crosslinkers, hydroxide radical footprinting, fluorescent probes)

-Br leaving group attached to linker and probe



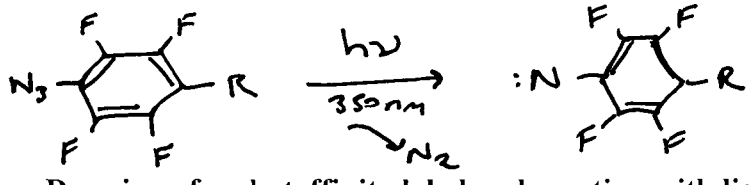
Example of attachment of a probe

The probe can be a photoaffinity label, fluorophore, BABE (generates hydroxyide radical)…



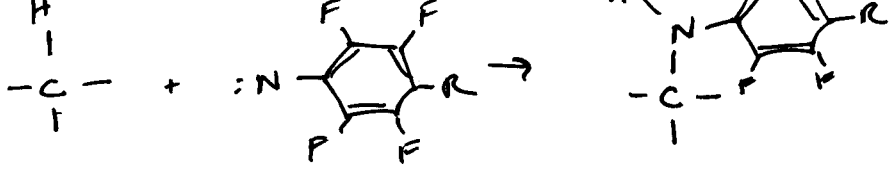
Malaimide provides another example of method for attachment of a probe

a) photoaffinity labels (developed by Westheimer)
-shine light, generate a reactive species that will react with whatever is nearby'

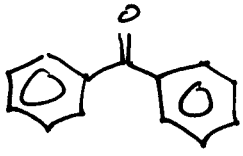


Drawing of a photaffinity label and reaction with light to give reactive nitrene

The nitrene is an electron deficient nitrogen, inserts into a C-H bond to give a C-N bond, which is chemically stable

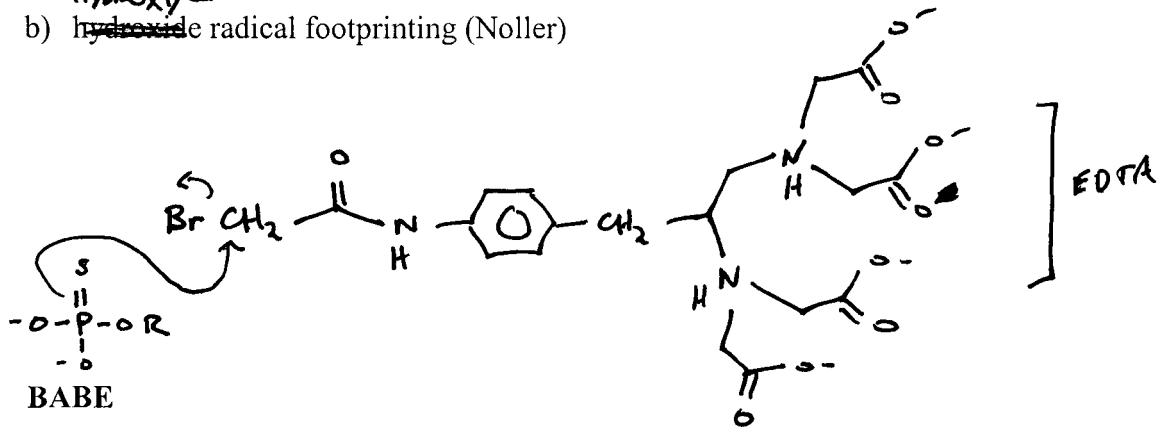


Nitrene reaction



Benzophenone, anther example of a photoaffinity label, absorbs at ~360nm

b) Hydroxyl ~~hydroxide~~ radical footprinting (Noller)



BABE

Acts as an iron chelator

Fe^{2+} reacts with hydrogen peroxide (H_2O_2) to generate Hydroxyl ~~hydroxide~~ radical (extremely reactive species)

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

Gives us information on a short distance scale

Intensity is proposed to be correlated with the distance from the probe

•OH (Hydroxyl ~~hydroxide~~ radical) is <u>diffusible,</u> will react with whatever it hits

Noller experiments looked at 16S RNA (1542nt)

Used 361-1542nt, labeled at 361 with BABE and ^{32}P

Read Noller methods paper!