Lecture #17

Lecture 17
3/12/04

Review for exam: Tuesday 16th 7-8:30pm, Room TBA
Exam: Wednesday 17th 7:30-9:30pm, Room TBA

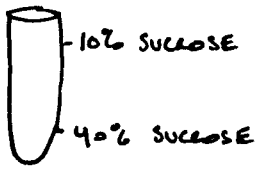
CHEMICAL METHODS for studying translation and the ribosome

1. Reconstitution (discussed last lecture)
    a) clone, purify, express all proteins
    b) make RNA (Uhlenbeck's method)
    c) reassemble ribosome – self-assembly

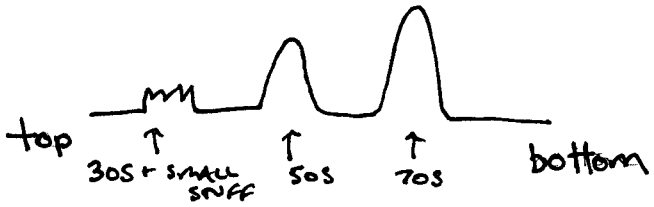
HOW DO YOU KNOW you've put it back together correctly??

Combine 30S (reconstituted)
50S (natural, isolated), difficult to reconstitute effciently
mRNA (polyU) –codes for protein, polyphenylalanine
the reconstitution requires Mg^{2+}

Purify reassembled system with sucrose gradient ultracentrifugation
Tube with a linear gradient of sucrose, gradient varies from 10% to 40% sucrose
Spin down, centrifugal field separates 30S, 50S, 70S ribosome
(heavier, closer to the bottom)



Picture of tube



Peaks of separation

How good is this reconstitution?? –Assay for activity

Compare natural and reconstituted ribosome
Radiolabeled tRNA charged with Phe is assayed for binding
Also, assay for formation of poly-phenylalanine (when all protein factors are included)

	PolyU dependent	PolyU independent	Poly-Phe
Natural	100%	20%	100%
Reconstituted	48%	12%	34%

The polyU independent binding is the result of tRNA binding to mRNA that was isolated with the ribosome (you can't get rid of all the contaminating mRNA)

As you can see, the reconstituted system is not perfect- but we do have tRNA binding and formation of polyphenylalanine chain = ACTIVITY!

It is not surprising given the complexity of the system that the reconstitution is not perfect. We are still learning about the mechanisms of RNA folding- major area of research
What is surprising is that we are actually able to reconstitute some activity!
Allows us to do further studies on the mechanism of translation, now with reconstituted system where we can label things and control variables

2. Antibiotics

Natural products (or man-made products) that have noncovalent interactions with RNA
Freeze out different states of the ribosome (using different antibiotics)
Allows us to look at the structure – take a "snapshot" of translation (cryoEM, Xray)
p. 17 handout 3a – structures of antibiotics

A. Chloramphenicol- see page 18 handout 3a (all base #s are from E.coli- standard #ing)

Structure from D.radiodurans

Binds the A-site of the ribosome
1)Notice the H-bonding interactions
2) Mg^{2+} is required for binding (RNA has 100s of Mg^{2+} bound- required for reconstitution of the ribosome and more specifically for RNA folding)
There is no way we could have known the importance of Mg^{2+} in CA interactions without the x-ray structure- Mg^{2+} is spectroscopically silent)
3) No protein anywhere near the binding site (no protein involved in binding!)
4) binding of chloramphenicol predicted by hydroxyl footprinting (Noller, methods paper)
5) structural data explains drug resistance
   a. enzyme uses acetyl CoA and can acetylate the 3'-OH of chloramphenicol- disrupts binding -> resistance
   (need more drug to get it to bind)
   b. methylation of cytosine 2452 (by SAM!), also disrupts binding->resistance

B. erythromycin

structure explains biochemical data

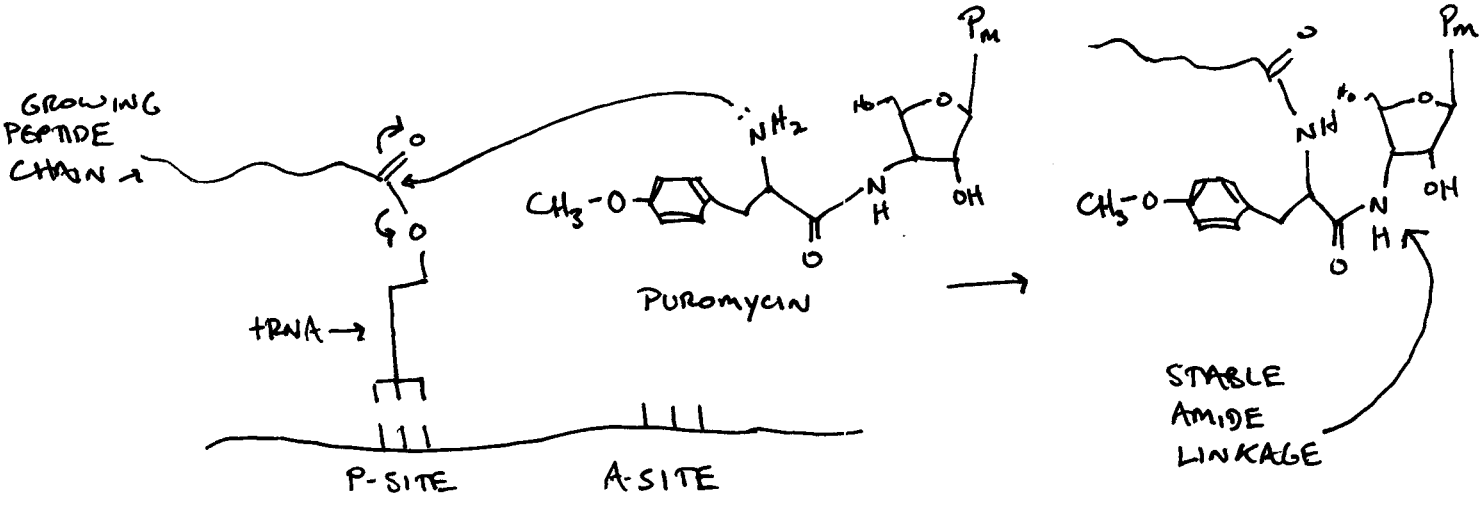
See p. 18 handout 3a for a cartoon drawing of where erythromycin, clindamycin and chloramphenical bind

Experimentally- you can still isolate a polypeptide of 6-7 amino acids with erythromycin

- erythromycin blocks the exit tunnel from the P-site, limiting the peptide chain length that can be formed

- resistance caused by methylation of adenine in the EM binding site

C. puromycin
di-methylated adenine attached to tyrosine analogue (mimics the end of a tRNA, the HO of tyrosine is methylated)
amide in place of an ester at the 3' position (chemically less reactive)
binds in A site like the end of a charged tRNA
Functions as a CHAIN TERMINATOR



Proposed mechanism for chain termination
Puromycin can form a peptide linkage with the growning peptide chain in the P site, but then it cannot undergo further peptide bond formation as the amide bond at 3' to the tRNA cannot be cleaved- you're stuck!

Example of the use of antibiotics to study function in conjunction with structure.
Kirromycin, blocks translation such the EF-Tu-tRNA-nucleotide complex is stuck.
A cryo EM at 10 angstoms resolution has been obtained. We have structures at atomic resolution of EF-Tu-tRNA-nucleotide complex. One can then use this atomic resolution structure to fit observed electron density in EM
f rom paper TIBS (2003) **28**, p259-266
Based on a combination of EM and Xray structures
-working hypothesis is that tRNA must be bent at this specfic stage in the translation process.

Antibiotics are important tools!
They allow us to think about structure and conformational changes in the ribosome