Lecture #16

Lecture 16
3/10/04

MODULE 3
References
Decoding: TIBS (2003) **28**, p259-266
Reassembly: Nomura, Nature (1970) **226**, p1214 (no online copy, available through Barton)
Noller, Methods in Enzymology, 318, p446-60 (print version in library)

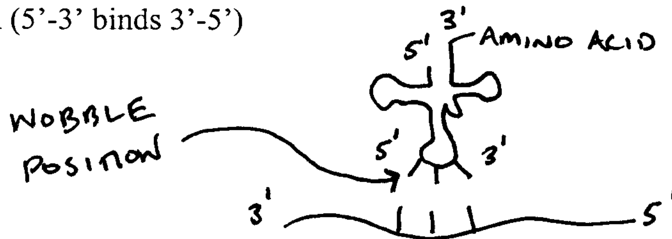
CARTOON OVERVIEW of translation (continued)
Remember from last lecture:

-GTP plays a role in initiation, elongation, and release
-Initiation: IF2-GTP somehow delivers the first charged tRNA (formylmethionine) to Psite
F-met can bind before mRNA- order not required
Shine-Dalgarno sequence of mRNA (purine rich) interacts with pyrimidine rich sequence of 16S RNA in small ribosomal subunit

ELONGATION
Factors: EF-Tu (GTP), EF-TS, EF-G (GTP)
EF-Tu
-represents 5% of a cell's protein
- involved in fidelity and proofreading
role of GTP- is it a switch or a motor??
EF-Ts
-guanine nucleotide exchange factor (GEF or NEF)

See p.11 handout 3a
EF-Tu (GTP) binds charged tRNA-> exists as a tertiary complex inside the cell
Loads tRNA into the A-site, hydrolysis of GTP and loss of Pi, left with EF-Tu-GDP
EF-Ts is required to dissociate GDP from EF-Tu (see p.16 handout 3a)
GTP is at mM concentration in the cell, rapidly binds the now free EF-Tu
(Ef-Tu is recycled)

Note: the amino acid is attached at the 3' end of the tRNA
The codon of the mRNA and the anticodon of the tRNA must be ANTI-PARALLEL for interaction (5'-3' binds 3'-5')



Drawing of interaction between codon of mRNA and anticodon trNA

Peptide bond formation
-prior to bond formation, check for fidelity
-peptide bond formation is thermodynamically easy (5 kcal/mol)
-rate constants for peptide bond formation are 10-50 s^{-1} (fairly fast)
-you can get peptide bond formation in absence of the 30 S subunit and in the absence of EF-Tu! But, it is very slow- chemically competent but kinetically incompetent (demonstrates the importance of localization of the substrates for catalysis)
-no protein in peptide-bond forming site!

See p. 12-13 handout 3a

Peptide bond formation occurs and the growing chain is now in the A site

Need to move the tRNAs and mRNA along so that the process can be repeated
(empty tRNA moves to E site, tRNA w/ growing chain moves from the A site to the P site)
EF-G (GTP) catalyzes mRNA translocation and tRNA movement
What is the role of GTP here? Switch or motor?

Hybrid model (see p. 9 handout 3a)
Elongation is more complicated than direct transfer of tRNAs into the sites

->ALL of the factors experience conformational changes
Complex movement

(model from Noller's footprinting work)

Finally a new tRNA binds the A site and the elongation process is repeated.

TERMINATION

See p. 9 handout 3a

Release factors: RF1, RF2, RF3(GTP)

3 stop codons: stopping does not have a specific tRNA with stop anti-codon associated with the stopping process

RF1 and RF3 together are thought to interact with the stop codon and mimic a tRNA(see structural models)

-RF1 active site allows water to attack (instead of an amino group from an amino acid) to hydrolyze the finished protein chain
-the ribosome is dissembled releasing the mRNA and protein

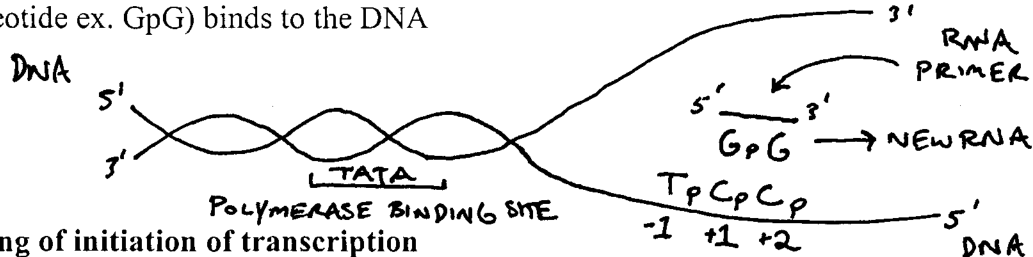
II. METHODS

1. Self-assembly: reconstitution experiment
    a) clone, express, purify all 21 of the proteins
    b) make RNA- Uhlenbeck's method makes unmodified RNA

Uhlenbeck's method
DNA plasmid containing gene for 16 S RNA (for example) and T7 promoter
Transcribe to RNA with RNA polymerase to yield 15-100 mg quantities of RNA

How does RNA polymerase actually work?

Binding of the polymerase to its promoter causes a bubble in the DNA where it unwinds at the site of initiation of transcription.. A ribonucleotide primer (can be a single or dinucleotide ex. GpG) binds to the DNA



Drawing of initiation of transcription

Polymerase adds on NTPs to make a new strand of RNA

The primer can be replaced with a modified G that interacts specifically w/ a probe (like a cross-linker or a photoaffinity label) -> make RNA with probe attached

c) reconstitution of players
How does RNA fold? Can it self assemble? Are proteins involved?

Order of addition for reconstitution of 30S subunit worked out phenomenologically by Nomura in the 1970s

Protocol
Add 16 S RNA (made by Uhlenbeck's method) 45deg C for 15 min
↓
Add group 1 proteins (54,7,8,15,17,20) and incubate at 42 deg for 20 min
↓
Add group 2 proteins (5,6,9,11,12,13,16,18,19) and incubate at 42 degree for 20 min
↓
Add group 3 proteins (2,3,10,14,21) same conditions as above
↓
Forms 30S ribosome subunit!!

KEY: How can you know experimentally that it is assembled correctly??

We can now put probes anywhere in RNA and then reassemble the system!