3.1. Goals of Initiation

- load a specialized tRNA directly into the P site
  - ↑ initiator tRNA
- recruit the ribosome to the start codon(s) on mRNA

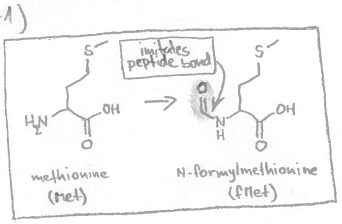
initiation vs. elongation
- similar goals
- different steps

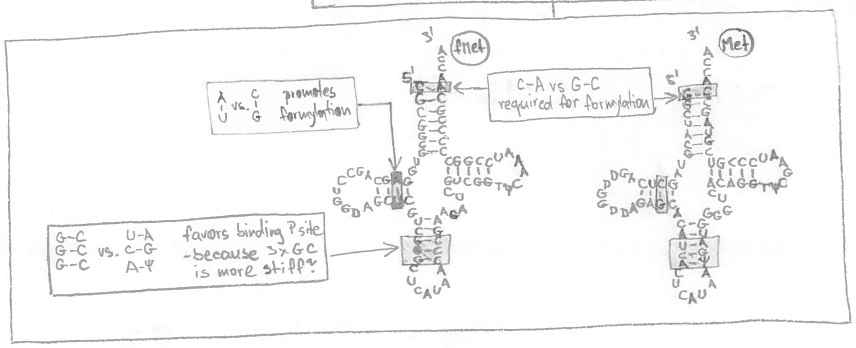
3.2. Bacterial Initiation Factors and Initiator tRNA

- 3 auxiliary factors: IF1, IF2, IF3 (IF = Initiation Factor)
- small ribosomal subunit (30S)
  - mRNA (binds simultaneously w/ fMet-tRNA)
  - either — large ribosomal subunit (50S) ← 30S and 50S dissociate after termination
- E(X) site — IF3 (blocks 50S from binding to 30S)
- A site — IF1 — IF2-GTP — fMet-tRNA (recruited by IF2 AFTER binding to IF1)

contrast w/ EF-Tu, which binds tRNA BEFORE binding to ribosome

$Met + tRNA \xrightarrow{charging} Met\text{-}tRNA \xrightarrow[\text{(enzymatic modification)}]{formylation} fMet\text{-}tRNA$





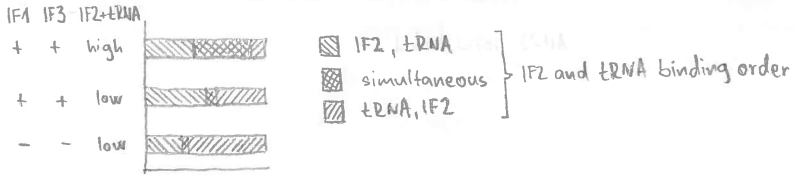
3.3 Bacterial Initiation Continued

- 16S rRNA (part of small ribosomal subunit: 30S): CCUCC ⟷ GGAGG : mRNA ← positions AUG start codon near the P site
- initiator fMet-tRNA: UAC ⟷ AUG : mRNA ← locks-in the reading frame

see ch. 1.3 pg 1.1

- IF3 binding is (slightly) destabilised by tRNA/mRNA base pairing
- IF2-GTP recruits large ribosomal subunit (50S) ← see ch 3.4 ???
  - sarcin-ricin loop (factor-binding center) catalysis hydrolys of IF2-bound GTP
- IF2-GDP dissociates from IF1
  - 50S binds more tightly to 30S
  - IF3 and IF1 are displaced from 30S by 50S

3.4. Experimental Ordering of Initiation Events



- there will be different results for different transcripts
  - eg. depending on number of nts b/w RBS and AUG
    - ~7nt: optimum positioning, easier tRNA binding, even w/o TF2
    - 3nt: relatively bad positioning, harder tRNA binding (w/ or w/o TF2?)

! TF2 is NOT required for recruiting tRNA !

3.5. Eukaryotic Initiation, Pt. I

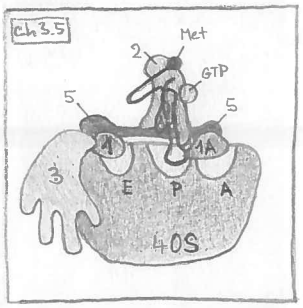
- initiator $tRNA_i^{Met}$ always binds small subunit BEFORE mRNA
- small subunit is recruited to mRNA by initiation factors (not base-pairing)
- start codon is identified by scanning from 5' end of the mRNA

↑ $tRNA_i^{Met}$ has to be already bound in order to recognize AUG

eukaryotes and archaea use Met instead of fMet
- fMet is recognized as foreign material by the immune system
- mitochondria and chloroplasts still DO use fMet

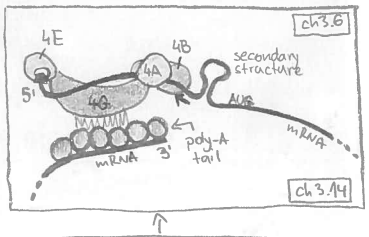
- loading the initiator tRNA to 40S (small ribosomal subunit)
  - E site: eIF1, eIF3, eIF5 bind (very rough equivalent of bacterial IF3)
  - A site: eIF1A binds (rough equivalent of bacterial IF1)

  → blocking A and E sites; displaces large ribosomal subunit (60S)
  - eIF2-GTP binds Met-$tRNA_i^{Met}$ (ternary complex)
  - eIF2-GTP-Met-$tRNA_i^{Met}$ binds small ribosomal subunit (40S) + eIF1,3,5,1A ← 43S pre-initiation complex (PIC)



3.6. Eukaryotic Initiation, Pt. II

- preparing mRNA for small ribosomal subunit (40S) recruitment
  - eIF4E binds 5' cap of mRNA
    - regulation: prevent eIF4A from interaction w/ other ↓ proteins (see discussion on regulation)
  - eIF4G + eIF4A bind to eIF4E-mRNA
    - eIF4F = eIF4G + eIF4A + eIF4E (historically, they have been purified as a complex, hence single name)
  - eIF4A (RNA helicase) removes secondary & tertiary structures from 5' end of mRNA
    - it has been thought that eIF4B stimulated activity of eIF4A
    - new data suggest that eIF4B plays more of a role in opening up the binding site for RNA in ribosome
    - → eIF4B stimulates binding of mRNA to ribosome, interaction w/ eIFA was incorrectly assumed
  - 43S pre-initiation complex binds mRNA/eIF4G/A/E/B ← 48S pre-initiation complex (PIC)
    - eIF3 binds eIF4E
    - eIF1A binds eIF4A



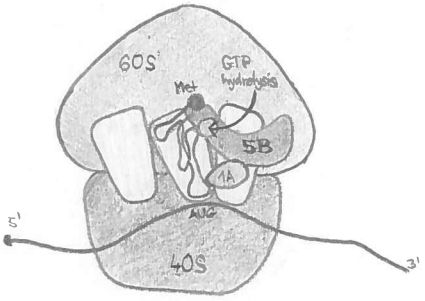
poly-A binding protein Pab1 (RRM2 domain) binds to eIF4G
ch 3.14

3.7. Eukaryotic Initiation, Pt. III

- identifying the start codon
  - 43S PIC scans 5'→3' until the $tRNA_i^{Met}$ base-pairs w/ AUG (ALMOST always the 5'-most AUG)
    - stimulated by eIF4A helicase activity
      - it would work also w/o eIF4A (randomly moving over mRNA until bp is made); eIF4A direct the search
      - eIF4A is not very "strong" helicase, bp w/ AUG is strong enough to hold against eIF4A's helicase activity
  - base-pairing b/w $tRNA_i^{Met}$ and mRNA (AUG) causes hydrolysis of GTP in eIF2-GTP
  - eIF2-GDP dissociates from its substrate
  - all other factors except eIF1A dissociate (eIF1/3/5/4B, eIF4E/G/A)
  - eIF5B-GTP binds to $tRNA_i^{Met}$ + eIF1A

3.8 Eukaryotic Initiation and Factor Comparison

- binding of large ribosomal subunit (60S)
  - eIF5B-GTP recruits large subunit
  - eIF5B hydrolyses GTP (catalyzed by factor binding site/sarcin-ricin loop)
  - eIF5B dissociates
  - large subunit binding displaces eIF1A



- factors: eukaryotic vs. bacterial
  - eIF1,3,5 ≈ IF3
  - eIF1A ≃ IF1
  - eIF2 ($tRNA_i^{Met}$ recruitment) } ≈ IF2
  - eIF5B (60S recruitment) }
  - eIF4E,G,A,B → no bacterial equivalent (bypassed by RBS)

3.9. Internal Ribosome Entry Sites (IRESs)

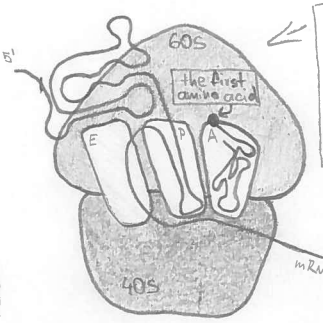
- not all euk. mRNAs require 5' cap
  - use internal ribosome entry sites (IRESs)
    - Group I: directly recruit 80S (full ribosome)
      - do not require any of euk. initiation factors (eIFs)
      - rare, all known examples are viral (e.g. knock off eIF4E → 5' caps no longer recognized → all ribosomes available to the virus as long as its mRNA can initiate w/o knocked off eIF4E)
    - Group II: recruits the small subunit (40S)
      - require subset of eIFs + Met-tRNA$_i^{met}$
    - Group III:
      - require IRESs specific binding factors (recognize IRES in similar way to txn factors recognizing their binding sites)
      - require (subset of) eIFs + Met-tRNA$_i^{Met}$

some viral mRNAs do not need 5' cap
- alternate set of factors, bypassing one or more steps in tnl initiation
- 5' end folds into cap-like structure, second codon in ORF serves as start codon

3.10. CrPV Example of IRES

- CrPV: Cricket Paralysis Virus
- 5' end of CrPV mRNA binds directly in P-site of full ribosome (80S)
- 5' end folded, mimicking
  - 5' cap
  - P tRNA (P/E hybrid state, w/ acceptor arm in E site)
    - self-basepairing with coding portion of mRNA
- the first codon AFTER the start codon encodes THE FIRST AA
  - resulting protein can start w/ any aa (not just Met)

not necessarily UAC AUG



Group I (CrPV)
- bypasses all initiation machinery
- still needs
  - termination machinery
  - elongation machinery

ch.2: eEFI (eEFIα), eEFII

! eEFs vs. eIFs !
ch2 ch3

3.11. More IRES Examples

Group 1&2: mainly viral
Group 3: mainly cellular

- Group 2
  - mRNA (IRES) ⟵ eIF4G : bypasses eIF4E (see ch.3.6, pg.3.2)
    - ⟵ eIF4A
- Group 3 (example: apoptosis - cell death)
  - eIF4E is destroyed in early stage of apoptosis (new proteins no longer synthesized)
  - still need to synthesize a few proteins
    - DAP5 (Death Associated Protein 5) - IRES dependent tnl associated factor (ITAF)
      - binds specific mRNA sequences in cellular RNAs: possibly in the middle of ORF (truncated protein - "intentionally", to e.g. bypass regulation)
      - mimics part of eIF4G
        - including: eIF4A binding region - continues normal process of tnl initiation
        - excluding: eIF4E binding region - not needed: eIF4E has been destroyed & is being bypassed

e.g. by removing inhibitory domain of a nuclease

3.12. Termination

- two key events
  - release protein from tRNA
  - release ribosome from tRNA/mRNA (ribosome recycling)
- stop codons recognized by proteins (not tRNAs)
  - mediated by class I release factors
    - mimic tRNA (shape and most importantly dimensions)
    - tRNA shape + extra region
    - bacteria
      - RF1: recognizes UAA, UAG ← Proline, Valine, Threonine (PVT)
      - RF2: recognizes UAA, UGA ← Serine, Proline, Phenylalanine (SPF)
        - the only thing ensuring specificity
    - eukaryotes - eRF1: recognizes UAA, UAG, UGA ← Asparagine, Isoleucine, Lysine, Serine (NIKS)
  - end of "acceptor arm" - hydrolysis motif - in RF1/RF2: Glycine, Glycine, Glutamine (GGQ)
    - stimulate peptide hydrolysis ("tricks" the ribosome into accepting water instead of amino group of aa, as the nucleophile)
    - some conserved bases of peptidyl transferase center (see ch.1.13, pg.1.4) are also involved in peptide hydrolysis

RF1 and RF2 are otherwise similar enough for modification in these 3 aas to cause change in specificity for particular stop codons

NOT analogous to RF1/RF2

see ch.2.6, pg.2.2

3.13. Bacterial Tnl Termination and Ribosome Recycling

! NOT GTP !

- RF3-GDP (Release Factor 3 - classII release factor) binds
  - GDP → GTP: conformational change, displaces RF1/RF2
  - GTP → GDP hydrolysis (sarcin-ricin loop): RF3-GDP dissociates
- ribosome: A site: empty, E and P sites: tRNAs, ! no protein (it was already released, see ch 3.12) !
  - EF-Tu+aa-tRNA - probes A site: no protein → "no deal"
  - RRF (Ribosome Recycling Factor) binds (in the way that looks like tRNA that undergone peptidyl transferase: half of A site empty - see ch.2.7., pg.2.3)
- EF-G binds
  - translocation
    - E-tRNA released
    - P-tRNA released
    - RRF: A → P site, no affinity for P site → released

RRF has affinity for A site only (different interactions w/ ribosome than tRNA

  - GTP → GDP hydrolysis (sarcin-ricin loop): EF-G-GDP dissociates

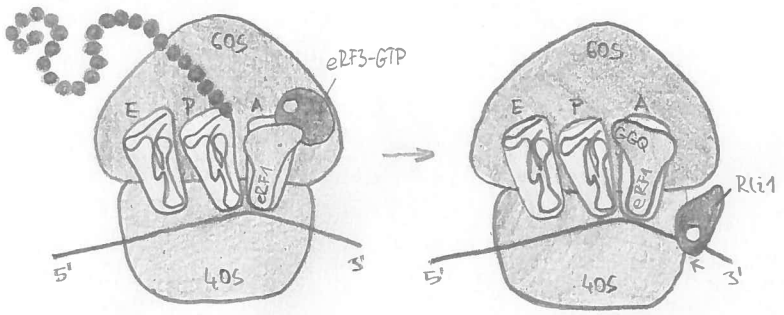
ch 3.2, onwards

- ribosome: A, P, E sites empty, mRNA still present
  - IF3 (see ch.3.2, pg 3.1) binds ← need IF1 to bind and continue w/ next tnl cycle
  - ribosome subunits separated
  - mRNA released

3.14. Eukaryotic Tnl Termination and Ribosome Recycling

GTP
eRF3 binds to eRF1 (see ch.3.12, pg. 3.3) and delivers it to stop codon
- eRF3 ≈ eEF1/EF-Tu
- if the stop codon is recognized by NIKS motif → factor binding center (sarcin-ricin loop) catalyses eRF3-GTP → eRF3-GDP hydrolysis
  - eRF3-GDP is released
  - eRF1 undergoes accomodation-like (B3→B4, ch 2.3, pg 2.1) event
    - GGQ hydrolysis motif (ch 3.12, pg 3.3) is positioned to stimulate peptide hydrolysis
      - conserved b/w prokaryotes and eukaryotes

Rli1-ATP binds mRNA and forces ribosomal subunits apart (helicase activity), releasing all factors/tRNAs/mRNA in process
- ! NOT WELL UNDERSTOOD !
  - using ATP hydrolysis

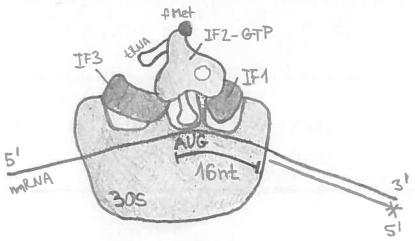


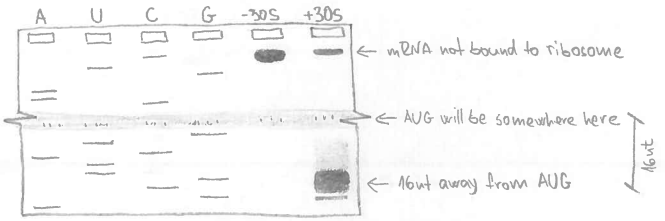
- happens more frequently w/ small mRNAs
- small mRNAs are in general better translated (than long mRNAs)

- eukaryotic mRNA
  - tends to be in circular state: eIF4G binds poly-A tail of mRNA (see ch.3.6, pg 3.2)
  - places stop codon near start codon → may make it easier to restart ttn after termination
  - circular state of mRNA is fact, tnl optimization ↑ is not clear though !!!

3.15 Toeprinting Assay

- measures whether the ribosome has been correctly positioned over start codon





assemble the initiation complex
- process
  - substrate (30S, factors, tRNA, mRNA, ...)
  - labelled primer
  - reverse transcriptase
    - extends labelled primer
    - can not access template protected by ribosome (16nt from P site)
  - separate on denaturing acrylamide gel
    - RNA not labelled → will not show
    - extended primer labelled

3.16 Hydroxyl Radical Footprinting

- identifies binding sites of proteins on ribosome

	traditional footprinting	hydroxyl radical footprinting
cleavage of nucleic acid by	nuclease	•OH
cleavage detected via	end-labelling of nucleic acid	primer extension on nucleic acid template

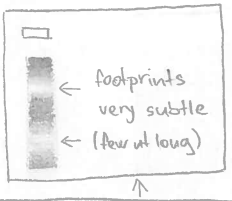
Fenton Reaction
$Fe^{2+} + H_2O_2 \rightarrow$ •OH + $^-$OH
↘ cleaves ribose of RNA/DNA

•OH is very small (MUCH smaller than nucleases) ← DNase/RNase too big to get to the binding site

after cleavage around bound protein
- anneal primer (labelled)
- extend w/ reverse transcriptase

need more primers to start extending close to the cleavage site

end-labelling would not work on rRNA (too long RNA → false stops before reaching the site of protein binding)

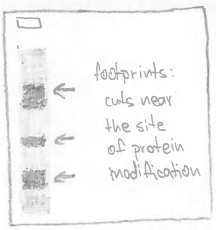


- protein-dependent hydroxyl radical cleavage
  - modify protein w/ Fe^{2+} binding chemical
    - in a unique position: usually done by using single-Cystein-derivative of the protein
    - need to be close enough for radicals to act at the correct place (they are very short lived)
    - need to make sure that protein still binds and is functional (mutations close to its active site)

vs. not a problem there, because there is a lot of radicals there

•OH radical very small
- only very tightly bound protein protects RNA
- RNA not fully protected (some signal present even from footprints)
- ribose is being cut
  - base-pairing will NOT protect RNA
  - only backbone interactions protect RNA

Protein INdependent hydroxyl radical cleavage



3.17 Antibiotics and Analogs

ANTIBIOTIC	TARGET	EFFECT
puromycin	peptidyl transferase centre	terminates chain
chloramphenicol	large subunit A-site	prevents tRNA binding to A-site
erythromycin	peptide exit tunnel	stalls protein synthesis
edeine	small subunit E-site	prevents IF3 binding, inhibiting initiation
tetracyclin	small subunit A-site	prevents tRNA accomodation

← - mimics tRNA in A-site of large subunit
- undergoes peptidyl transferase reaction
- unfinished peptide is released (puromycin is not bound to mRNA and is very small)

↑ can be used as a measure of peptidyl transferase activity

- antibiotics can be used in various assays (apart of their use in medicine)
- non-hydrolyzable GTP analogs
  - GTP-γ-S
    - has sulphur instead of oxygen on the γ phosphate
    - dramatically slows down (or even completely inhibits) GTP hydrolysis
    } strong inhibitor
  - GMP-PCP
    - has carbon instead of oxygen b/w last two (β,γ) "phosphates"
    - completely non-hydrolyzable
    } true non-hydrolyzable analog

vs.: GMP-PCP has different bond distances and angles ⇒ will not bind in some cases

- used for determining steps before/after GTP hydrolysis