4.1. Amino Acid Incorporation Assay

measures bulk translation using radiolabelled amino acids

- process
  - add labelled aa precursors
    - almost always ^{35}S-labelled Met and/or Cys
    - historically: tritiated aas
  - take time points - measure incorporation of labelled aas

usually include Met ⇒ at least the first aa will be labelled ← unless it is post-processed

either cell extract or living cells ← may have consequence on cellular growth/metabolism
starve cells of Met/Cys → they will take up Met/Cys from solution

SDS-PAGE
- SDS = Sodium Dodecyl Sulfate - linearizes protein and imparts negative charge
- PAGE = Polyacrylamide Gel Electrophoresis
- hard to find out overall protein content (separates out the proteins ← need to reintegrate)

TCA precipitation - TCA = Tri Chloro Acetic acid (CCl_3COOH) - very strong acid
- add TCA (10-20%) to translation reaction/crude cell lysate ←
  - proteins: precipitate
  - aas: remain soluble
- collect precipitate on filter paper
- measure radioactivity (scintillation counting)

usually do not need to lysate - most cells lyse at pH ~2, induced by presence of TCA

linearizes proteins (by disabling H-bonds) ⇒ exposes hydrophobic regions ⇒ interaction of hydrophobic regions causes protein precipitation; aas just go to ⊕ charged state appropriate to pH ~2

- best way to get amount of total protein
- hard to get amount of particular protein

but there are better ways nowadays → ← can be done by immunoprecipitation followed by SDS-PAGE

4.2. Polysome Profiling

measures bulk tln by quantifying the ribosomes bound to mRNA

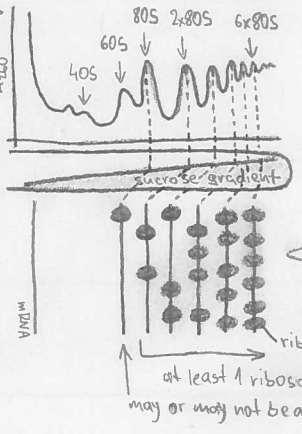
- polysome - mRNA bound to multiple ribosomes → being actively translated

in vivo assay ← includes cell extracts!

monosome: ribosome can be e.g. arrested at the initiation step
polysome: it is very hard to have multiple ribosomes bound to mRNA w/o any of them actively translating

- process
  - incubate cells w/ elongation inhibitor (optional) - arrest translation
    - e.g. cycloheximide (interferes w/ translocation step)
    - need to keep ribosome bound to mRNA
  - lyse cells
  - fractionate lysate by ultracentrifugation
    - sucrose gradient (velocity gradient)
    - 'big' particles sediment faster
  - monitor RNA levels accross the gradient by spectrometry
    - A_{260nm}
    - most cellular RNA is rRNA
  - RT-PCR (Reverse Transcriptase PCR) can be used to get information on individual RNA
    - fractionate sediment
    - identify RNAs present in individual fractions

- sufficient for measuring changes in the level of tln (globally within the cell)
- higher number of heavy polysomes ⇒ high overall efficiency



one ribosome may (or may not) be arrested at the initiation step

at least 1 ribosome actively replicating
may or may not be actively replicating

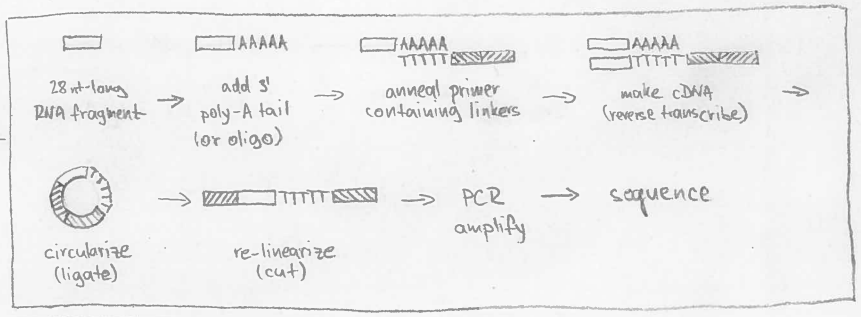
80S, 2x80S, ... 6x80S, ...

information on how actively is given mRNA being translated

4.3. Ribo-Seq

measures genome-wide tln, provides mRNA-specific data

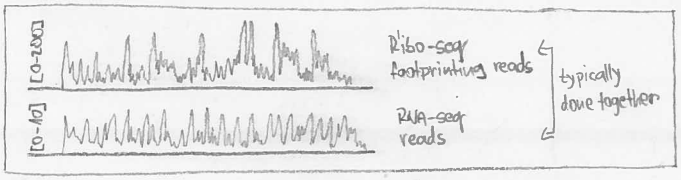
- process
  - treat polysomes w/ RNase
  - isolate 80S ribosomes using sucrose gradient
  - isolate (typically) 28nt-long mRNA fragments
  - convert to DNA and sequence



- # of copies of a protein per mRNA
  - varies widely
  - can be partially explained by translational efficiency

~1000-fold differences down to ~20-fold differences

protein expression regulation at
- transcription
- translation



$\frac{\text{Ribo-seq}}{\text{RNA-seq}}$ = Translational Efficiency (TE)

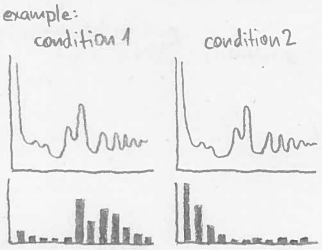
4.4. Translational Regulation

- translational efficiency: number of proteins made per mRNA per unit of time ← unit of time varies: e.g. bacterial tnl is faster than euk. tnl → minutes vs. hours
- translational control
  - basal TE (↑) of each mRNA (which varies b/w different mRNAs)
  - regulated changes in TE of an mRNA
- translational vs. transcriptional regulation (is translational regulation needed? isn't transcriptional regulation enough?)
  - faster at changing protein levels
    - increase - ✓
    - decrease - it is much faster to directly degrade the protein
  - allows regulation when there is no transcription (e.g. early embryonic development)
  - allows to control where in the cell is the protein made - localize site of translation ← especially euk. - all txn is localized to nucleus
    - direct mRNA to specific location
    - localize translation activators/repressors
  - allows to differentially regulate production of different proteins from the same polycistronic mRNA ← bacterial specific
  - can rapidly inhibit bulk translation → prevent catastrophic protein misfolding (e.g. under stressful conditions)

→ would be very difficult even in prokaryotes (most chromosome tethering has to do with replication - oriC & termination sites)

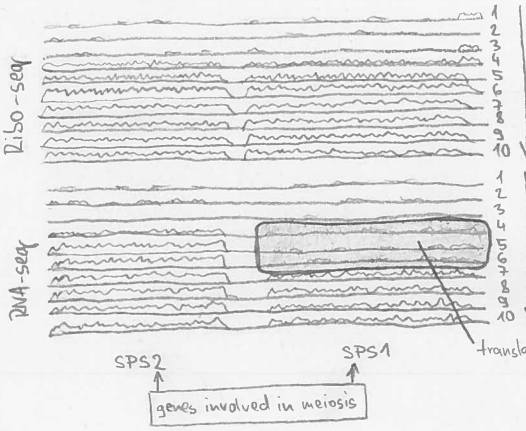
4.5. Examples of Assaying for Translation Regulation

- example:



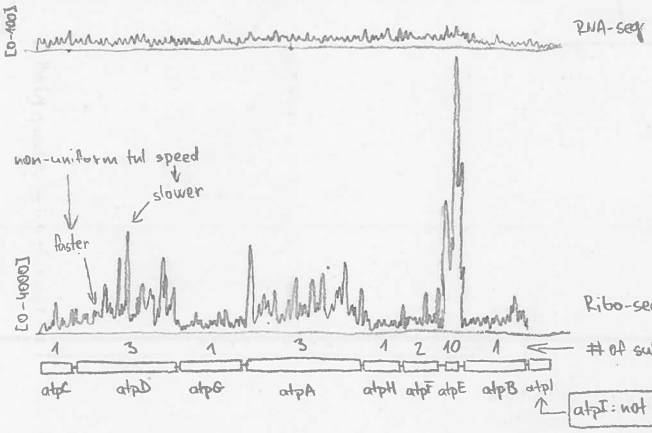
  - roughly the same amount of mRNA under both conditions → txn largely unaffected
  - much lower translation levels under condition 2

- example: cells going through meiosis in a synchronous manner



limitations: cannot distinguish
- change in protein degradation vs. translation
- change in initiation vs. elongation of translation
- if the ribosome on mRNA is actively translating or paused

- example: ATP synthase complex encoded in single polycistronic mRNA



atpI: not part of ATP synthase, poorly expressed

4.6. Rate-Limiting Step

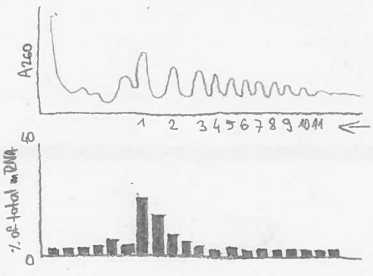
max ribosome density: ~ 1/35nt ← 28nt protected by ribosome + some additional space

- ribosome density: number of ribosomes per unit of length of ORF
  - low: limited by initiation step ←
  - high: limited by elongation step ←

usually 100nt (by convention: most of ORFs are longer than 100nt)

it either takes a long time to load new ribosome to mRNA, lowering overall ribosome density, or it takes a long time for initiated ribosome to move room for next one, while producing the protein (elongation), raising overall ribosome density

- polysome profiling: find out how many ribosomes are loaded on mRNA
  - need resolution high enough to be able to identify individual peaks (monosome, disome, trisome, ...)



(S. cerevisiae) ← only 72 genes had density ≥ 1 ribosome / 50 nt
average ribosome density: 1 ribosome / 154 nt
maximum ribosome density: 1 ribosome / 35 nt

↓

initiation is the rate-limiting step most of the time

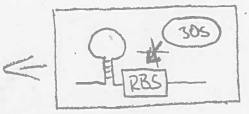
↑

there is not much evidence saying that elongation may be the rate limiting step

4.7. Bacterial Translation Regulation, Pt. I

- mostly regulated step: mRNA & fMet-$tRNA_i^{fMet}$ loading
- modulation of mRNA translation (basal levels)
  - alter
    - RBS sequence (GG-AGG - see ch 1.3, pg. 1.1)
    - distance b/w RBS and start codon (3-9nt, 6-7 is optimal)

4.8. Bacterial Translation Regulation, Pt. II



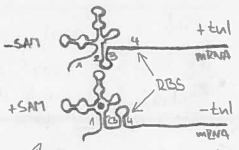
  - create 2° structures near or including the RBS
    - unlike eukaryotes w/ eIF4A helicase activity, there is no such mechanism in bacteria
    - experiment
      - synthesis of 154 GFP transcript variants
        - varied mRNA structure by introducing silent mutations in region 5nt upstream - 40nt downstream
      - measure GFP fluorescence (translation)
      - test effect on codon usage variation -> found no effect on translation

4.9. Bacterial Translation Regulation, Pt. III

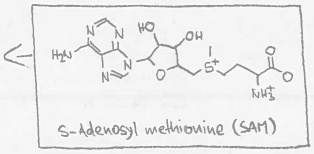
- methods to regulate the efficiency of individual mRNAs
  - protein dependent
    - a protein binding to mRNA near RBS, sterically preventing 30S binding
      - mRNA internal hybridization
      - typically upstream
      - or directly with RBS
      - binding motifs
      - different regulatory factors can bind different mRNAs
      - ↑ different mRNAs can have different sequences present in 5' non-coding region, near RBS
  - RNA dependent
    - polar effect - ORF hybridizes with binding motif near RBS, creating 2° structure that prevents 30S from binding
      - ribosome translating the ORF disrupts the secondary structure, allowing translation of the disabled ORF
      - example
        - ORF1 basepairs w/ RBS of ORF2 => ORF2 is disabled
        - ribosome translating ORF1 disrupts the basepairing => ORF2 is reenabled
        - allows co-regulation of ORFs on the same operon
        - by disabling ORF1, also ORF2 will be disabled
          - e.g. by inserting stop codon early in the transcript, such that the ribosome terminates before disrupting the 2° structure

4.10 Bacterial Translation Regulation, Pt. IV

- riboswitch dependent regulation
  - ↑ RNA molecules that change their folding in response to small molecule binding



example: mRNA encoding SAM



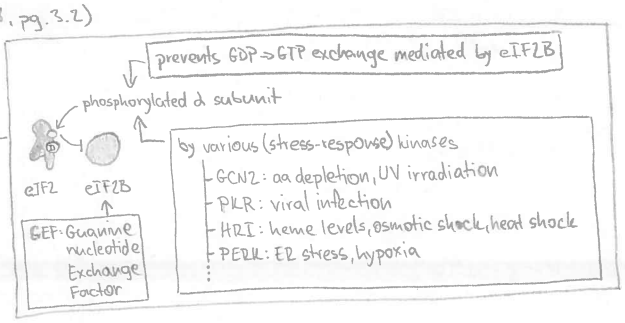
there are many of these riboswitches (the above is one example only)

  - small RNA regulation
    - bacterial sRNA typically 80-110nt
    - activating

    - repressing

4.11. Eukaryotic Translation Regulation, Pt. I

- targets of regulation (3 events in tln initiation)
  - binding of ternary complex (eIF2-GTP + Met-$tRNA_i^{Met}$) to 40S (see ch. 3.5, 3.7, 3.8, pg. 3.2)
    - global tln repression/activation (as opposed to per-mRNA regulation)
    - mechanism
      - eIF2 hydrolyses the GTP as part of its release process from 40S
      - eIF2B catalyzes GDP ⇒ GTP exchange
      - eIF2-GTP can form ternary complex and bind 40S again



4.12. Eukaryotic Translation Regulation, Pt. II

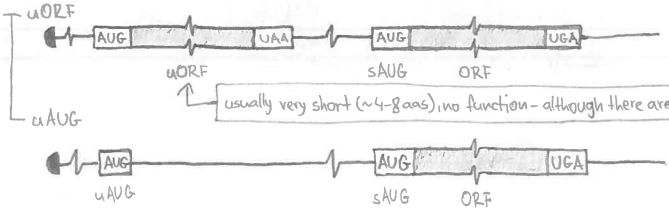
- assembling 5'-cap binding factors on mRNA (see ch 3.6, pg. 3.2)
  - global tln activation/repression (as opposed to per-mRNA regulation)
    - mechanism – 5' cap → eIF4E → eIF4G / 4E-BP (compete for binding (sterically)) ← kinases (growth-regulating) phosphorylate 4E-BP
      - 4E-BP: (eIF) 4E-Binding Protein
      - only NOT phosphorylated
  - local (i.e. individual mRNAs separately)
    - mechanism – 3'UTR (downstream of ORF) → Bruno → cup (4E-BP) ← achieves mRNA-specific regulation by tethering 4E-BP to particular mRNAs

4.13. Eukaryotic Translation Regulation, Pt. III

- scanning mRNA, recognizing the start codon
  - local (i.e. individual mRNAs separately)
    - mechanism
      - stabilizes 2° structures in 5'UTR (upstream of AUG) by binding of regulatory factors
      - eIF4A (helicase) can remove 2° structures from mRNA, but not ones stabilized
      - example: ferritin: "iron-scavenging" protein – 2° structure in 5'UTR stabilized by binding of IRP (Iron Regulatory Protein)
        - hairpin
        - does NOT bind, when bound to Fe^{2+}
        - ferritin expressed only in presence of free Fe^{2+}

4.14. Upstream ORFs, Pt. I

- WTF: ~50% of human genes do have an upstream AUG (uAUG)
  - can be used generally (as opposed to specifically), to modulate level of translation
  - uAUG/uORF – negative regulators



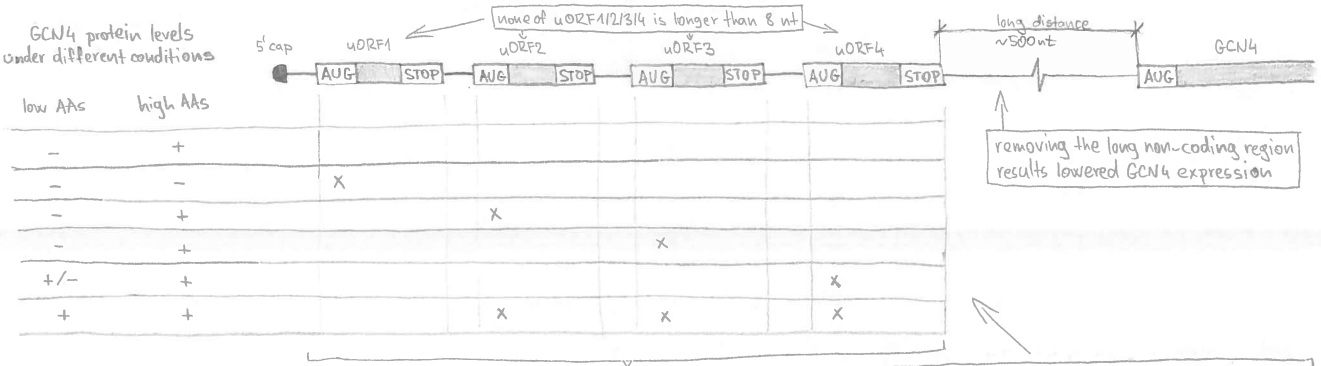
- usually very short (~4-8 aas), no function – although there are exceptions
- ← the ORF should not be translated (ribosome initiates on the first AUG and terminates at the end of uORF)
- !!! but see ch. 4.15 !!!
- N-terminal extensions usually do not have profound effect on protein function
  - in frame: resulting protein will have extra N-terminal extension
  - out of frame: protein resulting from ORF is usually not functional due to frame shift
- !!! but see ch. 4.15 !!!

4.15. Upstream ORFs, Pt. II

- methods to translate downstream AUG
  - bypass the 1st AUG (not 100% of the time, though → negative regulation)
    - Kozak consensus sequence G/ANNNAUGG (see ch. 1.4, pg. 1.2) → increases the probability of bypassing the first AUG
      - at the 2nd AUG
      - start
    - 2° structure near/overlapping the 1st AUG
  - reinitiate after termination at the end of uORF (not 100% of the time, though → negative regulation)
    - works only for short (~3-10 nt) ORFs – some of initiation factors remain associated w/ translating region for short while
    - 40S subunit remains associated w/ mRNA and continues scanning
    - influenced also by sequence downstream of stop codon

4.16. Upstream ORFs in GCN4

- example: GCN4 translation regulation under different conditions
  - ↳ transcription factor and "master regulator" for gene expression (regulates close to 1/10 of yeast genome)

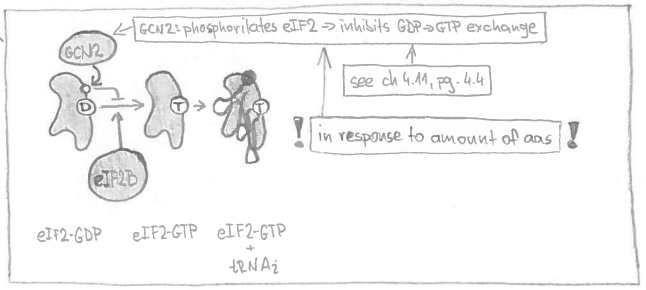


low AAs	high AAs	uORF1	uORF2	uORF3	uORF4	GCN4
–	+					
–	–	X				
–	+		X			
–	+			X		
+/–	+				X	
+	+		X	X	X	

x = mutation in respective AUG (i.e. disabling ORF) ← mutations inside uORF1/2/3/4 do not have any effect ↑ apart of mutations to start/stop codons

- uORF1 – ~40% of ribosomes that translate uORF1 will retain the 40 subunit after termination
  - continue scanning for AUG ← !!! no $tRNA_i$ => "flying blind" !!!
- uORF2/3/4 – do not show substantial reinitiation, after they have been translated
  - will be recognized only if $tRNA_i$ is present
  - "traps" ← if $tRNA_i$ arrives soon enough for uORF2/3/4 to be recognized, translation stops after termination on recognized uORF (no reinitiation)

! regulation is NOT based on amount of Met present in the cell →



4.17. Translation Optimization

- codon bias – e.g. E. coli Gly codons
  - GGU: ~34%
  - GGC: ~40%
  - GGA: ~11%
  - GGU: ~15%
  - } $tRNA_s$ abundance also reflects this
  - ↑ codon distribution
  - ← – no measurable effect in normal situations
  - – makes difference when e.g. using E. coli to produce large amounts of single protein
- ribosome pausing
  - codon independent
  - caused by presence of GGAGG sequence (inside ORF), that seems to interact w/ ribosome, slowing down translation
  - ↓ see ch. 13, pg 11; ch. 33, pg 31: RBS, 16S rRNA