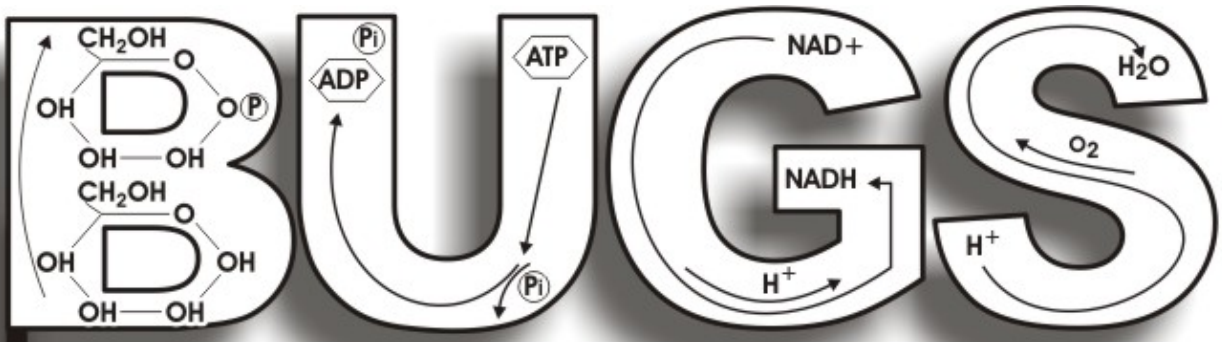
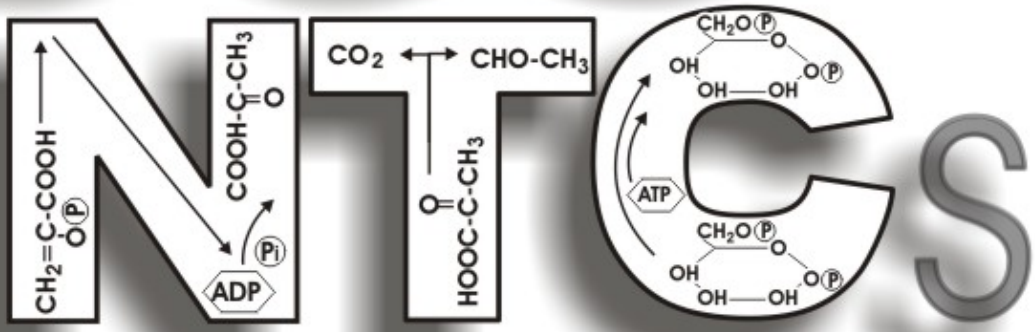




BIOC 312

SET # 8 Lectures 27-29 (03/19/07 –03/23/07)

ANNOUNCEMENTS

- VOTE for next year's BUGS council! Polls are open April 3rd to 4th in all Biochem classes. It takes 2 seconds and has an impact on your undergraduate career!
- **The BUGS office will be closing Thurs. April 5th. Pick up your NTCs, your NTC writing cheques, clothing or anything else you need from BUGS before this date!**

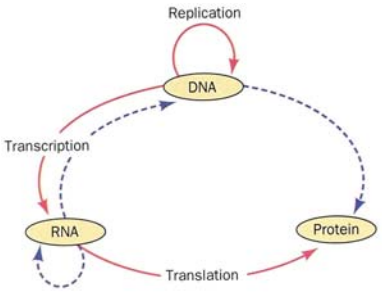
< Biochemistry Undergraduate Society - 5th Floor McIntyre rm. 511 >
< bugs@sus.mcgill.ca - (514) 398-5247>

03/19/2007

Lecture #27–RNA Processing & Stability

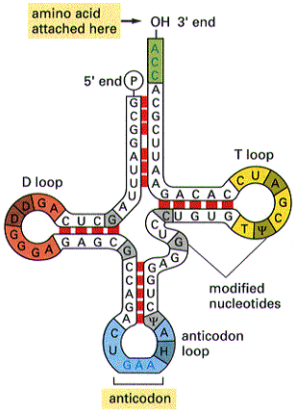
NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**

The "handout" can be found on WEBCT under "Course Content"
In the next 3 lectures we will discuss Translational control.



The Central Dogma of molecular biology

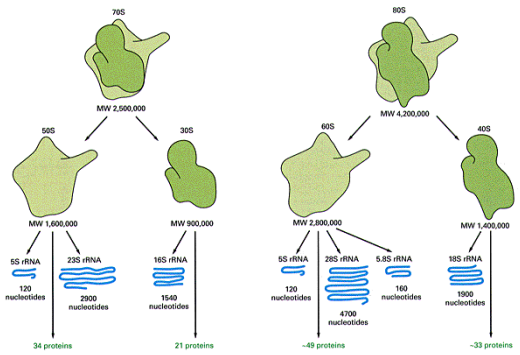
- Coined by Watson and Crick
- DNA → RNA → protein.
  - This was once thought to be only unidirectional, but later turned out that RNA → DNA is possible via reverse transcriptase
  - RNA is a large source of genetic information
  - But protein → RNA is not possible due to the complexity of the machinery involved
- It is now believed that RNA was the first type of genetic material to exist.



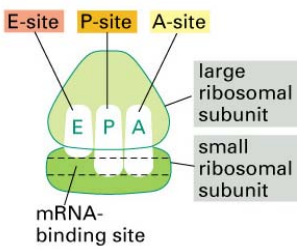
Transfer RNA (tRNA)

- Acts as the "go between" for AAs and mRNA
- tRNA base pairs with its specific codon (codon-anti-codon interaction) and recognizes a cognate **aminoacyl tRNA synthetase** bound to the specific AA encoded by the codon, which transfers this amino acid onto the tRNA.
- It is the first RNA ever sequenced (*figure 2*, right); due to its short sequence.
  - Characteristic clover-leaf structure with **anti-codon** region and **3'OH** where AA is esterified and connected to the tRNA.
- All tRNAs have their own identity specified by their terminal 5' and 3' sequences.

Ribosomes



- The ribosome is the largest cellular molecular machine.
- All ribosomes have one small subunit and one large subunit.
- Eukaryotic ribosomes are bigger than prokaryotic ribosomes due to their protein strucutre and longer rRNA composition.
  - Both ribosomes perform the same function; however the **eukaryotic ribosome is subject to more regulation.**
- Image at right: Prokaryotic (left) versus Eukaryotic (right) Ribosomes
- Researchers have thoroughly analyzed the prokaryotic ribosomal subunits and the different binding sites.
  - What still must be researched are the dynamics of translation via ribosomes, which occurs very quickly (in minutes).
- Ribosomal binding sites (*figure 5*, right).
- **A** = acceptor, where aminoacyl tRNA binds.
- **P** = peptidyl, occupied by newly elongated peptidyl-tRNA.



- **E** = exit, where tRNA leaves the ribosome.

Eukaryotic vs Prokaryotic Initiation

- there is a large difference in initiation translation although the elongation phase is very similar.
- the reason for this difference is due to the increased levels of control in the eukaryotic translation system

Structure of Eukaryotic and Prokaryotic mRNA

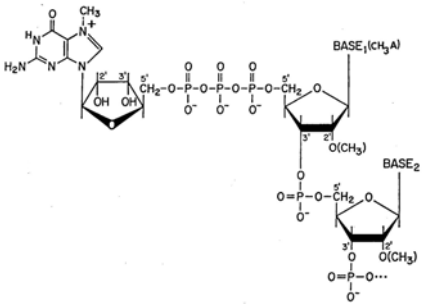
- Prokaryotic mRNAs have a 5' triphosphate while eukaryotic have **5' cap** structure.
- Most eukaryotic mRNAs have **polyA tail** (with exception of histone mRNAs), while some prokaryotic mRNAs have a stretch of adenines.
  - The stretch of adenines in prokaryotic mRNAs is involved in degradation whereas eukaryotic polyA tail is important for stability and translation.
- Prokaryotic mRNAs are very labile (half-life of minutes), whereas eukaryotic mRNAs are more stable with longer half-lives (hours to weeks).
- There is no nucleus in prokaryotes (thus simultaneous transcription and translation occurs), mRNAs are modified differently than in eukaryotes.
- There was a paper in Science which claimed that eukaryotic mRNAs can be translated in the nucleus, but since the experiment couldn't be repeated, this is not a widely accepted theory.
- Many prokaryotic mRNAs are **polycistronic**; they have different initiation sites so several proteins can be translated from one mRNA encoding several genes.
  - There may be simultaneous translation at multiple reading frames.
- Eukaryotic mRNA are monocistronic with some exceptions which shall be discussed later.

Steps in mRNA Synthesis

- Transcription in eukaryotes is initiated by RNA pol II
- The cap is added very early in transcription, following the transcription of the first 10 nucleotides.
- Pre-mRNA is cleaved; determined by a cleavage signal
- Then the polyA tail is added; which is composed of 250-300 adenosines
- Introns are spliced out; other methylations may occur before this step
- The mature mRNA is then exported out of the cytoplasm.
  - All eukaryotic mRNA synthesized in the nucleus by RNA pol II are capped.

Structure of the 5' Cap (*figure at right*).

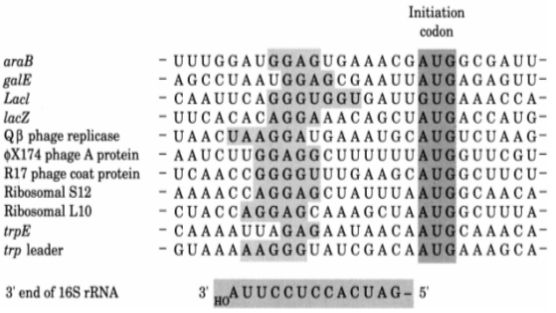
  - There is a methyl group is on the first base (guanine) to form 7-methylguanosine
  - This is linked via a triphosphate ester bond to the first mRNA ribonucleotide via a very unusual 5' - 5' linkage (usually 5' to 3' bond).
- Many mRNAs in higher eukaryotes have other modifications.
  - i.e., methyl groups can be located at positions 2' carbon of the ribose
  - The functions of these modifications include protection from 5' exonucleases, facilitating translation, nuclear export and splicing as well as other mRNA processing.
- Capping of mRNA:
  - RNA triphosphatase remove the phosphate group on the 5' nucleotide.
  - mRNA guanylyltransferase takes a GTP and incorporates it into the 5' end of the mRNA.
  - Different mRNA methyltransferases put methyl groups onto N7 of guanine and C2' of ribose using S-adenosylmethionine as the methyl donor



- Transcripts made by polymerase I or III are not capped; only pol II transcripts are capped.
- Polymerase II also makes small nuclear RNAs (snRNA) and microRNAs

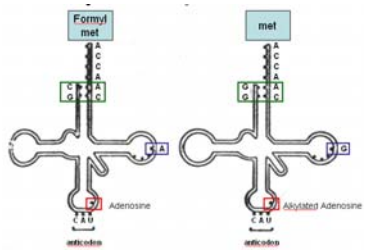
Translation Initiation in Prokaryotes: The Shine-Dalgarno Sequence

- There are many differences between initiation in prokaryotes and eukaryotes.
- Elongation and termination are similar in both.



In prokaryotes, the determinant factor in recruiting the ribosome is the **Shine-Dalgarno (SD) sequence.**

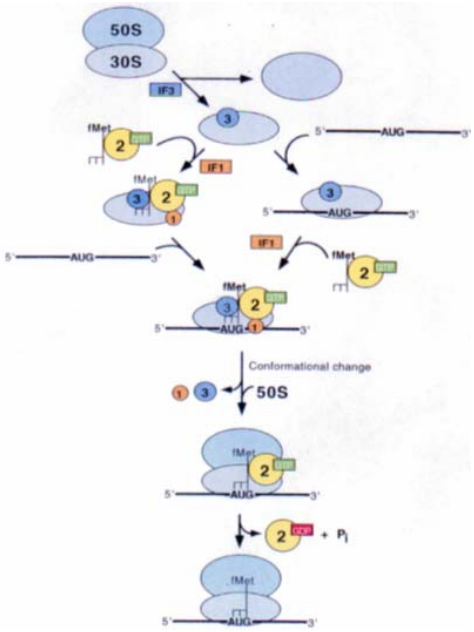
- Upstream of the start codon is a purine rich sequence, the Shine-Dalgarno sequence.
  - It base pairs with the 3' end of the 16S rRNA.
  - Base pairing is not sufficient for initiation but it is a necessary process. If there is a mutation in the, the ribosome will not be able to bind to mRNA.
  - A suppressor mutation can compensate for that mutation and will restore base pairing.
  - The fundamental sequence of the Shine-Dalgarno is not important, but its ability to base pair with the ribosome is
  - This mechanism does not apply to eukaryotic mRNA.



- It was determined that methionine corresponded to an AUG initiation codon
  - Sometimes there is a 1 base pair differentiation in the codon sequence, but the codon will still code for Methionine, which is necessary in order to initiate translation
- In prokaryotes, the methionine is formylated (CHO group) at nitrogen. Thus N-formyl methionine is the only form of methionine used for initiation. This is not seen in eukaryotes.
- formylMet and Met tRNAs have different structures. The two tRNAs are used for initiation and elongation, respectively. Their sequence is different in several places.
- fMet interacts with initiation factors (needed for initiation only), while Met tRNA interacts with elongation factors.

Prokaryotic Translation Initiation *(figure at right)*

- Involves three initiation factors that are each composed of one polypeptide
  - these are IF1, IF2, and IF3
- The purpose of IF3 is to keep the 50S and 30S ribosome subunits dissociated since initiation must occur with only the 30S ribosomal subunit.
- The fMet anticodon base pairs with AUG in complex with GTP
- A ternary complex composed of GTP, IF2, and fMet tRNA binds to the small ribosomal subunit which is already associated with IF3.

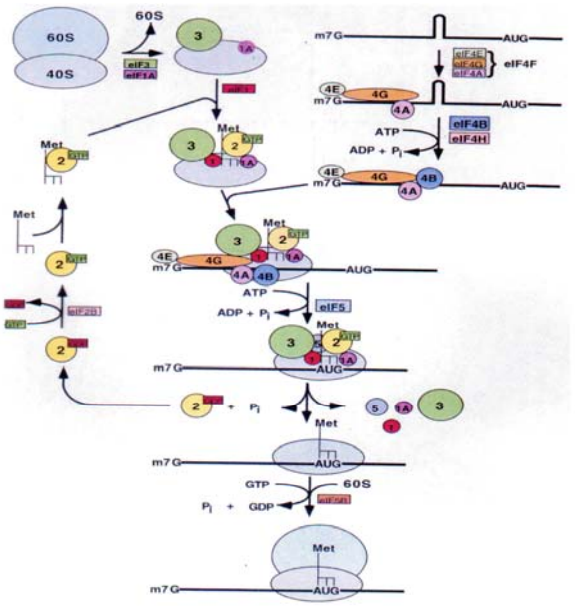


Two Pathways which Allow Ribosome to be Recruited to mRNA:

- 30S and IF3 bind directly to Shine-Dalgarno sequence via base pairing and then recruit ternary complex with the help of IF1
- 40S first binds to ternary complex then binds to mRNA with help of IF1
  - The IFs are released by conformational changes once the fMet is bound
- There is a conformational change when GTP is hydrolyzed to a GDP, which leaves bound to IF2. IF1

and IF3 also dissociate, which leaves the tRNA free to elongate

Eukaryotic Translation Initiation



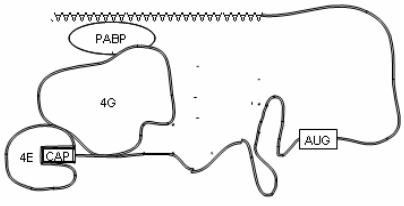
- The eukaryotic system is more complex because there is a lot more factors involved
    - There are at least 12 different translational factors recruited as opposed to the 3 seen earlier in prokaryotes
    - Many of the factors are composed of several subunits
- An example of multiple subunits appears in eIF2 which has 3 subunits (alpha, beta, gamma)
    - It is highly regulated by phosphorylation
- To begin translation the 60S must be dissociated from the 40S ribosome
- eIF3 is composed of 13 subunits (650 kDa) while in prokaryotes it only has one subunit. It has many functions. Primarily, it keeps the 40S and 60S ribosomal subunits apart, as in prokaryotes.
- eIF1 and eIF1A then associate with the 40S ribosome
- eIF4E is the cap-binding subunit
- When the ribosome reaches the initiation codon, the release of the initiation factors is mediated by eIF5.
- eIF5b repositions the 40S ribosome and aids in the joining of the last subunits

eIF4F

- The first component to bind the cap is the eIF4F, which consists of 3 subunits: eIF4E, eIF4G, and eIF4A
- **eIF4E** is the 5' cap-binding unit.
- **eIF4A** is an RNA helicase (first discovered in the "dead box family") that can disrupt hydrogen bonds. This unwinding always requires ATP
    - eIF4H and eIF4B help eIF4A but their mechanism of function is unknown
- **eIF4G** is a scaffolding protein, which brings the initiation factors to the mRNA. It contains binding sites for different IFs and kinases.
    - eIF4G is a Poly –A binding region protein (PABP) that forms a circular mRNA.

mRNA Circularization

- eIF4G can cause mRNA circularization
    - This results in more efficient initiation of translation because ribosomes, when they terminate, are closer to the cap structure causing an increase in the ribosomal concentration at the translation start site which increases initiation. Removing the PABP causes a decrease in the efficiency of translation
- Circularization is also important to prevent translation of accidentally cleaved mRNA
    - If an mRNA is cleaved prematurely, it produces a truncated mRNA that gives a truncated protein, which is extremely toxic to the cell.
    - If a truncated protein enters a complex; it will poison the complex by competing with the wild type protein. These truncated proteins are called *dominant negative mutations*, because it has a dominant effect on translation and a negative or damaging function/impact on the cell.
    - The incomplete mRNA cannot circularize and is not translated efficiently and therefore only a small amount of the dominant negative protein is synthesized

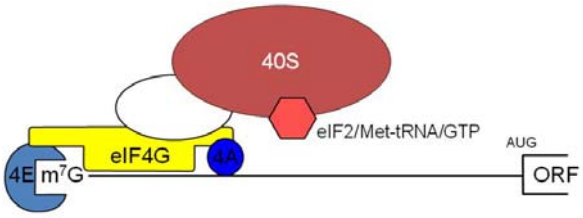


03/21/2007

Lecture #28–Translation Initiation in Prokaryotes

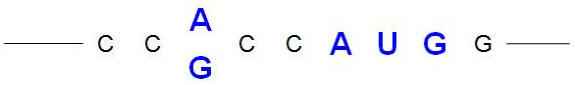
NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**

Cap-Dependent Ribosome Recruitment:



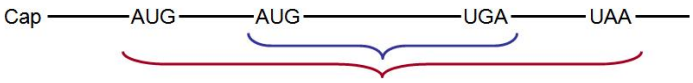
Ribosome recruitment is an ordered process involving a step wise addition of components to a complex. In the figure we see a capped mRNA which is bound by the eIF4 complex, which bound by the 4A (the helicase). This complex recruits eIF3 and this recruits the ribsome, the ternary complex and other factors. Therefore there is a large complex which assembles at the 5'-end of the mRNA. This complex moves in a unidirectional manner towards the AUG (initiator anticodon), which is called **scanning**. Then there is base pairing between the methionine anticodon and the mRNA, which allows the 40S subunit to stabilize.

Consensus Initiation AUG:



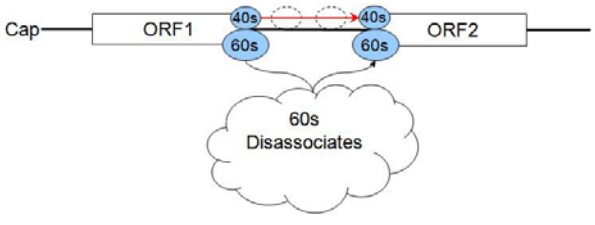
A long time ago scientists noticed that the initiator complex moved and when it reached the first AUG, translation was initiated. However this was only true for ~85% of sequences. In the other 15% of mRNA sequences, the first AUG was skipped. This happens because there is a consensus sequence for the recognition of an authentic AUG initiator. The most important factor is the **pyrimidine (C or U) in position 3**, which causes most of the ribosomes **to not recognize this AUG** and **continue to scan** until the next AUG. In **most mRNAs**, at this **position (-3) there is a purine (A or G)** and therefore they **start initiation** with the first AUG. When you have an upstream CCG/A sequence (as seen in the diagram) it favours initiation, but the most important conserved base pair is this *is the A/G…*

Leaky Scanning:



Because, the first AUG is not always used, the eukaryotic mRNA can become polycistronic (multiple proteins encoded by one mRNA). In this sequence, for example, the first AUG is not in a good consensus, so only 10-20% of the ribosomes start here, they will make protein which is terminated by the UAA (longer protein). The next start codon a ribosome can encounter is in a different reading frame (there are three possible frames), this will result in initiation at the second sequence terminating with the UGA. Therefore you have two different proteins arising from the same sequence, the process is known as **leaky scanning**, since the second AUG leaks over the upstream AUG.

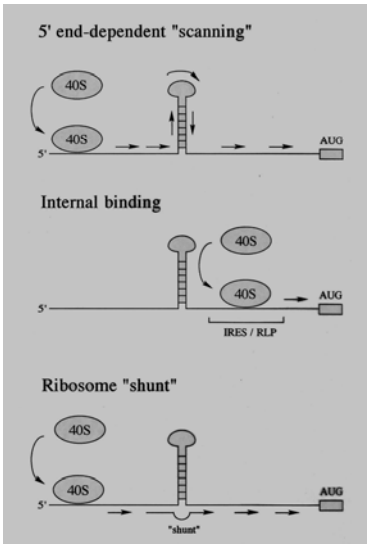
Termination-Reinitiation:



Another example of polycistronic mRNAs is seen in this situation:

- Two ORF are shown (right) and the distance between them is up to 50 or 60 nucleotides, the 60S ribosome will fall off at the termination codon of the first ORF, and the 40S remains attached to the mRNA and continues to scan. The 40S reaches the AUG of the second ORF and the 60S joins again. Therefore, there are two non-overlapping proteins from the same mRNA. This process is known as **termination reinitiation**.

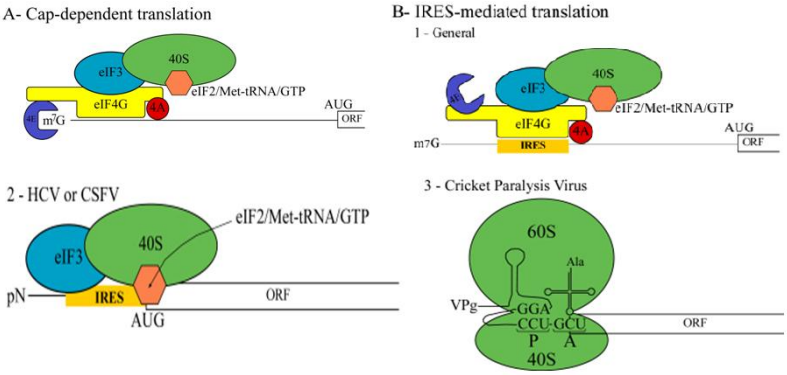
Translation Initiation for Polycistronic mRNA:

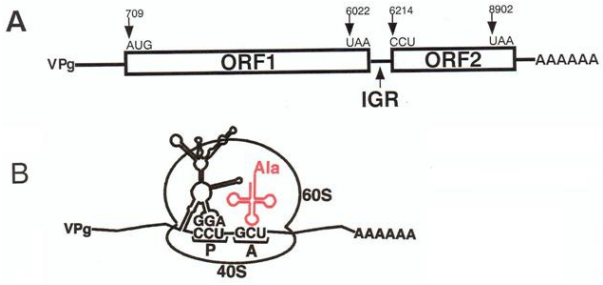


- There is a specific mechanism to get two proteins from one mRNA, which occurs by **internal initiation**, or **internal ribosome entry**. This is supported by the presence of the **internal ribosome entry site** (IRES).
- When researchers were studying a group of viruses which have mRNAs that are not capped, they found that the eIF4 complex in these viruses attaches internally to the 5'end and not through the cap structure.
  - This IRES sequence is what allows the 40S to continue and the 60S to re-bind. You don't necessarily have to initiate translation at the cap structure; the IRES sequence presents an alternative way.
- There are many mRNAs that are capped and use the IRES; the advantage of doing this, is that under certain conditions the cap translation is inhibited (such as during mitosis, under stress conditions and hypoxia), but the cell still needs certain proteins which are vital for cell survival. Therefore, these mRNAs use a cap-independent translation method.
- Another method is the **ribosome shunt**. Where the ribosome recognizes the cap structure, scans the mRNA, and when it comes to a stem-loop structure, it does not read the mRNA and shunts or skips that sequence and continues to scan.

IRES-Mediated Initiation:

The first method involves no eIF 4E-binding (no cap recognition) and a direct interaction between the eIF4G and the IRES. Another mechanism used by HCV (hepatitis C virus), during the course of HCV, involves binding of 40S to eIF3 and formation of the ternary complex on the IRES (very close to the AUG) this process does not require any other initiation factor.





Another mechanism, developed in certain insect viruses (such as cricket paralysis virus), involves no initiation factors. The mRNA does not have a cap at the 5'end, instead it has a protein called viral protein G (VPG) which is attached covalently to the mRNA. This mRNA has two ORFs, the first one starts with an AUG and the second uses an IRES sequence starting with a proline (CCU). The mRNA folds in such a way making a very complex stem-loop structure, as seen in the figure. There is a GGA which basepairs with a CCU at the P-site. Since the GGA is paired to the P-site no initiation Methionine is required.

Translation Control:

Reasons why cells and organisms are adapted translational control through phosphorylation:

1. **Directness and Rapidity** → any control applied in the nucleus takes time; there is a delay during to transcription and processing (15 minutes). Therefore, if you need the protein right away the mRNA must be ready to translate and if you need to shut off the protein, then it can be degraded easily. This can be done by phosphorylation, which can effect interaction of proteins and therefore affect many processes.

2. **Reversibility** → phosphorylation and dephosphorylation can occur in less than a second.
3. **Fine Control**
4. **Regulation of Large Genes** → certain genes with introns can be very big (sometimes up to two million basepairs), so transcription can take almost 24 hours.
5. **Systems that Lack Transcriptional Control** → this is important in development, since there is little transcription at this time. Therefore most of the control is based on the mRNAs that exists.
6. **Spatial Control** → this is control in different parts of the cell. This is especially important in the brain. A neuron has many dendrites, each with thousands of synapses. Each synapse responds to different stimuli and therefore the neuron must activate only certain parts without affecting the nucleus, through activation of local translation.
7. **Flexibility**

Translational control in bacteria is inhibited by antibiotics, such as puromycin. These antibiotics work on translation or by binding on the ribosomes. The antibiotics are based on similarities to the control on translational machinery; the molecules bind to the ribosomes because they are similar to something else.

- Example: puromycin inhibits both prokaryotic and eukaryotic transcription. Because of the similarity to amino-acyl tRNA, puromycin can enter the A-site and form a peptide bond with the nascent polypeptide, and gets incorporated into the peptide.

Diphtheria Toxin:

- This toxin is related to the bacterial disease diphtheria, which is a toxin produced by a bacteriophage.
- This toxin modifies and amino acid in the enzyme **diphthamide**. It is a potent toxin, because it only takes one molecule of the toxin to affect a cell's biochemical functions.

Translational Control in Bacteriophages:

- Very simple in structure, such as MS2 or Qβ, which encode three-four proteins from only one mRNA.
- When the virus is matured, the coat proteins coat the viral mRNA, thus protecting it.
- In order to replicate in the cell the virus must make a minus strand from its' plus strand, it uses a viral synthetase. Then to form more plus strands the minus strands are transcribed.
- Therefore, the viruses reproduce themselves and the cell lyses when there is a buildup of bacteriophages.
- There is no active translational control in the bacteriophage mRNA. But when the phage infects the bacteria, not all the ORF can be translated due to secondary structures of the mRNA. At the 5' end there is folding that obstructs the AUG of the first protein (protein A), the ORF that can be read and synthesized is the coat protein.
- When the secondary structure is disassembled, the synthetase protein can be translated. Once there is enough synthetase, the synthetase binds to a regulatory site and prevents further binding to the coat ORF.
  - The reason for this is to free the plus-strand RNA in order to increase transcription with the synthetase. Coat protein production must be stopped.
- The A protein can be made before the transcription can occur.
- This is how the phage has adapted the regulation of its cycle.

Translational Control in Eukaryotes:

<u>Picornaviruses:</u>

- These viruses do not have a cap-structure, they translate through IRES.
- Examples of picornaviruses are rhinovirus, poliovirus, hepatitis A virus and encephalomycocarditis virus.

Protein Synthesis in Polioviruses Infected Cells:

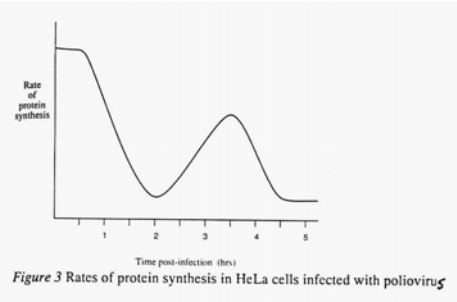
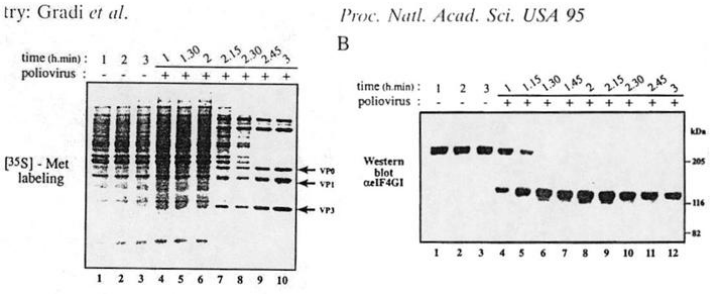


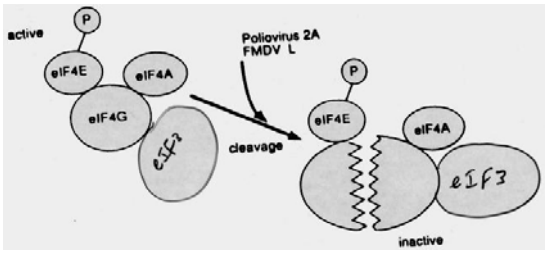
Figure 3 Rates of protein synthesis in HeLa cells infected with poliovirus

If we measure protein synthesis using radio-labelled methionine (S^{35}), we see a decrease in translation at the beginning of infection and then a surge in translation. This is known as the shuttle of protein synthesis. Many viruses do this.

If we run S^{35}-labelled proteins on a gel we can see after 2 hours that the cell proteins are still present, but after 2.5 hours mostly the viral proteins are seen and no cellular proteins. At 3 hours, these are mostly coat proteins. How is the virus doing this? Through the cap-recognition process, it is cleaving one of the components of the cap-binding protein eIF4G.



There is a major cleavage site in eIF4G. How does this explain the shutoff of the host protein translation?



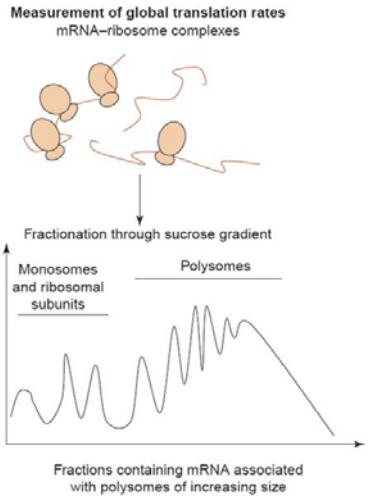
After the cleavage there are two halves of the complex, one half is inactive because it does not contain the eIF4E. But this portion is enough to translate the poliovirus. Therefore, the host mRNAs cannot be translated.

The protease that cleaves eIF4G is called **protease 2A**, this protease is required for it's own lifecycle. The poliovirus mRNA is quite long, the mRNA contains only one AUG start site and produces one large protein. One of the proteases which cleaves the viral polypeptide is protease 2A.

Translational Control:

Most of the translational control occurs at the level of initiation. This is so because when you want to control a process you will control it at the beginning, and the rate-limiting step in translational processes is initiation. Translation rates can be regulated by:

- Treatment of cells with mitogens, hormones or growth factors, which generally leads to an increase in translation
- Nutrient deprivation or environmental stresses such as heat shock, osmotic shock or UV irradiation generally reduce translation rates.



How do we measure the regulation at the initiation phase? This done by a test known as: the **polysome profile**. Polysomes are composed of an mRNA with many ribosomes, and they can be separated through density gradient centrifugation using a sucrose gradient. An mRNA with many ribosomes will be heavy and therefore separate to the bottom of the gradient and if the mRNA has only a few ribosomes it will sediment to the top of the gradient. When the rate of initiation is fast there will be many ribosomes attached to the mRNA. When the rate of initiation is slow, there will be only a few ribosomes on the mRNA.

Ferritin mRNA Translational Control:

The cellular concentration of iron has an impact on translational control. Iron is essential for cell survival (mitochondria need it or else we cannot breathe), however too much molecular concentration of iron is toxic to the cell. Therefore there is a protein that binds to iron and ensures it doesn't affect the cell. This protein is known as **ferritin**, which is made up of many light and heavy chains, which keep this ferritin attached to the iron. Therefore, ferritin levels in the cell must be highly regulated, and are dependant on the cellular concentration of iron.

03/23/2007

Lecture #29–Translation Regulation

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**

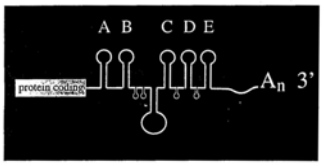
Last lecture, we saw that usually when we regulate translation, we do it at initiation level and we can detect levels of translation by looking at the polysome profile. So when translation initiation is inefficient, you have one or two ribosomes associated with messenger RNA and if its efficient you have many.

If you run this in a density gradient (sucrose gradient), you can measure by absorbance the density at 260nm. You can see where the 80s, 60s and 40s ribosomes are. If you have high density at the 80s level then you have efficient translation but if you have high absorbance at the 60s and 40s level then you have inefficient translation.

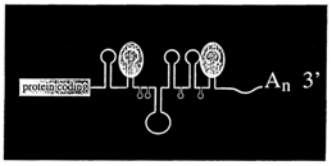
Iron Metabolism

- You can have control by a repressor protein (ferritin). Ferritin is a protein that is formed using many subunits to make a big complex. This complex binds iron in the cell. The level of ferritin in the cell is regulated at the level of translation and not at other processes like transcription, splicing or post-translational control.
- In the 5'UTR of the mRNA there is a stem and loop structure that binds a protein called IRP (Iron-regulated binding protein) (or Iron response element binding protein). This protein binds to the mRNA and its binding is dependent on iron concentration in the cell. High iron = want to makes lots of ferritin because iron is toxic in the plasma. So you remove IRP when the iron binds to it. Now you make lots of ferritin to remove the iron. When IRP is bound on the mRNA, it prevents translation by inhibiting binding of ribosomes.

- There are not many mechanisms that work this way in the cell.
- Another mRNA that is relevant to iron metabolism is the ferritin receptor which brings in ferritin that binds iron in the serum outside of the cell. The stability of this mRNA is regulated by its stability in the cell. It has a stem and loop structure in its 3' UTR and is almost exactly the same sequence as in the 5'UTR but this time its regulated at the level of stability (different than the mechanism at the 5' end).
- Here there is a step and loop structure that binds to IRP. There are heavy chains and light chains. The sequence of the stem and loop that is found in the ferritin receptor is very similar: CAGUGCAGUG.



IRE-BP absent : mRNA degradation (IRP)



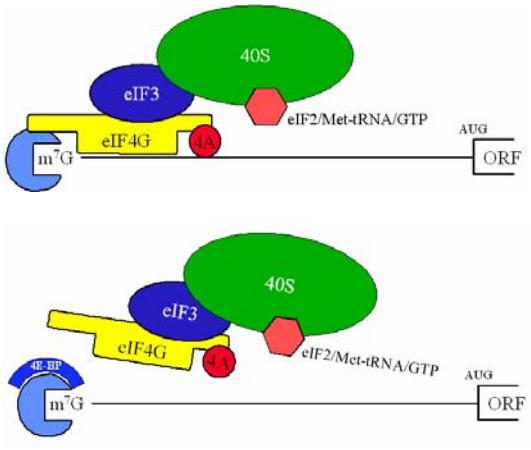
IRE-BP bound : mRNA stabilized (IRP)

- This control needs to be fast and efficient which is why we use this mechanism.

Phosphorylation

- This mechanism is efficient because it's fast: you can phosphorylate with a kinase and phosphatases remove the phosphates. Many of the initiation factors are phosphorylated on multiple sites. We don't understand all the phosphorylation but we know some really important ones (eIF2 for example).
- Phosphorylation is regulated by signalling pathways. For example there is the Ras pathway and the PI3Kinase pathway which both affect signalling molecules in the cell. These in turn affect splicing, transcription, and cytoskeleton and as well if affects translation.
- Certain proteins that are phosphorylated in translational regulation are: eIF4G, eIF4B, eIF4E (CAP binding protein). These are downstream of signalling pathways.
- One important phosphorylation event occurs with 4EBPs, which are proteins that bind to the eIF4E protein that recognize the 5' cap structure. They compete with eIF4G for binding to eIF4E and in such they inhibit CAP-dependent translation.

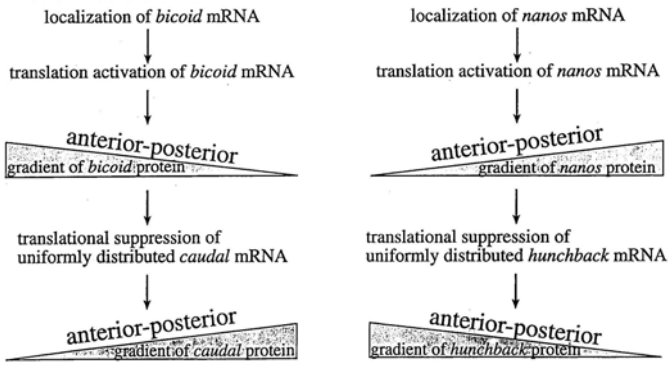
Mechanism



- A complex assembles at the 5'end: eIF4E, eIF4G, and eIF3. The complex of 4A, 4G, and 4E is often referred to as the 4F complex. The 4EBPs bind on the surface of the eIF4E exactly where eIF4G binds so they directly compete with them. This will inhibit cap-dependent translation but not IRS dependent translation because it doesn't require any eIF4E.
- This inhibition is reversible. The 4EBPs become hyperphosphorylated in response to extracellular stimuli (mitogens, growth factors and even viruses). When they become phosphorylated, they dissociate from eIF4E and you have an increase in translation.
- The pathway that leads to this is through PI3K and mTOR, which are involved in phosphorylating 4EBPs and can be targetted in many cancers.
- We know 4EBPs regulate translation and the regulation is at the level of inhibition of eIF4E, which is controlled by the state of phosphorylation.
- The mechanism has been elucidated at the molecular level with crystallography experiments and NMR experiments.

Important control mechanism in early development

- During early development there is no transcription so there is no transcriptional control → the control is mainly translation. Most of this study is done with flies because there are great to work with (biochemistry and genetics techniques).
- When you have an egg in early development it's symmetrical (oval shaped).

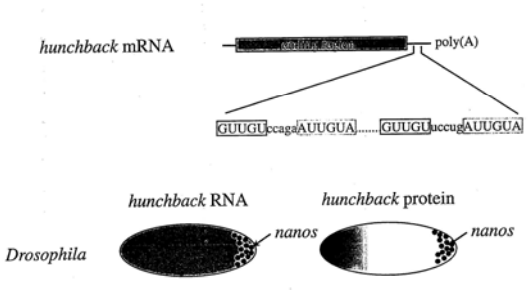


How do you establish polarity (head, tail, back (dorsal), front (ventral))?

- This is done with transcription factors that are morphogenic. They determine the morphology. There is a cascade of TFs that are expressed at different levels of development. This is all controlled at the level of translation.
- All of the mRNA necessary will be deposited there. It is deposited uniformly all over the oocyte but then translation of mRNA is confined to certain parts of the egg. For example you make gradients of the different morphogens of TFs. If you want to make head (anterior), you localize a protein TF (bicoid

mRNA) with a gradient from the anterior to the posterior. You activate this and bicoid mRNA is then translated in a gradient form anterior to posterior.

- Bicoid is a translational repressor so it represses the translation of another mRNA TF called caudal, which is uniformly distributed. But because bicoid is distributed in a gradient, it will repress more in the anterior than in the posterior (tail).
- Another example involves nanos and hunchback, which have same mechanism. Hunchback mRNA is distributed uniformly in the egg. But nanos is concentrated in the posterior which binds to the hunchback mRNA at the 3'UTR and inhibits hunchback translation at the posterior. This creates a gradient of hunchback protein.



How does a protein that binds to 3'UTR prevent translation at 5'UTR?

What happens is what we have following case:

- 4EBP binds to 4E and competes with 4G and therefore prevents translation (pretty standard mechanism). In developmental case, there is a similar competition of different proteins with the 4G for binding to 5'end of mRNA. In bicoid case, it binds to another similar protein to 4E called 4EHP, and the bicoid binds to the mRNA of caudal. At the same time it binds to the 4EHP, making a circle (important), which prevents the formation of the complex with 4G.
- There are several other examples, all of which involve competition with 4G for binding to 4E.

Phosphorylation of eIF2

- A very important translational control is the phosphorylation of eIF2, more specifically on the eIF2α subunit. eIF2 is the one that binds to initiator tRNA (met-tRNA) and what happens is that when eIF2-GDP is released during initiation from the complex (GTP is hydrolyzed to GDP).
- The complex of eIF2-GTP is about 100times more stable than the complex with GDP. There must be a mechanism that switches GDP and GTP. eIF2B has 5 subunits and it exchanges GDP for GTP (it's a guanine nucleotides exchange factor). If you phosphorylate one of the subunits of eIF2 (eIF2α), you inhibit this process, which inhibits translation.
- This is so important that 4 different kinases do exactly the same thing. They phosphorylate eIF2α on Serine51. This amino acid is conserved from yeast to humans and is phosphorylated by these 4 kinases: GCN2, PERK, PKR, and HRI, which respond to different stimuli.

The following table shows these:

Name	Mr (kDa)	Conserved in:	Properties	Conditions for	
				activation	inhibition
HCR	70	Mammals	heme-regulated protein kinase	deficiencies of iron or hemin; heat shock	adequate hemin levels + normal physiological temperature
PKR	62-65	Mammals	ds-RNA-regulated protein kinase	low concentrations of dsRNA; treatment with heparin; others ?	high concentrations of dsRNA; small RNAs; p58 inhibitor protein; dsRNA sequestration by other proteins
GCN2	182	All Species	essential for translational induction of GCN4 in yeast homologs in *Drosophila* and mammals	nitrogen starvation	adequate availability of amino acids
PERK	123	Vertebrates	Transmembrane Endoplasmic Reticulum associated kinase	Glucose starvation; accumulation of unfolded proteins in ER; ultraviolet radiation	Adequate availability of glucose; interaction with ER chaperone BiP

So there is a wide array of different kinases that, once activated all phosphorylated eIF2α.

How was this inhibition of translation first discovered?

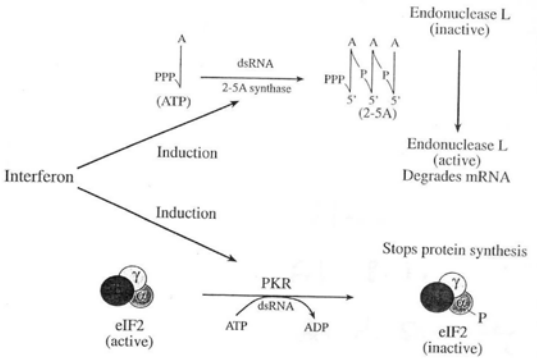
You can do translation in a reticulocyte lysate, which has efficient translation machinery. If you deplete the heme, the translation is going to level off because eIF2α is phosphorylated and when you add back guanine nucleotide exchange factor (GEF) (eIF2B), you get back the translation.

PKR activation

- Protein kinase (RNA-dependent) activity is very important in interferon response. Interferon was discovered in 1957, when researchers infected cells with Influenza virus (dsRNA), took medium from infected cells and placed them with uninfected cells. These uninfected cells became resistant to infection. Why?
- The infected cells secreted interferon (family of proteins characterized by their ability to bind to a specific receptor). When the cells were infected with dsRNA, they were stimulated to produce interferon, which binds to interferon receptors, which are internalized and go into the nucleus. There are phosphorylation events, which lead to the synthesis of anti-viral proteins (50 new proteins made in order to counteract virus infection).

Where does translation control fit into all this?

One of the proteins made and activated by dsRNA via increased levels of interferon is PKR. PKR is a protein that phosphorylates eIF2α. Now eIF2-GDP can't be recycled into eIF2-GTP and is inactive. All four kinases produce the same results.



Why do you want to inhibit translation?

Once you have an infected cell you want to kill this cell so this cell can't make a lot of progeny virus, which would infect other host cells. By inhibitng translation and protein synthesis the cell will die.

Viruses have survived millions of years and some have found ways to counteract PKR.

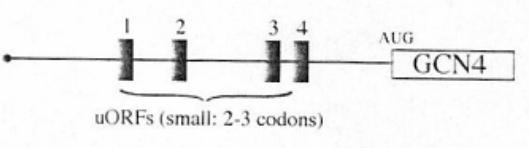
- Influenza has a protein (p58) that binds to PKR and inhibits it.
- A small RNA called VA (virus associated RNA), which binds to PKR, which can no longer bind to dsRNA and is thus prevented from being activated.
- Other viruses have a mechanism to bind dsRNA and prevent PKR from binding to it.
- Different viruses have found different mechanisms to counteract the effects of PKR.

GCN4

- This work started in yeast in the early 80s and established a widespread model for translational control. GCN4 is a TF that is involved in the synthesis of amino acids. So during amino acid starvation in yeast, the yeast has to synthesize its required amino acids. This synthesis is regulated using one TF involved in the transcription of 30 genes for 11 amino acid biosynthetic pathways.
- They found that GCN4 mRNA levels remain the same regardless of environmental conditions, so it is not under transcriptional control. It is actually under translational control.

What did researchers find using genetic experiments?

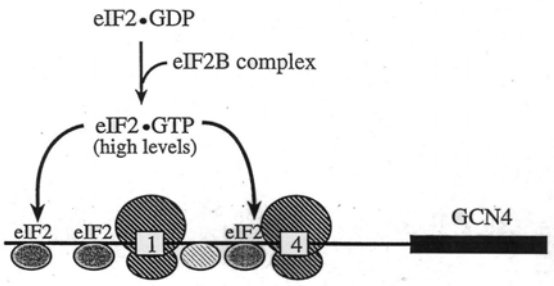
- 5'UTR is about 600nts long (compared to usual 50nts in yeast).
- It also has this small upstream open reading frame.
- They are all really small (2-3 codons).
- When this 5' UTR was removed, GCN4 always has derepressed



translation.

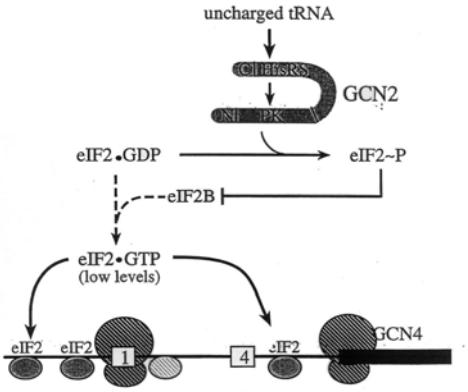
- The ORFs act as a kind of repressor under normal conditions but when there is a lack of amino acids, you stimulate GCN4. How exactly does this happen?
- ORF 1 and 2 work similarly and 3 and 4 work similarly.
- Recall: → GCN4 is expressed in amino acid starvation; but when there is plenty of amino acid, GCN4 is repressed.
- The model is based on termination/reinitiation.

Non starvation conditions:



- There are high levels of eIF2-GDP.
- You get translation of ORF1.
- You terminate and the 40S continues scanning.
- It reaches ORF4 then you translate this and once you terminate after ORF4 there is a termination signal that prevents scanning.
- When the 40S resumes scanning, after translating ORF1, it has no initiation factors, so it must pick them up.
- When you have high enough levels of eIF2-GTP (ternary complex), they are easily recruited so that when you reach another ORF, the complex is competent for reinitiation.

Starvation conditions:



- You have uncharged tRNA because there are no amino acids.
- There is activation of GCN2, which now phosphorylates eIF2α.
- Now eIF2-GDP can't be transferred to eIF2-GTP
- There are now low levels of eIF2-GTP
- So when the 40S ribosome scans and reaches the AUG of ORF4, it doesn't have enough met-tRNA-eIF2-GTP, it continues scanning in a different mode and continues until it reaches the GCN4 to translate it.
- **In this model phosphorylation is actually stimulating translation.**
- This is actually not a unique case → 5% of proteins have 5'UTR that are subject to this type of regulation.

Regulation of ATF4 in Learning and Memory

ATF4 is important in learning and memory. It has an upstream open reading frame and likely works by the same mechanism as above. So when you phosphorylate eIF2α, you get an increase in ATF4.

Experiment in learning and memory:

- In order to have learning and memory, there needs to be an increase in translation/protein synthesis. When you memorize something there are molecular several steps to the process.
- Short-term memory is the first step.
- To consolidate this to long-term memory, you need to repeat this.
- This involves the hippocampus region, which consolidates short-term memory to long term memory. There is protein synthesis involved in this process.

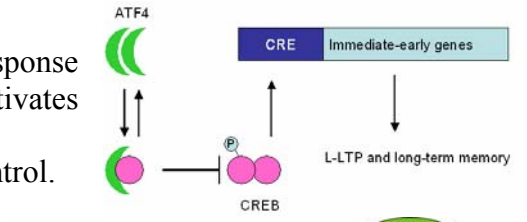
How do you measure memory in a qualitative way?

- One way is to study spatial memory in a mouse uses; the Morris Water Maze.
- There is a bath of water with a platform underwater; the mouse has to find the platform and use spatial cues in the room to remember where this platform is.

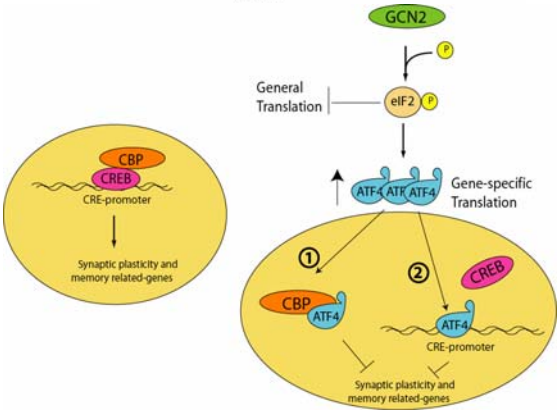
- You repeat this 3x/day and after a week, the mouse will improve its time taken to find the platform (typically after 6 days the mouse will improve and it will take it half the time).

How does this relate to ATF4?

- ATF4 was discovered to be a repressor of memory.
- A protein involved in this pathway is CREB (cAMP Response Element Binding Protein) that binds to a promoter and activates immediate-early genes involved in long-term memory.
- ATF4 is a TF that is like GCN4 in terms of its translation control.



- It binds to the CREB and prevents it from being phosphorylated.
- You can delete the GCN2 from the cell (knockout) and without it, you can't increase the ATF4, which means you can't inhibit memory.
- So a GCN2 knockout should remember the maze better.
- This was actually observed in mice.
- Knock-in experiments were also performed, where a phosphorylation site (serine51) on eIF2α was replaced for alanine.
- Now it can't be phosphorylated so ATF4 can't be increased and these mice also had a better memory.



MicroRNA

- siRNA are 21-23nt small RNAs processed from a genome-encoded hairpin
- First miRNA discovered in C. elegans (lin-4)
- They mediate translational control, sometimes cleavage, sometimes destabilization of target mRNA
- They constitute ~1% of the genome, and regulate up to 30% of the genes
- Govern cell division, differentiation, apoptosis, cell-specific roles, and are linked to viral and oncogene regulation
- It turns out that these siRNA bind to the 3' UTR of mRNA and inhibit cap-dependent translation (as seen in oocyte development)