Protein biosynthesis.

Normal and pathological protein metabolism

Lecture #22

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Introduction

- **Translation** is the RNA directed synthesis of polypeptides.
  - This process requires all three classes of RNA.
  - Although the chemistry of peptide bond formation is relatively simple, the processes leading to the ability to form a peptide bond are exceedingly complex.
  - The template for correct addition of individual amino acids is the mRNA, yet both tRNAs and rRNAs are involved in the process.
  - The tRNAs carry activated amino acids into the ribosome which is composed of rRNA and ribosomal proteins.
  - The ribosome is associated with the mRNA ensuring correct access of activated tRNAs and containing the necessary enzymatic activities to catalyze peptide bond formation.

Historical Perspectives

- Early genetic experiments demonstrated:
  1. The colinearity between the DNA and protein encoded by the DNA.
     1. *Yanofsky* showed that order of observed mutations in the E. coli tryptophan synthetase gene was the same as the corresponding amino acid changes in the protein.
  2. Crick and Brenner demonstrated, from a large series of double mutants of the bacteriophage T4, that the genetic code is read in a sequential manner starting from a fixed point in the gene, the code was most likely a triplet and that all 64 possible combinations of the 4 nucleotides code for amino acids, i.e. the code is degenerate since there are only 20 amino acids.

Experiments

- The above mentioned experiments only indicated deductive correlations regarding the genetic code.
  - The precise dictionary of the genetic code was originally determined by the use of in vitro translation systems derived from E. coli cells. Synthetic polyribonucleotides were added to these translation system along with all 20 amino acids. One amino acid at a time was radiolabeled.
  - The first demonstration of the dictionary of the genetic code was with the use of poly(U). This synthetic polyribonucleotide encoded the amino acid phenylalanine, i.e. the resulting polypeptide was poly(F).
- The utilization of a variety of repeating di- tri- and tetra polyribonucleotides established the entire genetic code.
  - These results of these experiments confirmed that some amino acids are encoded for by more than one triplet codon, hence the degeneracy of the genetic code.
  - These experiments also established the identity of translational termination codons.

RNA direction and protein synthesis

- An additional important point to come from these early experiments was that the 5' end of the RNA corresponded to the amino terminus of the polypeptide.
  - This was important since previous labeling experiments had demonstrated that the N-terminus is the beginning of the elongating polypeptide.
  - Therefore, in vitro translation experiments established that the RNA is read in the 5' to 3' direction.
- Crick first postulated that translation of the genetic code would be carried out through mediation of adapter molecules.
  - Each adapter was postulated to carry a specific amino acid and to recognize the corresponding codon.
  - He suggested that the adapters contain RNA because codon recognition could then occur by complimentarity to the sequences of the codons in the mRNA.

Important recognition steps

- During the course of in vitro protein synthesis and labeling experiments it was shown that the amino acids became transiently bound to a low molecular weight mass fraction of RNA.
  - This fraction of RNAs have been termed transfer RNAs (tRNAs) since they transfer amino acids to the elongating polypeptide.
  - These results indicate that accurate translation requires two equally important recognition steps:
  1. The correct choice of amino acid needs to be made for attachment to the correspondingly correct tRNA.
  2. Selection of the correct amino acid-charged tRNA by the mRNA.
     - This process is facilitated by the ribosomes which we will discuss below.

Summary of Experiments to Determine the Genetic Code

1. The genetic code is read in a sequential manner starting near the 5' end of the mRNA.
   - This means that translation proceeds along the mRNA in the 5' ---> 3' direction which corresponds to the N-terminal to C-terminal direction of the amino acid sequences within proteins.
2. The code is composed of a triplet of nucleotides.
3. That all 64 possible combinations of the 4 nucleotides code for amino acids, i.e. the code is degenerate since there are only 20 amino acids.

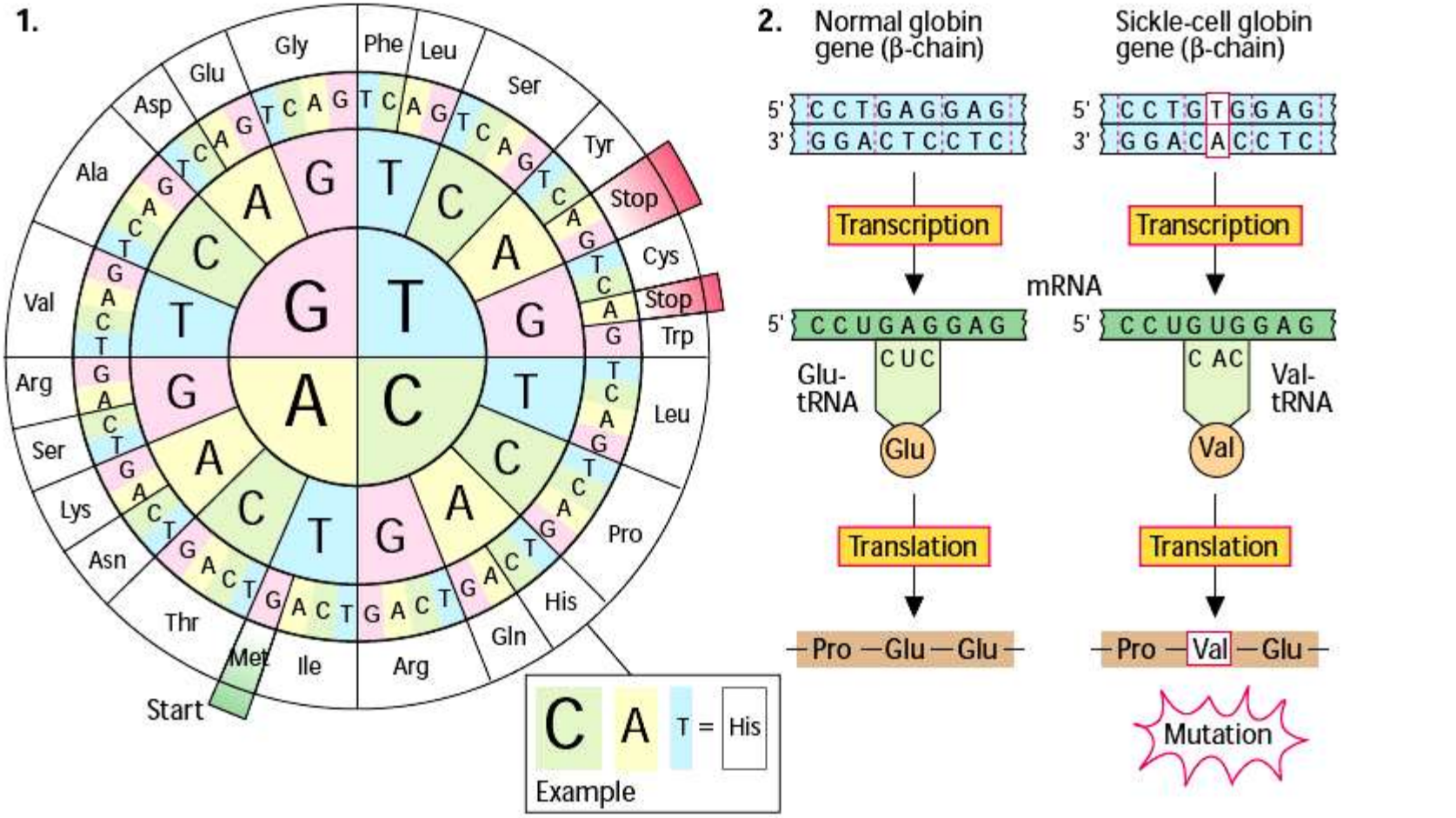
Dictionary of the Genetic Code

- The precise dictionary of the genetic code was determined with the use of in vitro translation systems and polyribonucleotides.
  - The results of these experiments confirmed that some amino acids are encoded by more than one triplet codon, hence the degeneracy of the genetic code.
  - These experiments also established the identity of translational termination codons.

The Genetic Code

	U	C	A	G
U	UUU Phe UUC Phe UUA Leu UUG Leu	UCU Ser UCC Ser UCA Ser UCG Ser	UAU Tyr UAC Tyr UAA End UAG End	UGU Cys UGC Cys UGA End UGG Trp
C	CUU Leu CUC Leu CUA Leu CUG Leu	CCU Pro CCC Pro CCA Pro CCG Pro	CAU His CAC His CAA Gln CAG Gln	CGU Arg CGC Arg CGA Arg CGG Arg
A	AUU Ile AUC Ile AUA Ile AUG Met	ACU Thr ACC Thr ACA Thr ACG Thr	AAU Asn AAC Asn AAA Lys AAG Lys	AGU Ser AGC Ser AGA Arg AGG Arg
G	GUU Val GUC Val GUA Val GUG Val	GCU Ala GCC Ala GCA Ala GCG Ala	GAU Asp GAC Asp GAA Glu GAG Glu	GGU Gly GGC Gly GGA Gly GGG Gly

The Genetic Code



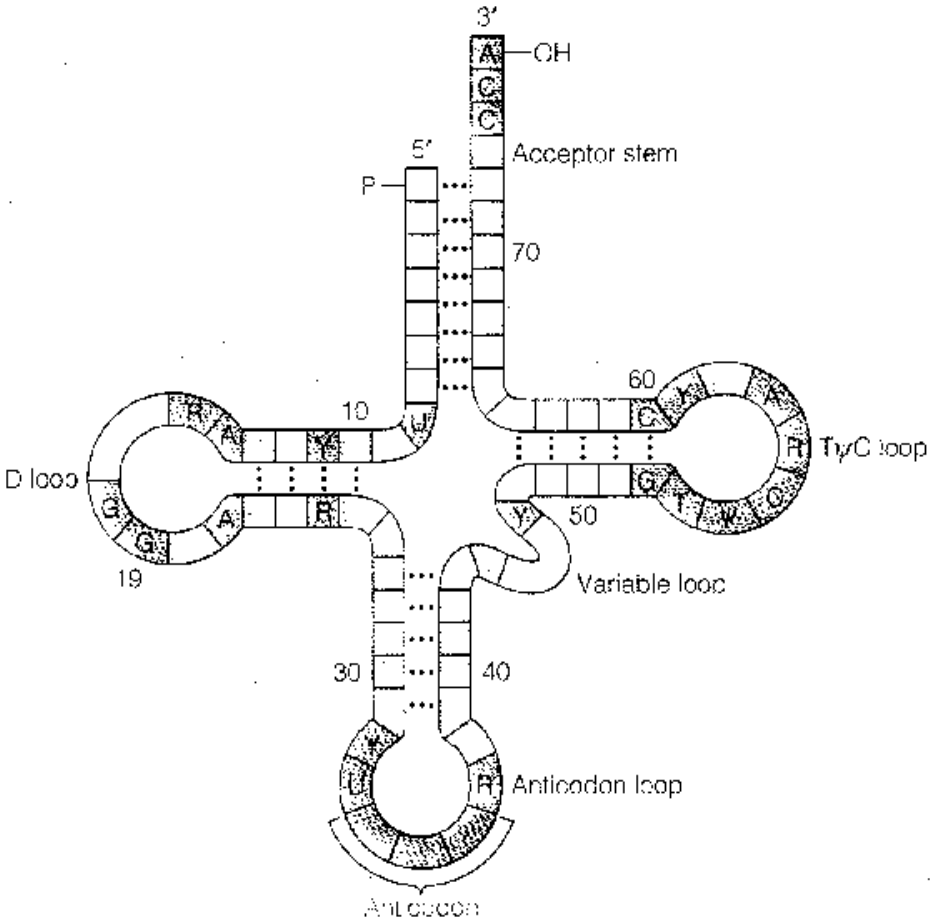
Characteristics of tRNAs

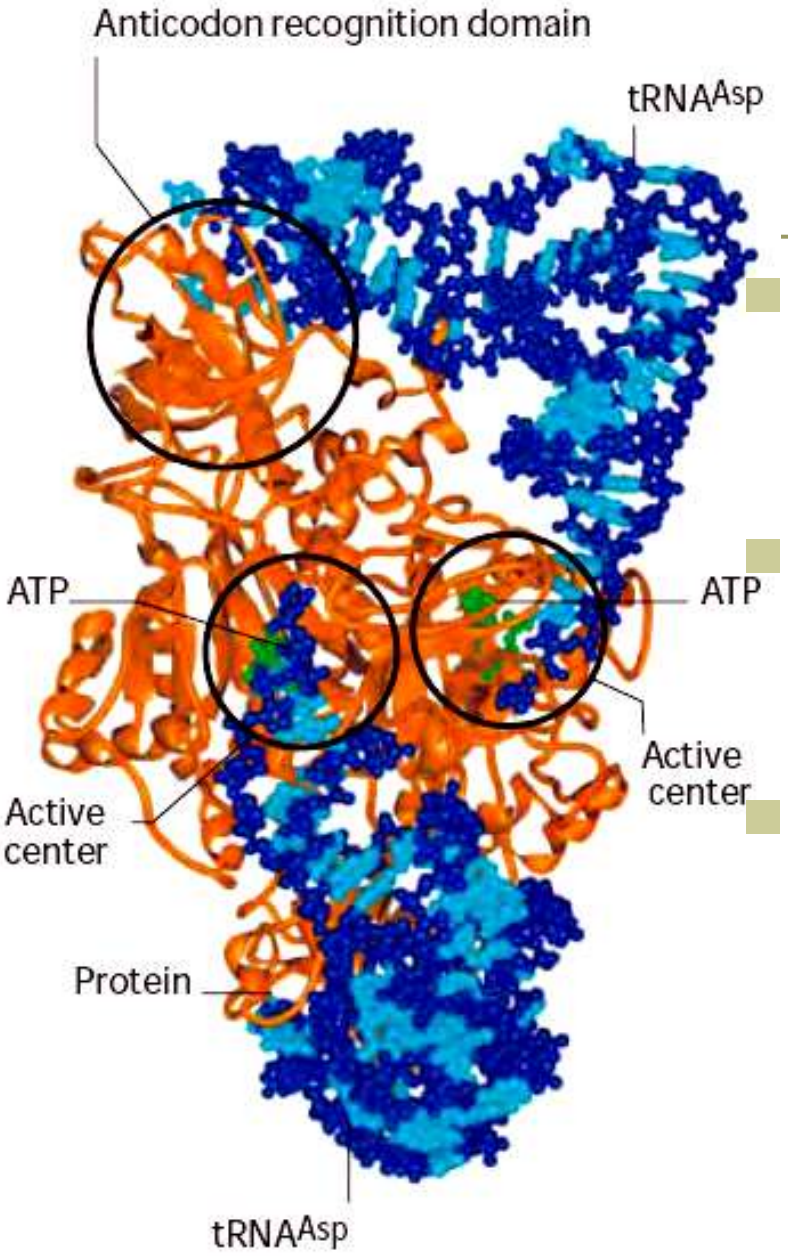
- More than 300 different tRNAs have been sequenced, either directly or from their corresponding DNA sequences.
  - tRNAs vary in length from 60 - 95 nucleotides (18 - 28 kD).
  - The majority contain 76 nucleotides.
  - Evidence has shown that the role of tRNAs in translation is to **carry** activated amino acids to the elongating polypeptide chain.

All tRNAs:

1. exhibit a cloverleaf-like secondary structure.
2. have a 5'-terminal phosphate.
3. have a 7 bp stem that includes the 5'-terminal nucleotide and may contain non-Watson-Crick base pairs, e.g. GU. This portion of the tRNA is called the **acceptor** since the amino acid is carried by the tRNA while attached to the 3'-terminal OH group.
4. have a D loop and a TφC loop.
5. have an anti-codon loop.
6. terminate at the 3'-end with the sequence 5'-CCA-3'.
7. contain 13 invariant positions and 8 semi-variant positions.
8. contain numerous modified nucleotide bases.

Typical cloverleaf diagram of a tRNA

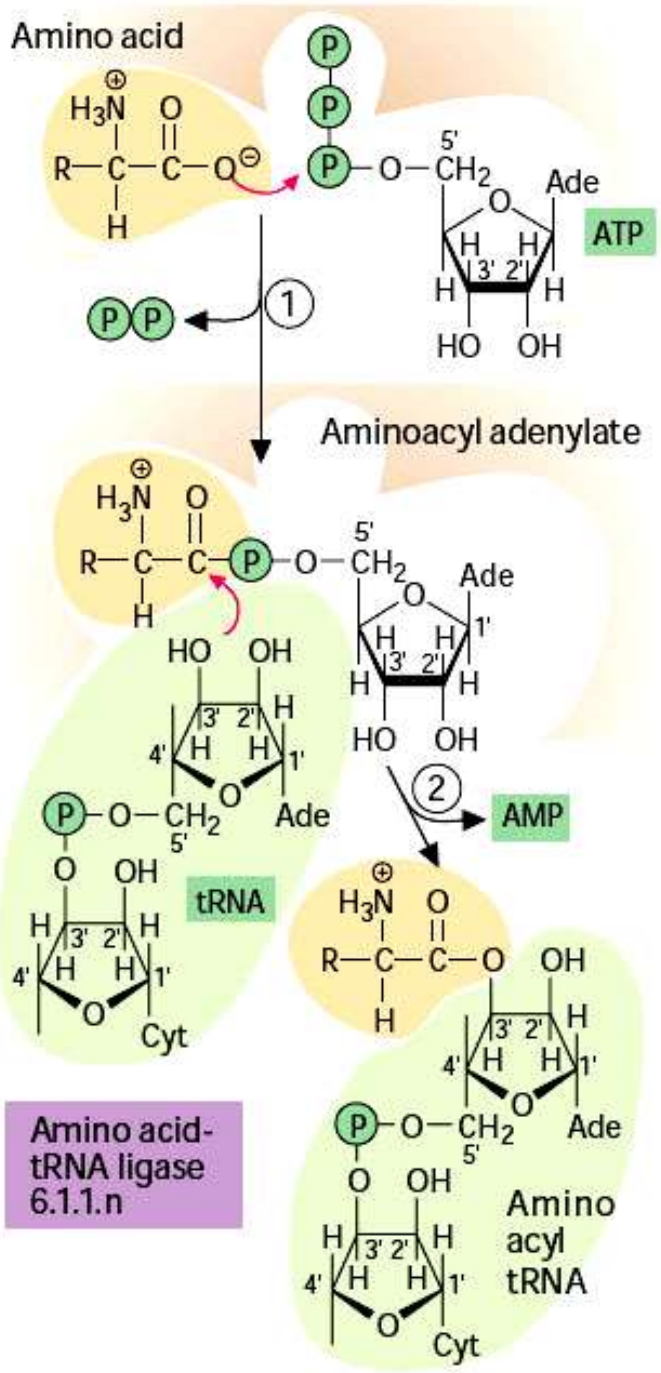




Activation of Amino Acids

- Activation of amino acids is carried out by a two step process catalyzed by **aminoacyl-tRNA synthetases**.
- Each tRNA, and the amino acid it carries, are recognized by individual aminoacyl-tRNA synthetases.
- This means there exists at least 20 different aminoacyl-tRNA synthetases, there are actually at least 21 since the initiator met-tRNA of both prokaryotes and eukaryotes is distinct from non-initiator met-tRNAs.

The Mechanism of Amino Acid Activation



- Activation of amino acids requires energy in the form of ATP and occurs in a two step reaction catalyzed by the **aminoacyl-tRNA synthetases**.
  - First the enzyme attaches the amino acid to the α-phosphate of ATP with the concomitant release of pyrophosphate. This is termed an **aminoacyl-adenylate intermediate**.

The Mechanism of Amino Acid Activation (cont'd)

- In the second step the enzyme catalyzes transfer of the amino acid to either the 2'- or 3'-OH of the ribose portion of the 3'-terminal adenosine residue of the tRNA generating the activated aminoacyl-tRNA.
- Although these reaction are freely reversible, the forward reaction is favored by the coupled hydrolysis of PP_i.

Correct Recognition

- Accurate recognition of the correct amino acid as well as the correct tRNA is different for each aminoacyl-tRNA synthetase.
- Since the different amino acids have different R groups, the enzyme for each amino acid has a different binding pocket for its specific amino acid. It is not the anticodon that determines the tRNA utilized by the synthetases.
- Although the exact mechanism is not known for all synthetases, it is likely to be a combination of the presence of specific modified bases and the secondary structure of the tRNA that is correctly recognized by the synthetases

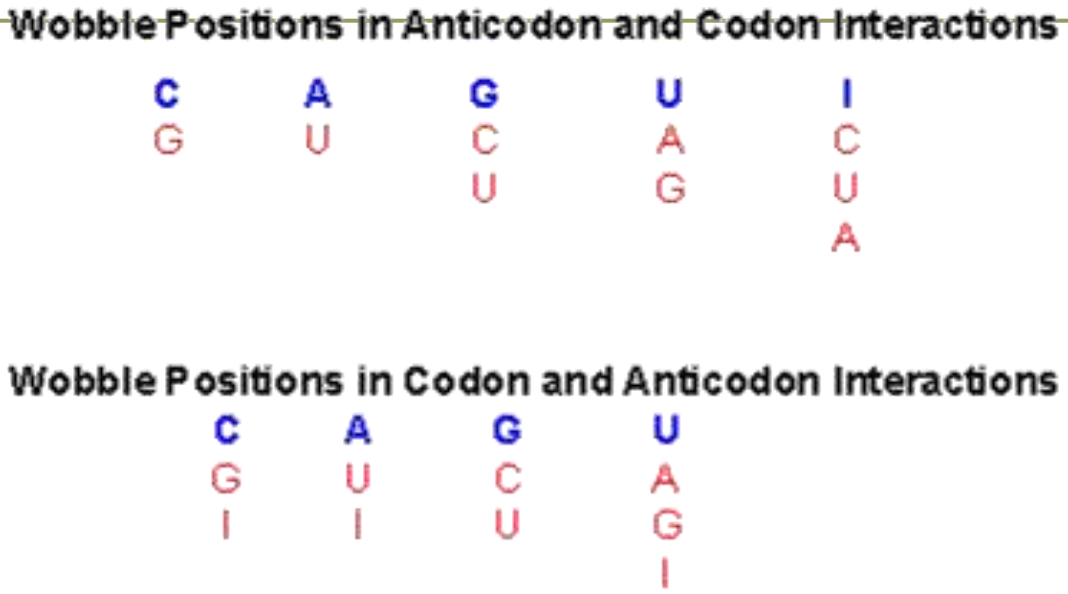
Experiments with aminoacyl-tRNAs

- It is absolutely necessary that the discrimination of correct amino acid and correct tRNA be made by a given synthetase prior to release of the aminoacyl-tRNA from the enzyme.
- Once the product is released there is no further way to proof-read whether a given tRNA is coupled to its corresponding tRNA.
  - Erroneous coupling would lead to the wrong amino acid being incorporated into the polypeptide since the discrimination of amino acid during protein synthesis comes from the recognition of the anticodon of a tRNA by the codon of the mRNA and not by recognition of the amino acid.
  - This was demonstrated by reductive desulfuration of cys-tRNAcys with Raney nickel generating ala-tRNAcys. Alanine was then incorporated into an elongating polypeptide where cysteine should have been.

The Wobble Hypothesis

- As discussed above, 3 of the possible 64 triplet codons are recognized as translational termination codons.
  - The remaining 61 codons might be considered as being recognized by individual tRNAs.
  - Most cells contain isoaccepting tRNAs, different tRNAs that are specific for the same amino acid, however, many tRNAs bind to two or three codons specifying their cognate amino acids.
    - As an example yeast tRNAphe has the anticodon 5'-GmAA-3' and can recognize the codons 5'-UUC-3' and 5'-UUU-3'.
    - It is, therefore, possible for non-Watson-Crick base pairing to occur at the third codon position, i.e. the 3' nucleotide of the mRNA codon and the 5' nucleotide of the tRNA anticodon.
    - This has phenomenon been termed the wobble hypothesis.

Wobbling illustration



- Diagram showing the various modified nucleotides of tRNAs that are found in the wobble position in the anticodon. The top half shows the wobble nucleotides of the anticodon in blue and the various nucleotides (in red) of the wobble position of the codon that can be found in non-Watson-Crick base-pairs. The lower panel illustrates the opposite showing the wobble nucleotides of the codon in blue and the associated wobble nucleotides of the anticodon in red.

Ribosomes are Major Constituent of the Cells

- Now that we have charged aminoacyl-tRNAs and the mRNAs to convert nucleotide sequences to amino acid sequences we need to bring the two together accurately and efficiently.
  - This is the job of the ribosomes.
  - Ribosomes are composed of proteins and rRNAs.
- All living organisms need to synthesis proteins and all cells of an organism need to synthesize proteins, therefore, it is not hard to imagine that ribosomes are a major constituent of all cells of all organisms.
  - The make up of the ribosomes, both rRNA and associated proteins are slightly different between prokaryotes and eukaryotes.

Order of Events in Translation

- The ability to begin to identify the roles of the various ribosomal proteins in the processes of ribosome assembly and translation was aided by the discovery that the ribosomal subunits will self assemble in vitro from their constituent parts.
- Following assembly of both the small and large subunits onto the mRNA, and given the presence of charged tRNAs, protein synthesis can take place.
  - To reiterate the process of protein synthesis:
  1. synthesis proceeds from the N-terminus to the C-terminus of the protein.
  2. the ribosomes "read" the mRNA in the 5' to 3' direction.
  3. active translation occurs on polyribosomes (also termed polysomes). This means that more than one ribosome can be bound to and translate a given mRNA at any one time.
  4. chain elongation occurs by sequential addition of amino acids to the C-terminal end of the ribosome bound polypeptide.

Three Steps in Translation

- Translation proceeds in an ordered process.
  - First accurate and efficient **initiation** occurs, then chain **elongation** and finally accurate and efficient **termination** must occur.
  - All three of these processes require specific proteins, some of which are ribosome associated and some of which are separate from the ribosome, but may be temporarily associated with it.

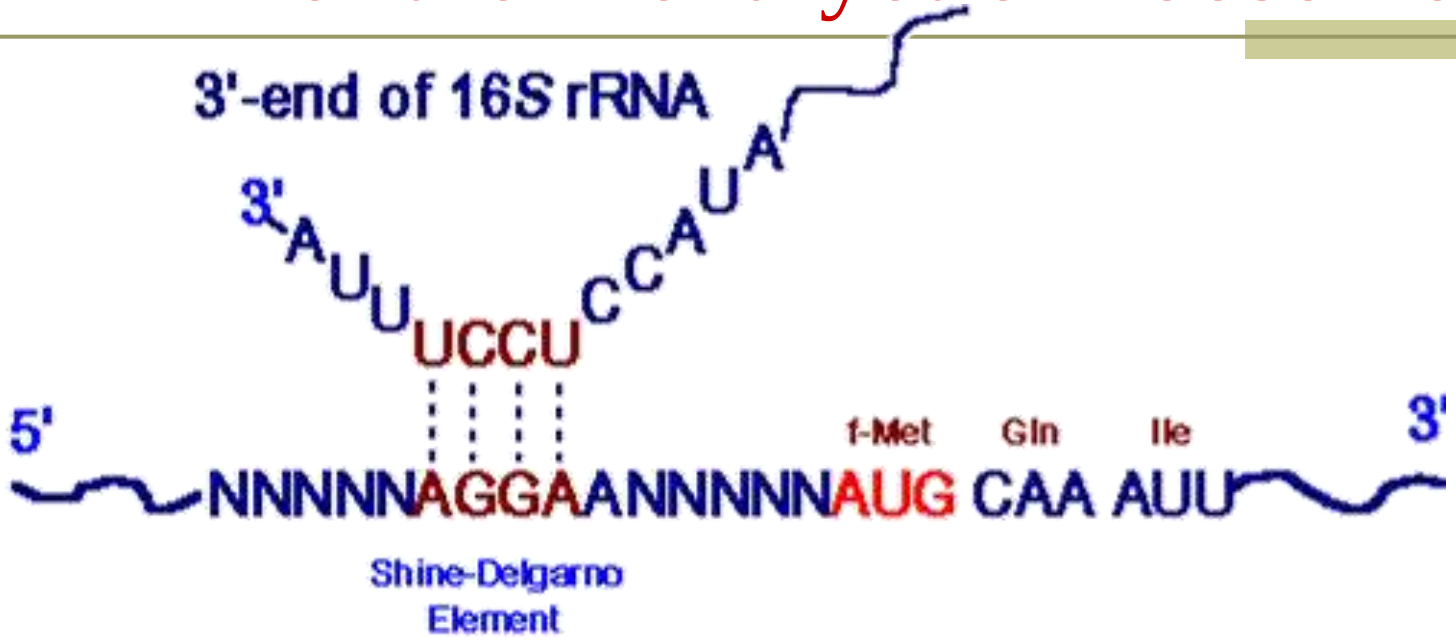
Initiation

- Initiation of translation in both prokaryotes and eukaryotes requires a specific initiator tRNA, $tRNA^{met}_{i}$, that is used to incorporate the initial methionine residue into all proteins.
  - In E. coli a specific version of $tRNA^{met}_{i}$ is required to initiate translation, [$tRNA^{fmet}_{i}$] .
  - The methionine attached to this initiator tRNA is formylated.
  - Formylation requires N^{10}-formy-THF and is carried out after the methionine is attached to the tRNA.
  - The fmet-$tRNA^{fmet}_{i}$ still recognizes the same codon, AUG, as regular $tRNA^{met}$.
  - Although $tRNA^{met}_{i}$ is specific for initiation in eukaryotes it is not a formylated $tRNA^{met}$.

AUG Codon and Shine-Delgarno Element

- The initiation of translation requires recognition of an **AUG codon**.
  - In the polycistronic prokaryotic RNAs this AUG codon is located adjacent to a **Shine-Delgarno element** in the mRNA.
  - **The Shine-Delgarno element** is recognized by complimentary sequences in the small subunit rRNA (16S in *E. coli*).
  - In eukaryotes initiator AUGs are generally, but not always, the first encountered by the ribosome.
  - A specific sequence context surrounding the initiator AUG aids ribosomal discrimination.
  - This context is $^{A}/_{G}CC^{A}/_{G}CCAUG^{A}/_{G}$ in most mRNAs.

The Shine-Delgarno Element and 16S rRNA of the Prokaryotic Ribosome



- The Shine-Delgarno element is found at the 5' side of each initiator AUG codon in prokaryotic polycistronic mRNAs.
- This element is complementary to sequences present near the 3'-end of the 16S rRNA of the prokaryotic ribosome.

Eukaryotic Initiation Factors and Their Functions

- The specific non-ribosomally associated proteins required for accurate translational initiation are termed initiation factors.
  - In *E. coli* they are **IF**s
  - in eukaryotes they are **eIF**s.
- Numerous **eIF**s have been identified.

Eucariotic Initiation Factors

Initiation Factor	Activity
eIF-1	repositioning of met-tRNA to facilitate mRNA binding
eIF-2	ternary complex formation
eIF-2A	AUG-dependent met-$tRNA_{met}^{i}$ binding to 40*S* ribosome
eIF-2B (also called GEF) guanine nucleotide exchange factor	GTP/GDP exchange during eIF-2 recycling
eIF-3 composed of ~10 subunits	ribosome subunit antiassociation, binding to 40*S* subunit

Eucariotic Initiation Factors (cont'd)

Initiation factor complex often referred to as eIF-4F composed of 3 primary subunits: eIF-4E, eIF-4A, eIF-4G and at least 2 additional factors: PABP, Mnk1 (or Mnk2)	mRNA binding to 40*S* subunit, ATPase-dependent RNA helicase activity, interaction between polyA tail and cap structure
PABP: polyA-binding protein	binds to the polyA tail of mRNAs and provides a link to eIF-4G
Mnk1 and Mnk2 eIF-4E kinases	phosphorylate eIF-4E increasing association with cap structure
eIF-4A	ATPase-dependent RNA helicase
eIF-4E	5' cap recognition
4E-BP (also called PHAS) 3 known forms	when de-phosphorylated 4E-BP binds eIF-4E and represses its' activity, phosphorylation of 4E-BP occurs in response many growth stimuli leading to release of eIF-4E and increased translational initiation

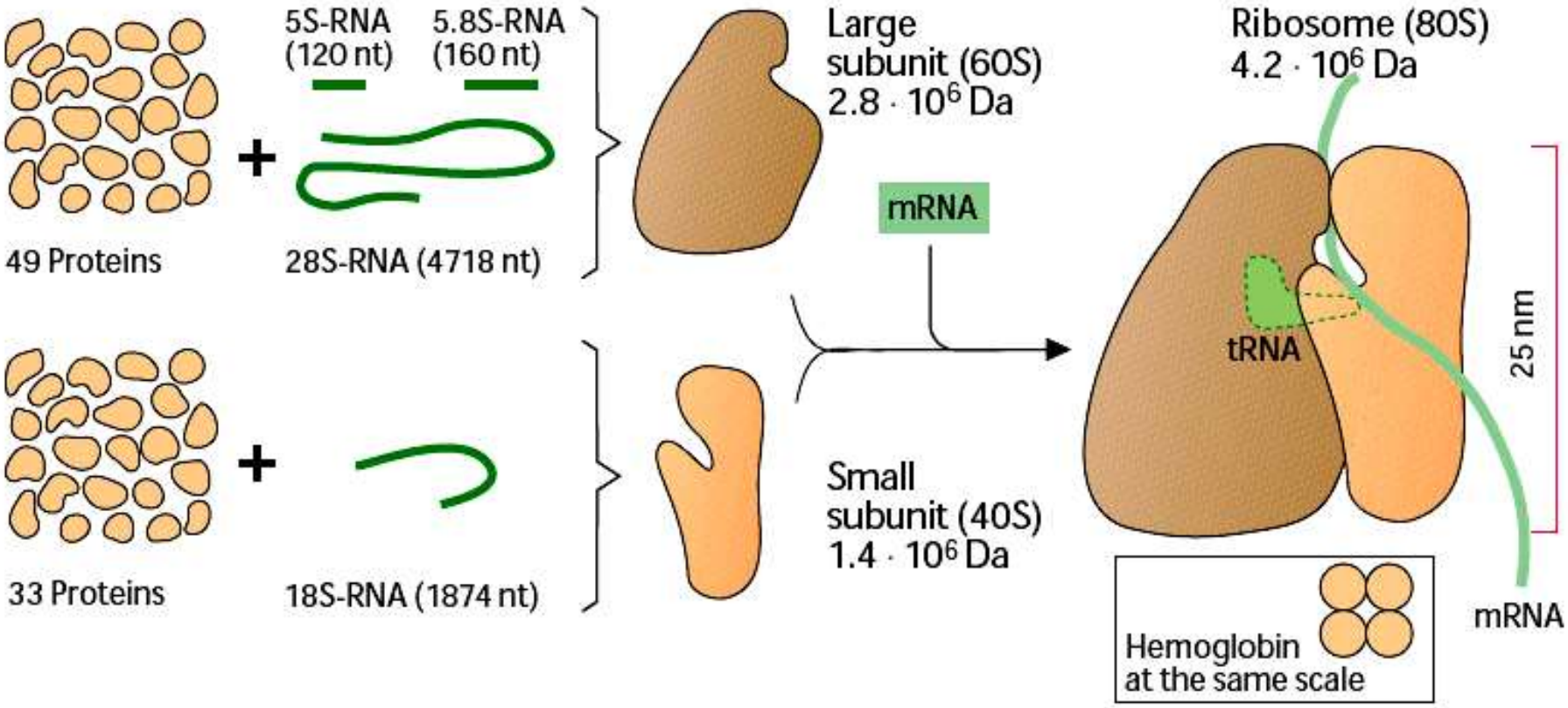
Eucariotic Initiation Factors (cont'd)

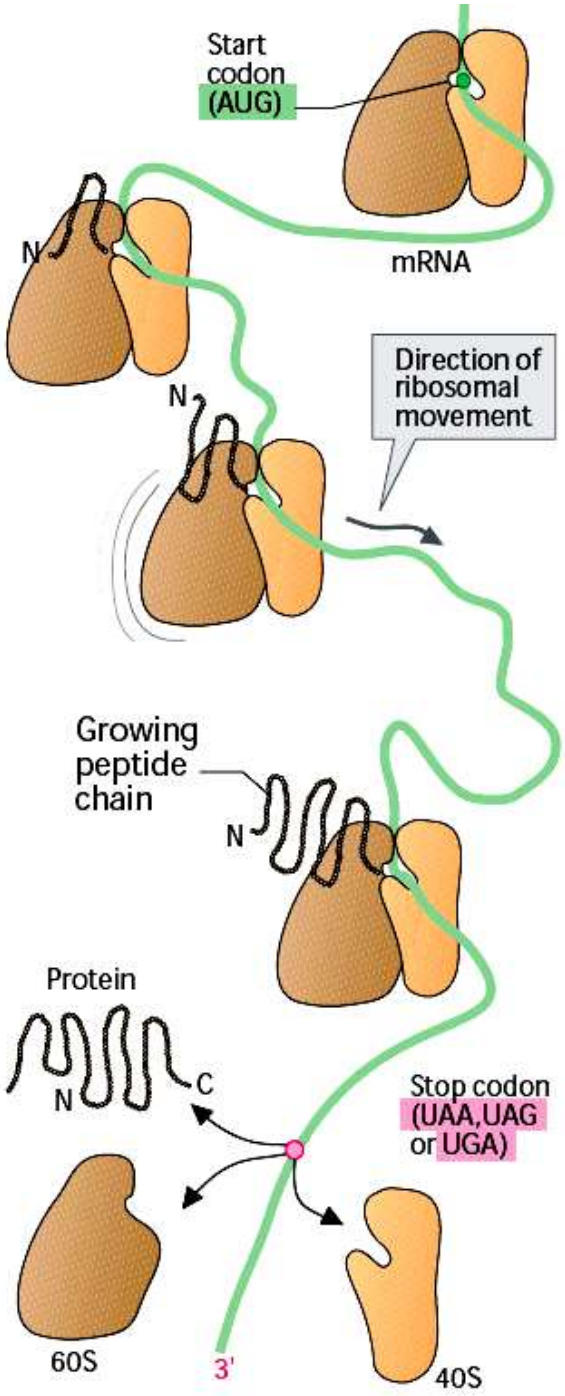
eIF-4G	acts as a scaffold for the assembly of eIF-4E and -4A in the eIF-4F complex, interaction with PABP allows 5'-end and 3'-ends of mRNAs to interact
eIF-4B	stimulates helicase, binds simultaneously with eIF-4F
eIF-5	release of eIF-2 and eIF-3, ribosome-dependent GTPase
eIF-6	ribosome subunit antiassociation

Specific Steps in Translational Initiation

- Initiation of translation requires 4 specific steps:
  1. A ribosome must dissociate into its' 40S and 60S subunits.
  2. A ternary complex termed the preinitiation complex is formed consisting of the initiator, GTP, eIF-2 and the 40S subunit.
  3. The mRNA is bound to the **preinitiation complex**.
  4. The 60S subunit associates with the preinitiation complex to form the 80S initiation complex.

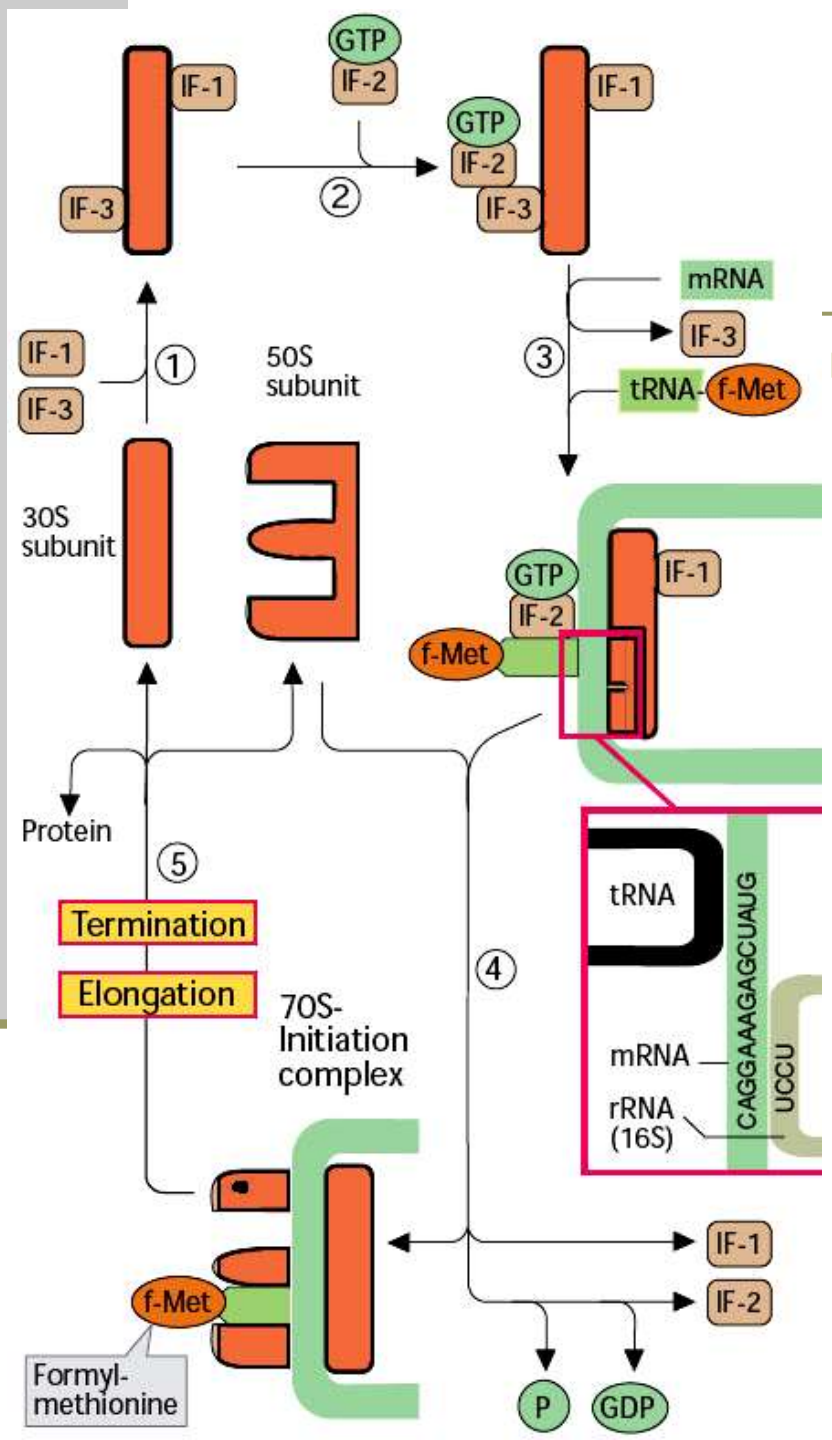
Structure of Eukaryotic Ribosomes





Polysomes

- In cells ribosomes are often found in a linear arrangement like a string of pearls: **polysomes**.
  - several ribosomes are translating a single mRNA molecule simultaneously.
- The ribosome first binds near the start codon (AUG) at the 5' end of the mRNA (top).
- During translation, the ribosome moves in the direction of the 3' end until it reaches a stop codon (UAA, UAG, or UGA).
- At this point, the newly synthesized chain is released, and the ribosome dissociates again into its two subunits.



Initiation of translation in E. coli

- The first phase of translation, initiation, involves several steps:
  - two proteins, initiation factors IF-1 and IF-3, bind to the 30 S subunit (1).
  - Another factor, IF-2, binds as a complex with GTP (2).
  - This allows the subunit to associate with the mRNA and makes it possible for a special tRNA to bind to the start codon (3).
  - In prokaryotes, this starter tRNA carries the substituted amino acid N-formylmethionine (fMet).
    - In eukaryotes, it carries methionine.
  - Finally, the 50 S subunit binds to the above complex (4).
  - During steps 3 and 4, the initiation factors are released again, and the GTP bound to IF–2 is hydrolyzed to GDP and P_i.

70 S Initiation Complex

- In the **70 S initiation complex**, formyl-methionine tRNA is initially located at a binding site known as the **peptidyl site (P)**.
- A second binding site, the **acceptor site (A)**, is not yet occupied during this phase of translation.
- Sometimes, a third tRNA binding site is defined as an *exit site (E),* from which uncharged tRNAs leave the ribosome again.

Preinitiation Complex Formation

- The initiation factors eIF-1 and eIF-3 bind to the 40S ribosomal subunit favoring antiassociation to the 60S subunit.
  - The prevention of subunit reassociation allows the preinitiation complex to form.
- The first step in the formation of the preinitiation complex is the binding of GTP to eIF-2 to form a binary complex. eIF-2 is composed of three subunits, α, β and γ.
  - The binary complex then binds to the activated initiator tRNA, met-tRNA^{met} forming a ternary complex that then binds to the 40S subunit forming the 43S preinitiation complex.
  - The preinitiation complex is stabilized by the earlier association of eIF-3 and eIF-1 to the 40S subunit.

Initiation Factor **eIF-4F**

- The cap structure of eukaryotic mRNAs is bound by specific eIFs prior to association with the preinitiation complex.
  - Cap binding is accomplished by the initiation factor **eIF-4F**.
  - This factor is actually a complex of 3 proteins; eIF-4E, A and G.
  - The protein eIF-4E is a 24 kDa protein which physically recognizes and binds to the cap structure. eIF-4A is a 46 kDa protein which binds and hydrolyzes ATP and exhibits RNA helicase activity.
  - Unwinding of mRNA secondary structure is necessary to allow access of the ribosomal subunits. eIF-4G aids in binding of the mRNA to the 43S preinitiation complex.

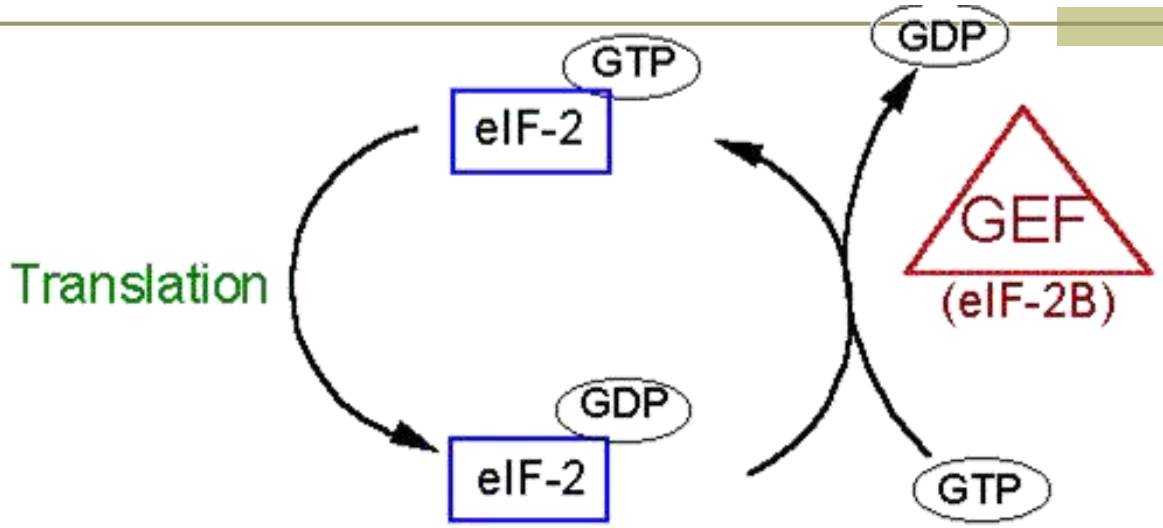
Initiation Complex Formation

- Once the mRNA is properly aligned onto the preinitiation complex and the initiator met-tRNAmet is bound to the initiator AUG codon (a process facilitated by eIF-1) the 60S subunit associates with the complex.
  - The association of the 60S subunit requires the activity of eIF-5 which has first bound to the preinitiation complex.
  - The energy needed to stimulate the formation of the 80S initiation complex comes from the hydrolysis of the GTP bound to eIF-2.
  - The GDP bound form of eIF-2 then binds to eIF-2B which stimulates the exchange of GTP for GDP on eIF-2.
  - When GTP is exchanged eIF-2B dissociates from eIF-2.
  - This is termed the eIF-2 cycle (see diagram below).
  - This cycle is absolutely required in order for eukaryotic translational initiation to occur.
  - The GTP exchange reaction can be affected by phosphorylation of the a-subunit of eIF-2.

P- and A-sites

- At this stage the initiator met-tRNA^{met} is bound to the mRNA within a site of the ribosome termed the P-site, for peptide site.
- The other site within the ribosome to which incoming charged tRNAs bind is termed the A-site, for amino acid site.

eIF-2 Cycle

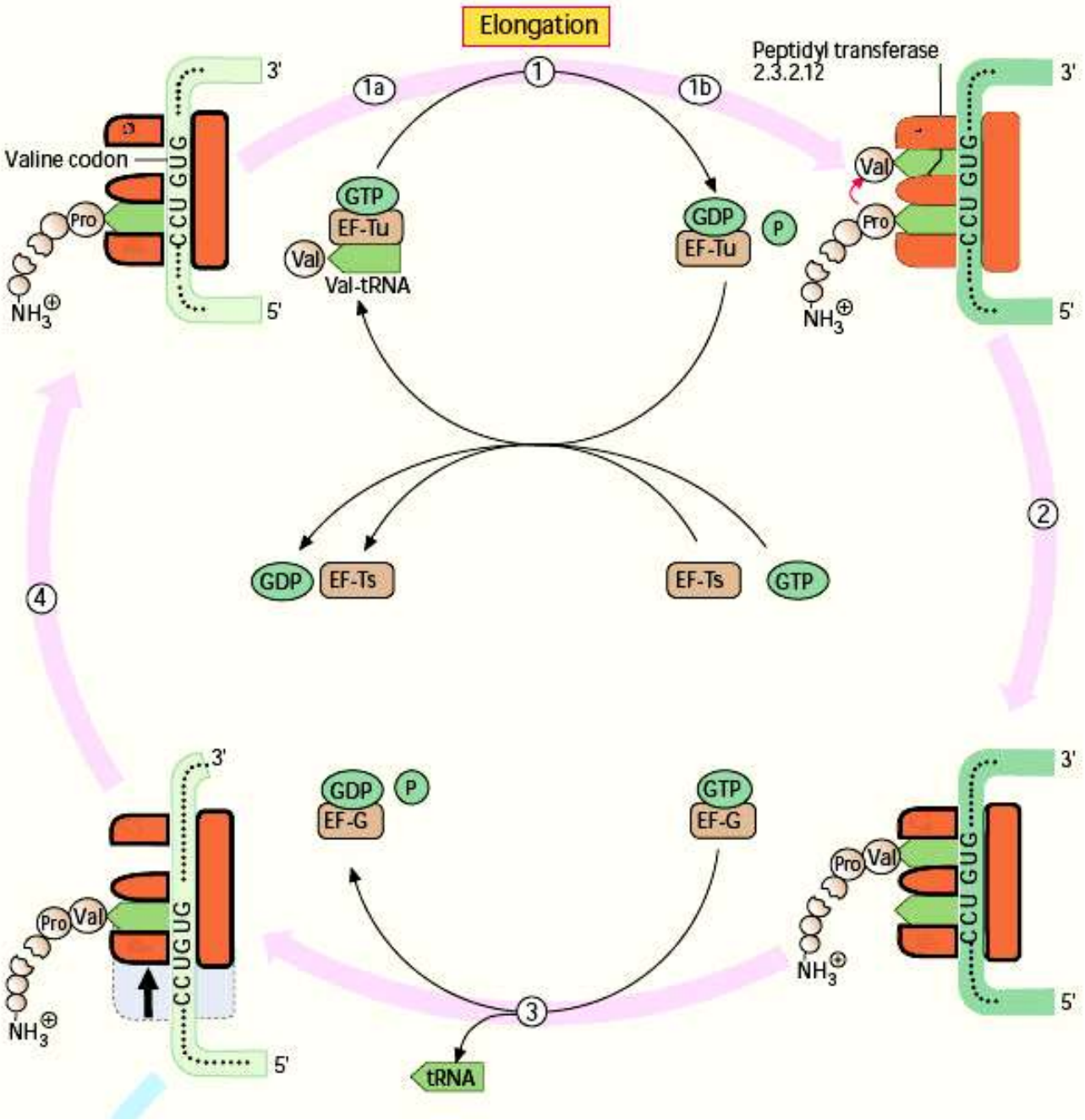


- The eIF-2 cycle involves the regeneration of GTP-bound eIF-2 following the hydrolysis of GTP during translational initiation.
- When the 40S preinitiation complex is engaged with the 60S ribosome to form the 80S initiation complex, the GTP bound to eIF-2 is hydrolyzed providing energy for the process.
- In order for additional rounds of translational initiation to occur, the GDP bound to eIF-2 must be exchanged for GTP.
- This is the function of eIF-2B which is also called guanine nucleotide exchange factor (GEF).

Elongation

- The process of elongation, like that of initiation requires specific non-ribosomal proteins.
  - In E. coli these are EFs and in eukaryotes eEFs.
  - Elongation of polypeptides occurs in a cyclic manner such that at the end of one complete round of amino acid addition the
  - A site will be empty and ready to accept the incoming aminoacyl-tRNA dictated by the next codon of the mRNA.
  - This means that not only does the incoming amino acid need to be attached to the peptide chain but the ribosome must move down the mRNA to the next codon.
  - Each incoming aminoacyl-tRNA is brought to the ribosome by an eEF-1a-GTP complex.
  - When the correct tRNA is deposited into the A site the GTP is hydrolyzed and the eEF-1a-GDP complex dissociates.
  - In order for additional translocation events the GDP must be exchanged for GTP.
  - This is carried out by eEF-1bg similarly to the GTP exchange that occurs with eIF-2 catalyzed by eIF-2B.

Elongation


Elongation
1
1a
1b
2
3
4
Peptidyl transferase
2.3.2.12
Valine codon
C C U G U G
Pro
Val
NH3
3'
5'
GTP
EF-Tu
Val-tRNA
GDP
P
EF-Ts
GTP
EF-G
tRNA

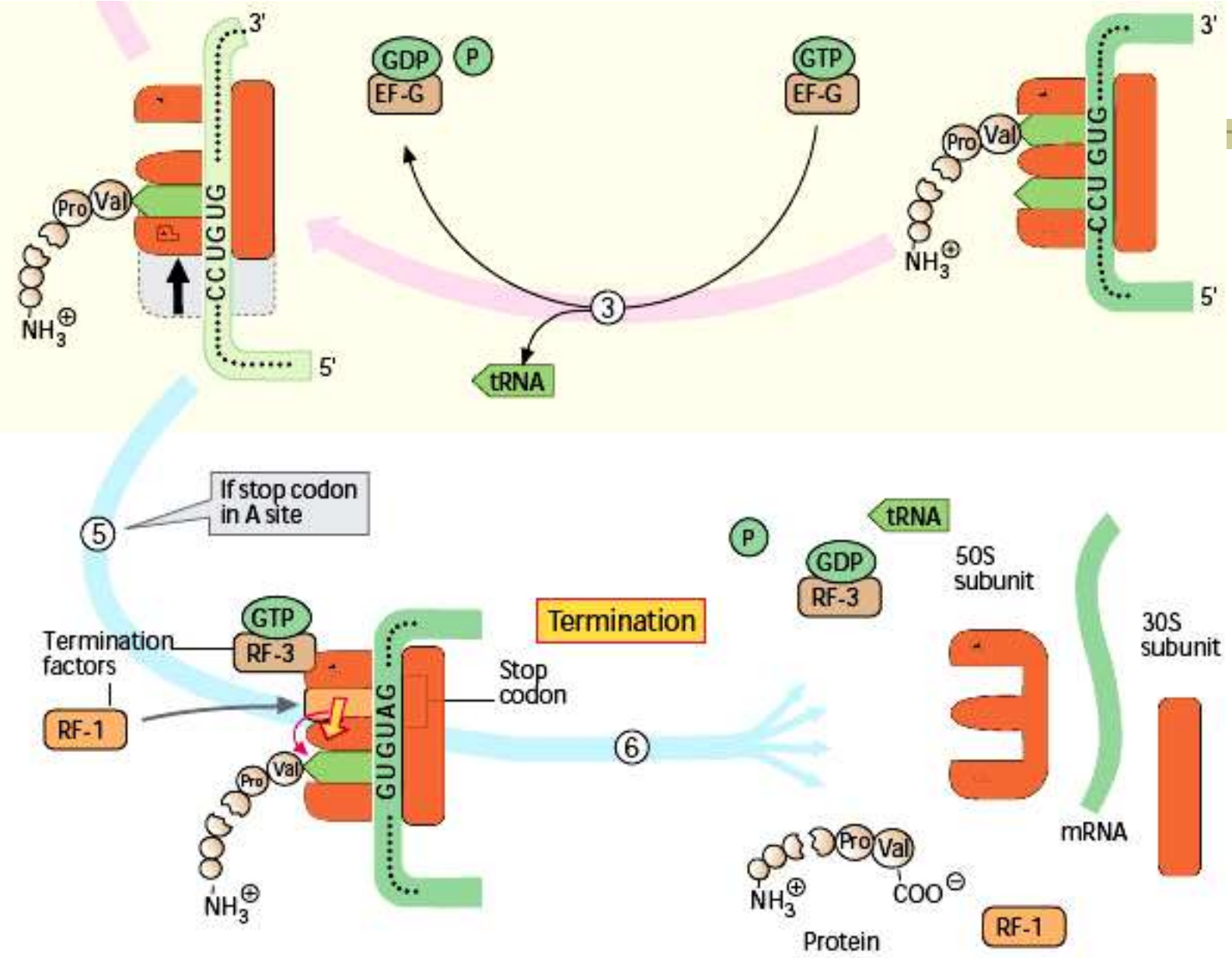
Steps in Elongation

- The peptide attached to the tRNA in the P site is transferred to the amino group at the aminoacyl-tRNA in the A site.
  - This reaction is catalyzed by ***peptidyltransferase***.
  - This process is termed **transpeptidation**.
  - The elongated peptide now resides on a tRNA in the A site.
    - The A site needs to be freed in order to accept the next aminoacyl-tRNA.
- The process of moving the peptidyl-tRNA from the A site to the P site is termed, **translocation**.
  - Translocation is catalyzed by eEF-2 coupled to GTP hydrolysis.
  - In the process of translocation the ribosome is moved along the mRNA such that the next codon of the mRNA resides under the A site.
  - Following translocation eEF-2 is released from the ribosome.
- The cycle can now begin again.

Termination

- Like initiation and elongation, translational termination requires specific protein factors identified as releasing factors, RFs in E. coli and eRFs in eukaryotes.
  - There are 2 RFs in E. coli and one in eukaryotes.
  - The signals for termination are the same in both prokaryotes and eukaryotes.
  - These signals are termination codons present in the mRNA.
  - There are 3 termination codons, UAG, UAA and UGA.
- In E. coli the termination codons UAA and UAG are recognized by RF-1, whereas RF-2 recognizes the termination codons UAA and UGA.
  - The eRF binds to the A site of the ribosome in conjunction with GTP.
  - The binding of eRF to the ribosome stimulates the peptidytransferase activity to transfer the peptidyl group to water instead of an aminoacyl-tRNA.
  - The resulting uncharged tRNA left in the P site is expelled with concomitant hydrolysis of GTP.
  - The inactive ribosome then releases its mRNA and the 80S complex dissociates into the 40S and 60S subunits ready for another round of translation.

Termination



Selenoproteins

- Selenium is a trace element and is found as a component of several prokaryotic and eukaryotic enzymes that are involved in redox reactions.
- The selenium in these selenoproteins is incorporated as a unique amino acid, selenocysteine, during translation.
  - A particularly important eukaryotic selenoenzyme is glutathione peroxidase.
  - This enzyme is required during the oxidation of glutathione by hydrogen peroxide (H_2O_2) and organic hydroperoxides.

Incorporation of Selenocysteine

- Incorporation of selenocysteine by the translational machinery occurs via an interesting and unique mechanism.
  - The tRNA for selenocysteine is charged with serine and then enzymatically selenylated to produce the selenocysteinyl-tRNA.
  - The anticodon of selenocysteinyl-tRNA interacts with a stop codon in the mRNA (UGA) instead of a serine codon.
  - The selenocysteinyl-tRNA has a unique structure that is not recognized by the termination machinery and is brought into the ribosome by a dedicated specific elongation factor.
  - An element in the 3' non-translated region (UTR) of selenoprotein mRNAs determines whether UGA is read as a stop codon or as a selenocysteine codon.

Regulation of eIF-4E Activity

- The cellular levels of eIF-4E are the lowest of all eukaryotic initiation factors which makes this factor a prime target for regulation. Indeed, at least 3 distinct mechanisms are known to exist that regulate the level and activity of eIF-4E. These include regulation of the level of transcription of the eIF-4E gene, post-translational modification via phosphorylation and inhibition by interaction with binding proteins.

- Although the exact mechanisms used to upregulate the transcription of the eIF-4E gene are not yet well understood, it is known that exposure of cells to growth factors as well as activation of T cells leads to increased expression of eIF-4E. The proto-oncogene MYC is believed to play a role in the transcriptional activation of eIF-4E as 2 functional MYC-binding sites have been found in the promoter region of the eIF-4E gene. Of significant note is the finding that cells that are stably over-expressing the MYC gene also have enhanced levels of eIF-4E. Quite strikingly it has been shown that promiscuous elevation in the levels of eIF-4E lead to tumorigenesis placing this translation factor in the category of proto-oncogene.

- Numerous extracellular stimuli (e.g. insulin, EGF, angiotensis II and gastrin) that exert a portion of their effects at the level of enhanced translation do so by affecting the state of eIF-4E phosphorylation. However, it should be noted that not all signals that lead to increased eIF-4E phosphorylation lead to increased rates of translation. Changes in eIF-4E phosphorylation correlate well with progression through the cell cycle. In resting (G0) cells eIF-4E phosphorylation is low, it increases during G1 and S phase and then declines again in M phase. Phosphorylation of eIF-4E occurs at one major site which is Ser209 (in the human and mouse proteins).

- The primary signal transduction pathway leading to eIF-4E phosphorylation is that involving the RAS gene. Many growth factors stimulate activation of RAS in response to binding their cognate receptors. Subsequently, RAS activation leads to the phosphorylation and activation of MAP-interacting kinase-1 (Mnk1) which in turn phosphorylates eIF-4E. Although the exact effect of eIF-4E phosphorylation is not clearly defined, it may be necessary to increase affinity of eIF-4E for the mRNA cap structure and for eIF-4G.
- The principal mechanism utilized in the regulation of eIF-4E activity is through its interaction with a family of binding/repressor proteins termed 4E-BPs (4E binding proteins) which are widely distributed in numerous vertebrate and invertebrate organisms. In mammalian cells 3 related 4E-BPs have been found where 4E-BP1 and 4E-BP2 are also identified as PHAS-I and PHAS-II (PHAS refers to properties of heat and acid stability).

- Binding of 4E-BPs to eIF-4E does not alter the affinity of eIF-4E for the cap structure but prevents the interaction of eIF-4E with eIF-4G which in turn suppresses the formation of the eIF-4F complex (see Table of Initiation Factors above). The ability of 4E-BPs to interact with eIF-4E is controlled via the phosphorylation of specific Ser and Thr residues in 4E-BP. When hypophosphorylated, 4E-BPs bind with high efficiency to eIF-4E but lose their binding capacity when phosphorylated. Numerous growth and signal transduction stimulating effectors lead to phosphorylation of 4E-BPs just as these same responses can lead to phosphorylation of eIF-4E.

- There are several signal transduction pathways whose activations lead to phosphorylation of 4E-BPs. These include pathways that lead to activation of phosphatidylinositol 3-kinase (PI3K), the Akt Ser/Thr kinase which is also called protein kinase B, PKB (Akt was originally identified as a virally encoded oncogene) and the FKBP12-rapamycin-associated protein/mammalian target of rapamycin (FRAP/mTOR) family of proteins. The mammalian TOR proteins are homologs of the yeast TOR proteins that were identified in a screen for yeast mutants resistant to rapamycin. Rapamycin is an immunosuppressant used primarily in the prevention of tissue rejection following organ transplantation. Rapamycin functions within cells by binding to the immunophilin (an intracellular protein that binds to immunosuppressive drugs, e.g. FK506 and rapamycin) FK506-binding protein 12 (FKBP12). The net effect of phosphorylation of 4E-BPs is that they are released from eIF-4E allowing eIF-4E to actively bind eIF-4G.

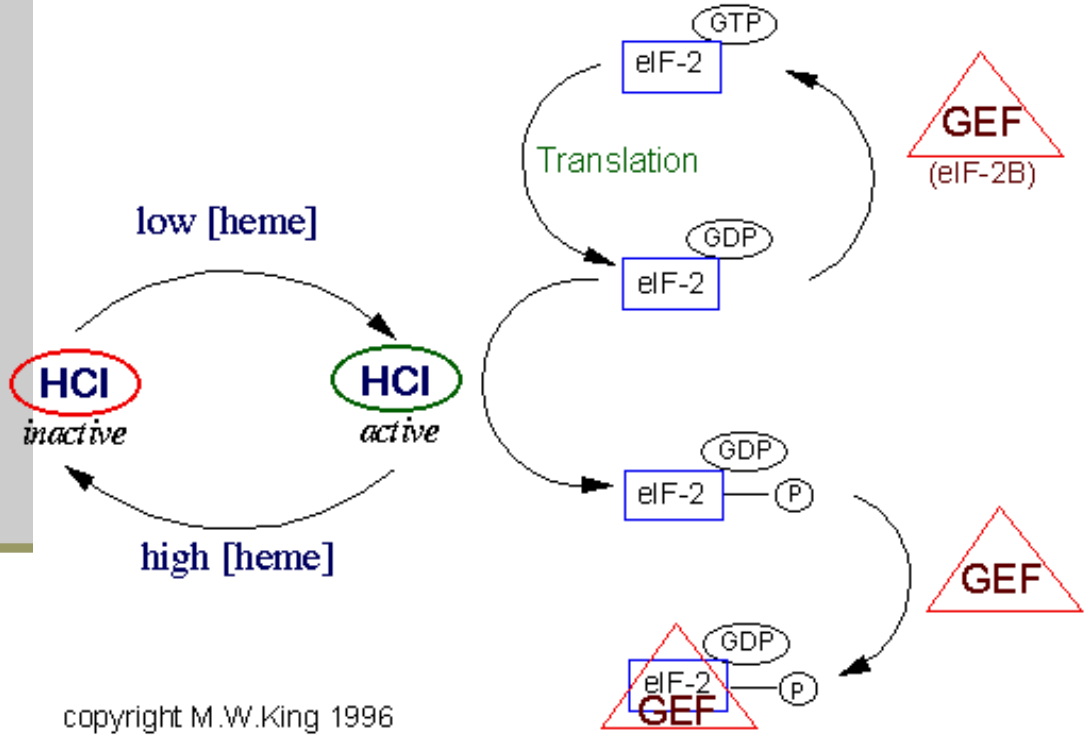
Heme Control of Translation

- Regulation of initiation in eukaryotes is effected by phosphorylation of a ser(S) residue in the a subunit of eIF-2. Phosphorylated eIF-2 in the absence of eIF-2B is just as active an initiator as non-phosphorylated eIF-2. However, when eIF-2 is phosphorylated the GDP-bound complex is stabilized and exchange for GTP is inhibited. The exchange of GDP for GTP is mediated by eIF-2B (also called guanine nucleotide exchange factor - GEF). When eIF-2 is phosphorylated it binds eIF-2B more tightly thus slowing the rate of exchange. It is this inhibited exchange that affects the rate of initiation.

Heme-controlled Inhibitor (HCI)

- The phosphorylation of eIF-2 is the result of an activity called heme-controlled inhibitor (HCI) which functions as diagrammed below.
  - HCI is generated in the absence of heme, a mitochondrial product.
  - Removal of phosphate is catalyzed by a specific eIF-2 phosphatase which is unaffected by heme.
  - The presence of HCI was first seen in in vitro translation system derived from lysates of reticulocytes.
  - Reticulocytes synthesize almost exclusively hemoglobin at an extremely high rate.
  - In an intact reticulocyte eIF-2 is protected from phosphorylation by a specific 67 kDa protein.

Regulation of Translation by Heme-controlled Inhibitor (HCI)



- Clinically important only in erythrocytes, which are enucleate and contain primarily globin mRNA.
  - As the level of heme falls the activity of HCI increases.
- HCI is a kinase which phosphorylates eIF-2.
  - When phosphorylated, eIF-2 still hydrolyzes bound GTP to GDP and still interacts with eIF-2B (GEF).
  - However, the rate of eIF-2B-mediated GTP exchange is greatly reduced. This renders eIF-2 incapable of being used to form an new ternary initiation complex and translational initiation is reduced.
- When the level of heme again rises the activity of HCI is reduced and translational initiation is once again active.

Interferon Control of Translation

- Regulation of translation can also be induced in virally infected cells. It would benefit a virally infected cell to turn off protein synthesis to prevent propagation of the viruses.
- This is accomplished by the induced synthesis of interferons (IFs).
- There are 3 classes of IFs: the leukocyte or α-IFs, the fibroblast or β-IFs and the lymphocyte or γ-IFs.
- IFs are induced by dsRNAs and themselves induce a specific kinase termed RNA-dependent protein kinase (PKR) that phosphorylates eIF-2 thereby shutting off translation in a similar manner to that of heme control of translation.
- Additionally, IFs induce the synthesis of 2'-5'-oligoadenylate, pppA(2'p5'A)n, that activates a pre-existing ribonuclease, RNase L.
- RNase L degrades all classes of mRNAs thereby shutting off translation.

Iron Control of Translation

- Regulation of the translation of certain mRNAs occurs through the action of specific RNA-binding proteins.
- Protein of this class have been identified that bind to sequences in either the 5' non-translated region (5'-UTR) or 3'-UTR.
  - Two particularly interesting and important regulatory schemes related to iron metabolism encompass RNA binding proteins that bind to either the 5'-UTR of one mRNA or the 3'-UTR of another.

Iron Metabolism Regulatory Schemes

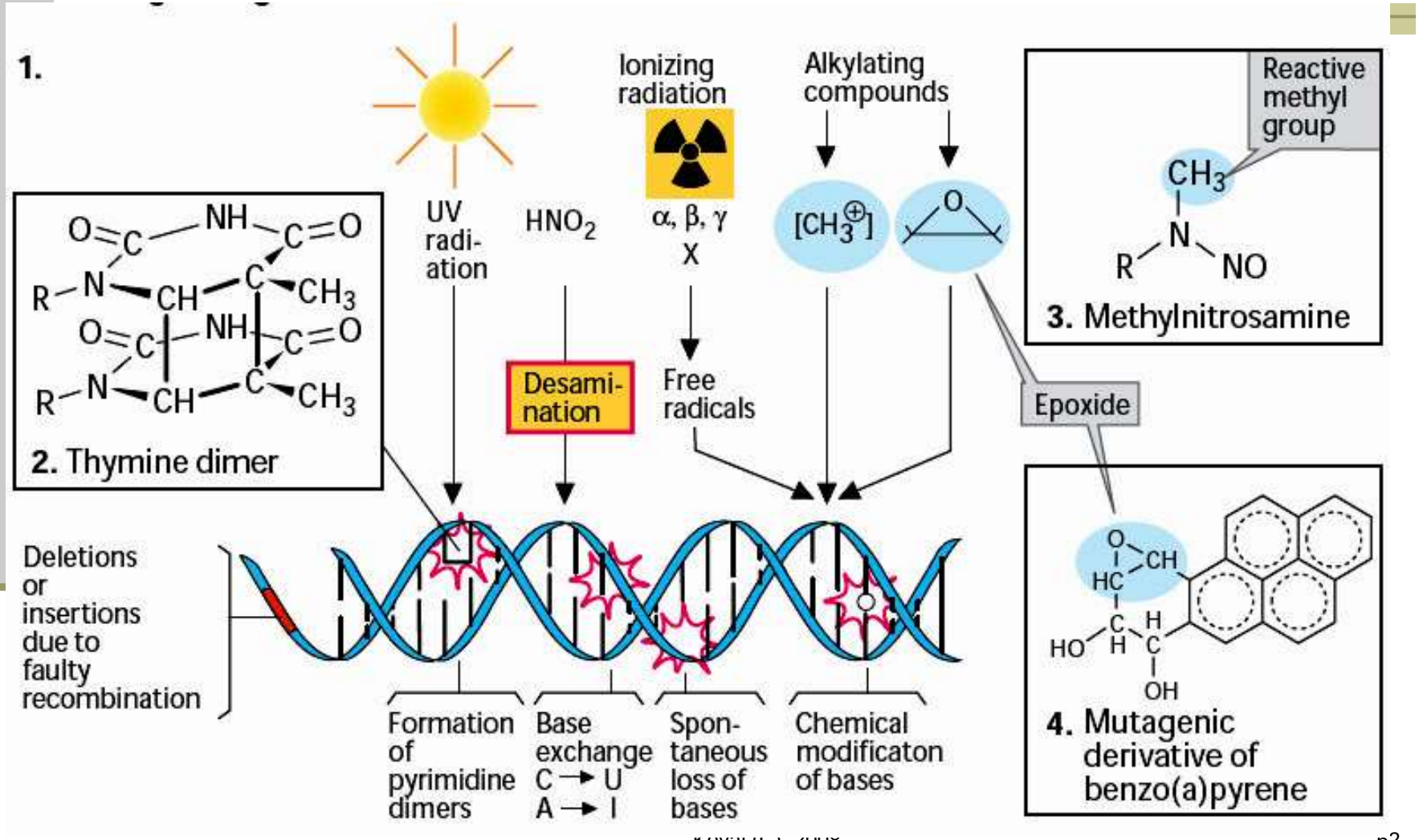
- The transferrin receptor is a protein located in the plasma membrane that binds the protein transferrin.
  - Transferrin is the major iron transport protein in the plasma.
  - When iron levels are low the rate of synthesis of the transferrin receptor mRNA increases so that cells can take up more iron.
- This regulation occurs through the action of an iron response element binding protein (IRBP) that binds to specific iron response elements (IREs) in the 3'-UTR of the transferrin receptor mRNA.
  - These IREs form hair-pin loop structures that are recognized by IRBP.
  - This IRBP is an iron-deficient form of aconitase, the iron-requiring enzyme of the TCA cycle.
  - When iron levels are low, IRBP is free of iron and can therefore, interact with the IREs in the 3'-UTR of the transferrin receptor mRNA.
- Transferrin receptor mRNA with IRBP bound is stabilized from degradation.
- Conversely, when iron levels are high, IRBP binds iron then cannot interact with the IREs in the transferrin receptor mRNA.
- The effect is an increase in degradation of the transferrin receptor mRNA.

- A related, but opposite, phenomenon controls the translation of the ferritin mRNA.
- Ferritin is an iron-binding protein that prevents toxic levels of ionized iron (Fe^{2+}) from building up in cells.
  - The ferritin mRNA has an IRE in its 5'-UTR. As with the transferrin receptor story, when iron levels are high, IRBP cannot bind to the IRE in the 5'-UTR of the ferritin mRNA. This allows the ferritin mRNA to be translated.
  - Conversely, when iron levels are low, the IRBP binds to the IRE in the ferrritin mRNA preventing its translation.

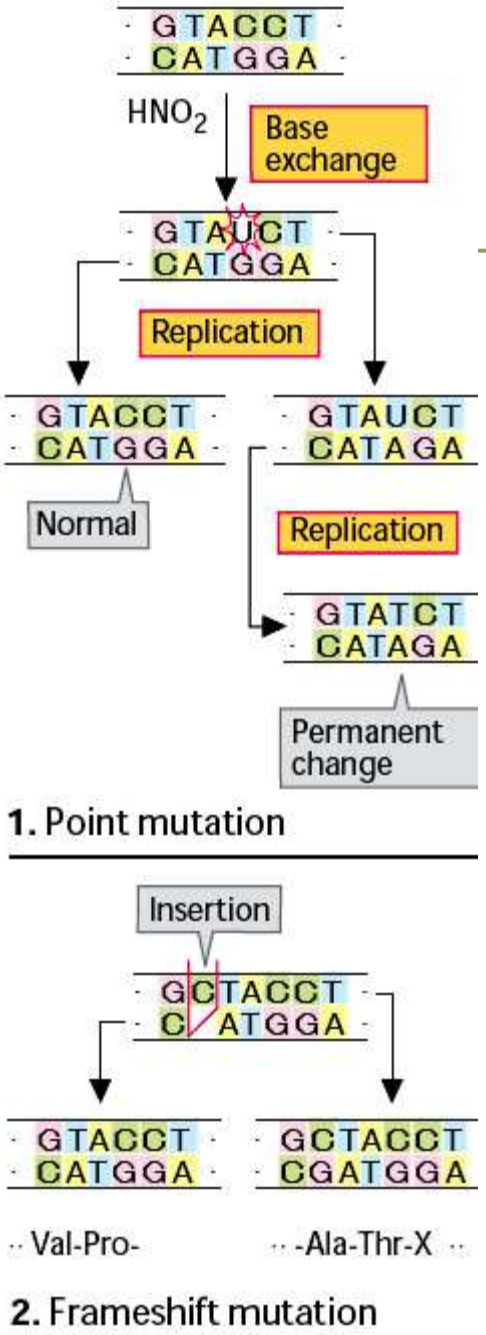
Mutation and Repair

- Genetic information is set down in the base sequence of DNA.
- Changes in the DNA bases or their sequence therefore have *mutagenic* effects.
- Mutagens often also damage growth regulation in cells, and they are then also *carcinogenic*.
- Gene alterations (**mutations**) are one of the decisive positive factors in biological evolution.
- On the other hand, an excessive mutation frequency would threaten the survival of individual organisms or entire species.
- For this reason, every cell has **repair mechanisms** that eliminate most of the DNA changes arising from mutations.

Mutagenic Agents

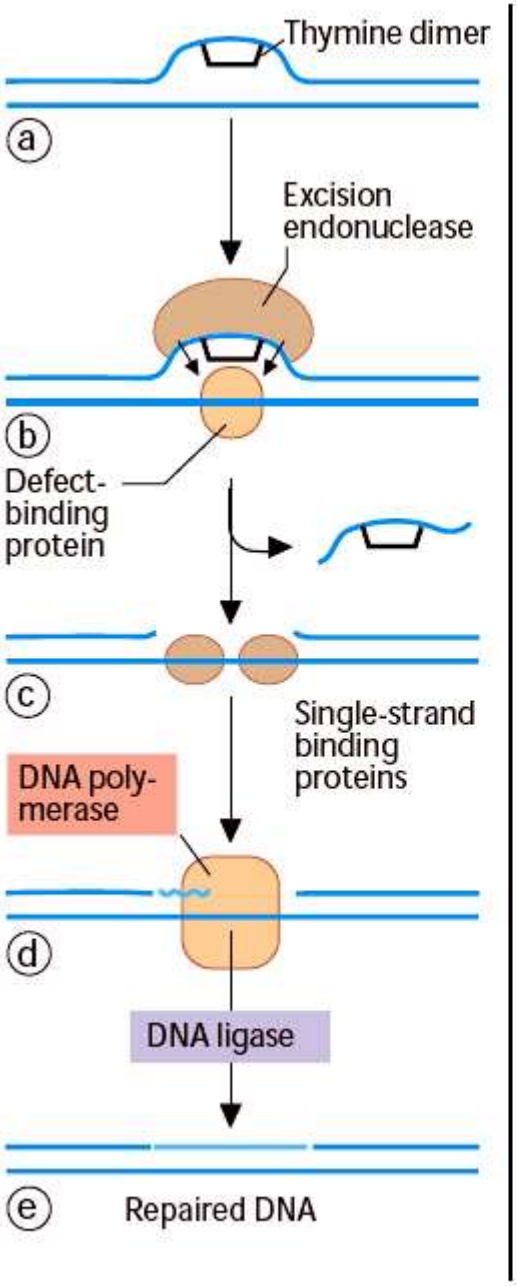


Mutations

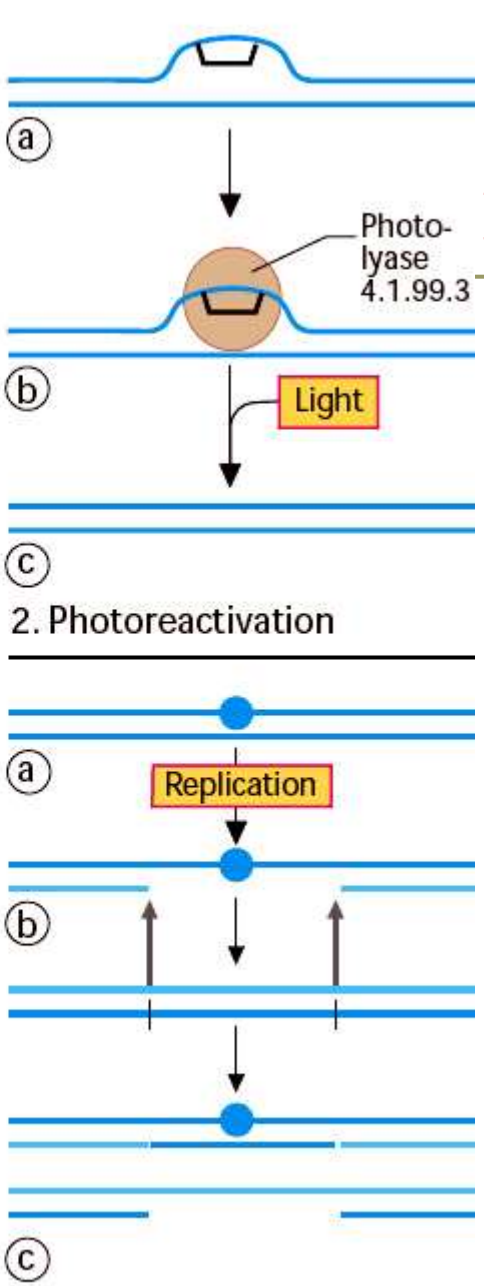


- Nitrous acid causes **point mutations** (**1**).
  - For example, C is converted to U, which in the next replication pairs with A instead of G.
  - The alteration thus becomes permanent.
- Mutations in which a number of nucleotides not divisible by three are inserted or removed lead to reading errors in whole segments of DNA, as they move the reading frame (**frameshift mutations**) (**2**).
  - From the inserted C onwards, the resulting mRNA is interpreted differently during translation, producing a completely new protein sequence.

Repair mechanisms



1. Excision repair



2. Photoreactivation

3. Recombinational repair

- An important mechanism for the removal of DNA damage is **excision repair** (**1**).
  - In this process, a specific *excision endonuclease* removes a complete segment of DNA on both sides of the error site. Using the sequence of the opposite strand, the missing segment is then replaced by a *DNA polymerase*. Finally, a *DNA ligase* closes the gaps again.
- Thymine dimers can be removed by **photoreactivation** (**2**).
  - A specific *photolyase* binds at the defect and, when illuminated, cleaves the dimer to yield two single bases again.

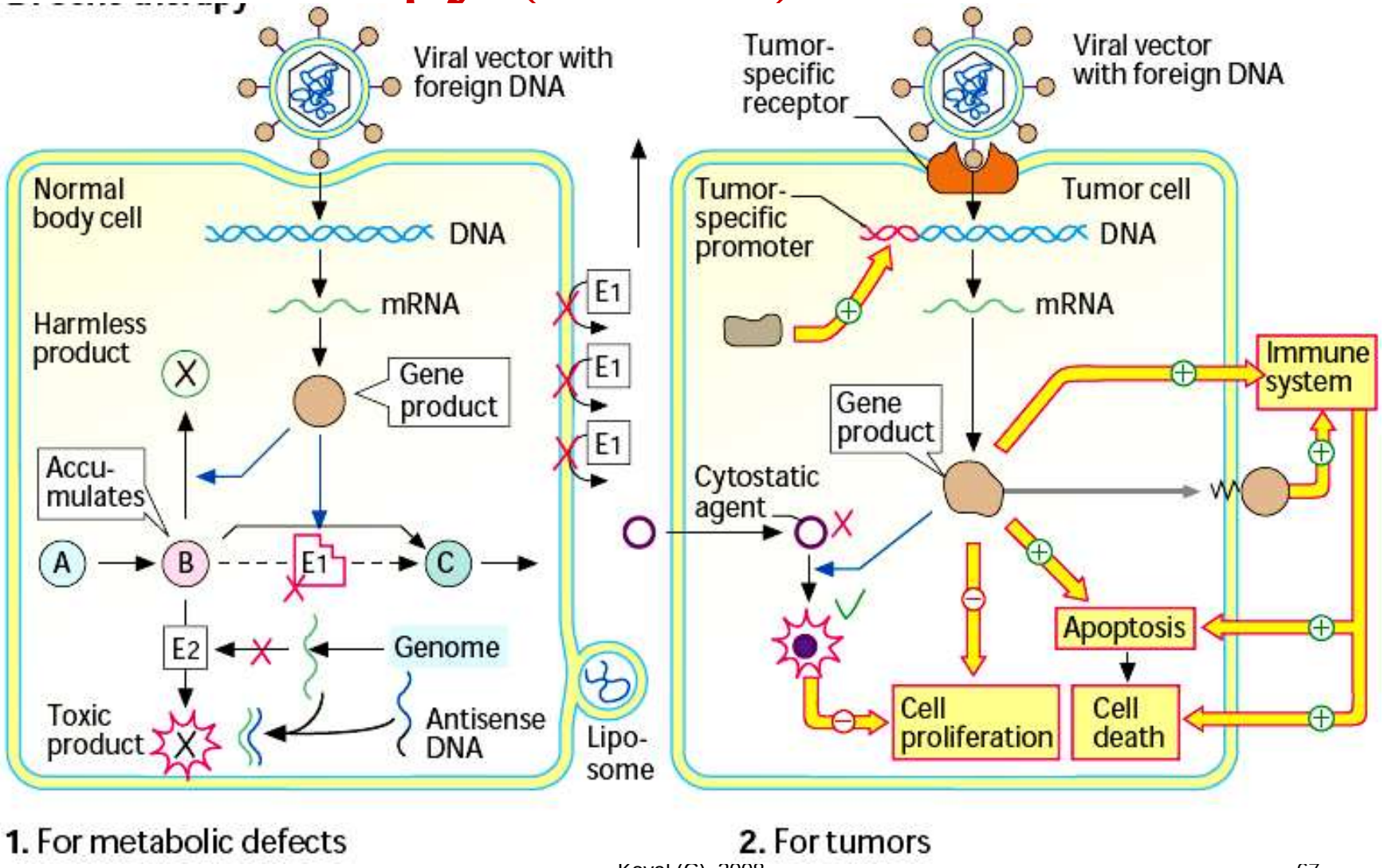
Recombination Repair

- A third mechanism is **recombination repair** (**3**, simplified).
- In this process, the defect is omitted during replication.
  - The gap is closed by shifting the corresponding sequence from the correctly replicated second strand.
  - The new gap that results is then filled by polymerases and ligases.
  - Finally, the original defect is corrected by excision repair **(1)**.

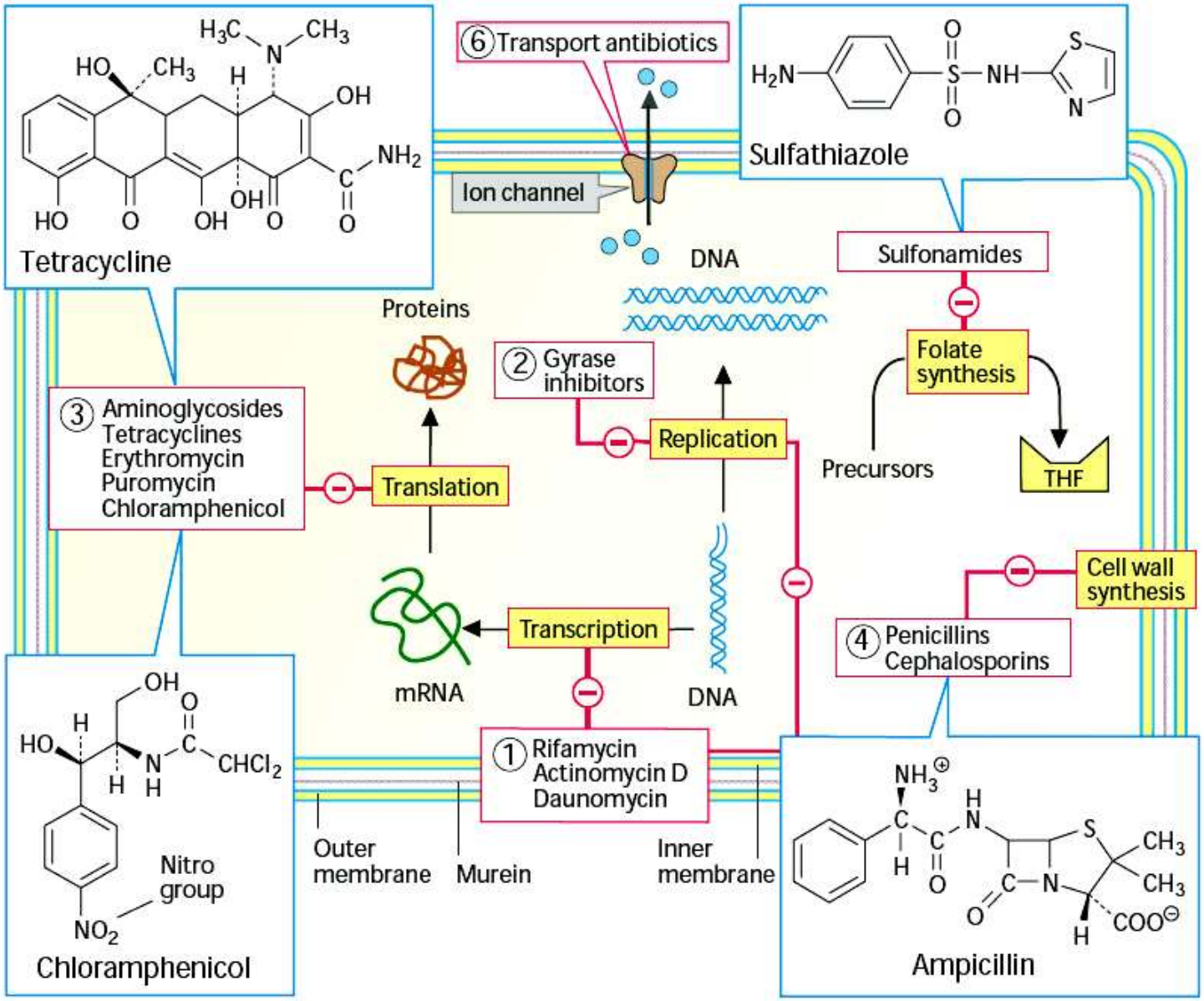
Gene therapy

- Many diseases, such as hereditary metabolic defects and tumors, can still not be adequately treated.
- About 10 years ago, projects were therefore initiated that aimed to treat diseases of this type by transferring genes into the affected cells (gene therapy).
- None of these procedures has yet become established in clinical practice.

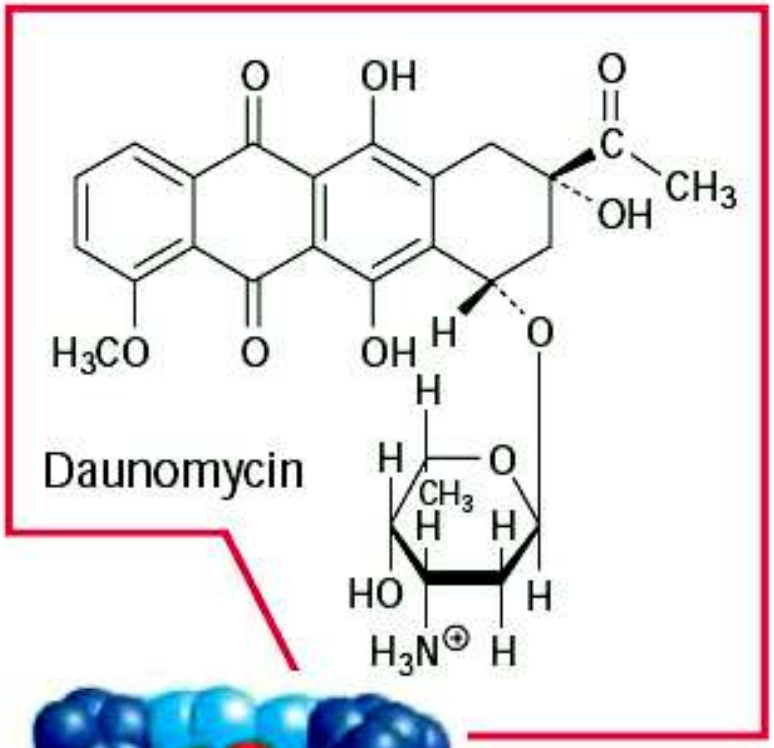
Gene therapy (cont'd)

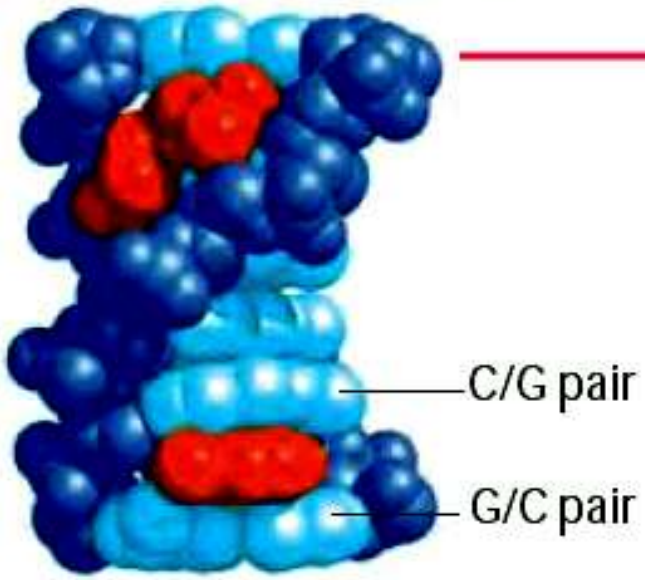


Antibiotics: Overview



Intercalators





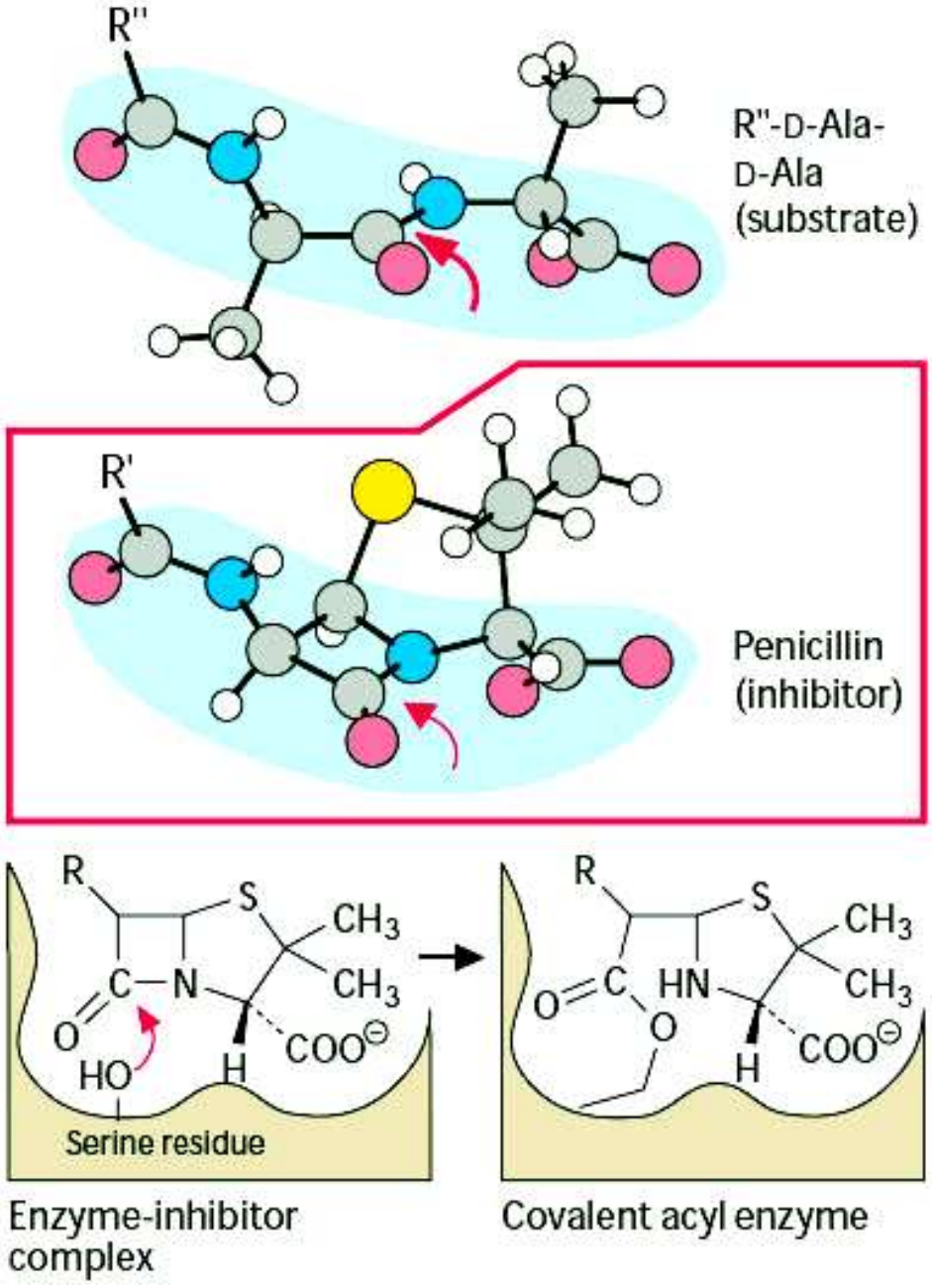
Daunomycin-DNA complex

- The effects of intercalators are illustrated here using the example of the **daunomycin–DNA complex**, in which two daunomycin molecules (red) are inserted in the double helix (blue).
- The antibiotic's ring system inserts itself between G/C base pairs (bottom),while the sugar moiety occupies the minor groove in the DNA (above). This leads to a localized change of the DNA conformation that prevents replication and transcription.

Penicillin as a "Suicide Substrate"

- The site of action in the β–lactam antibiotics is *muramoylpentapeptide carboxypeptidase,* an enzyme that is essential for cross-linking of bacterial cell walls.

Penicillin Action



- The antibiotic resembles the substrate of this enzyme (a peptide with the C-terminal sequence D-Ala–D–Ala) and is therefore reversibly bound in the active center. This brings the β-lactam ring into proximity with an essential serine residue of the enzyme.
- Nucleophilic substitution then results in the formation of a stable covalent bond between the enzyme and the inhibitor, blocking the active center.
- In dividing bacteria, the loss of activity of the enzyme leads to the formation of unstable cell walls and eventually death.

Protein Synthesis Inhibitors

- Many of the antibiotics utilized for the treatment of bacterial infections as well as certain toxins function through the inhibition of translation.
  - Inhibition can be effected at all stages of translation from initiation to elongation to termination.

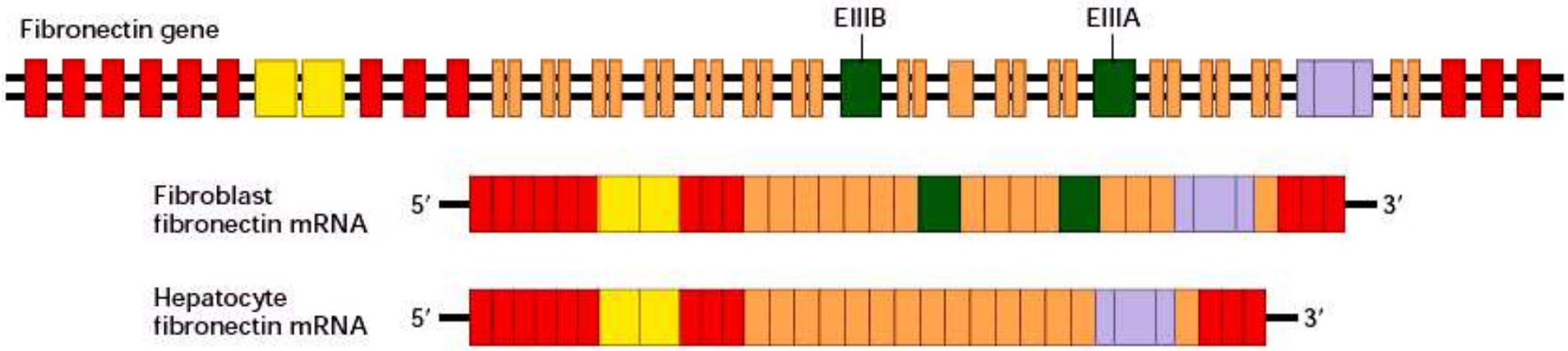
Several Antibiotic and Toxin inhibitors of Translation (1/2)

Inhibitor	Comments
Chloramphenicol	inhibits prokaryotic ***peptidyl transferase***
Streptomycin	inhibits prokaryotic peptide chain initiation, also induces mRNA misreading
Tetracycline	inhibits prokaryotic aminoacyl-tRNA binding to the ribosome small subunit
Neomycin	similar in activity to streptomycin
Erythromycin	inhibits prokaryotic translocation through the ribosome large subunit
Fusidic acid	similar to erythromycin only by preventing EF-G from dissociating from the large subunit

Several Antibiotic and Toxin inhibitors of Translation (2/2)

Inhibitor	Comments
Puromycin	resembles an aminoacyl-tRNA, interferes with peptide transfer resulting in premature termination in both prokaryotes and eukaryotes
Diptheria toxin	catalyzes ADP-ribosylation of and inactivation of eEF-2
Ricin	found in castor beans, catalyzes cleavage of the eukaryotic large subunit rRNA
Cycloheximide	inhibits eukaryotic ***peptidyltransferase***

Alternative RNA Splicing Increases the Number of Proteins Expressed from a Single Eukaryotic Gene



- **Cell type–specific splicing of fibronectin pre-mRNA in fibroblasts and hepatocytes.**
  - The ≈75-kb fibronectin gene *(top)* contains multiple exons.
  - The EIIIB and EIIIA exons (green) encode binding domains for specific proteins on the surface of fibroblasts. The fibronectin mRNA produced in fibroblasts includes the EIIIA and EIIIB exons, whereas these exons are spliced out of fibronectin mRNA in hepatocytes.
  - In this diagram, introns (black lines) are not drawn to scale; most of them are much longer than any of the exons.

Conclusion


Thank you
for your attention