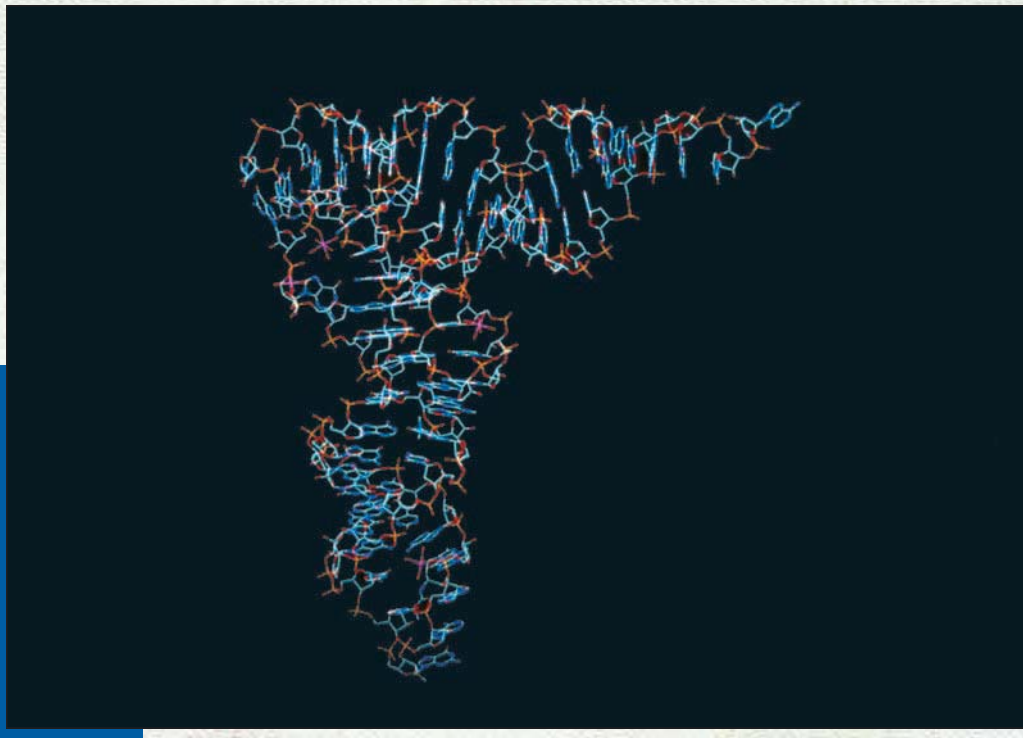
CHAPTER 17

The Mechanism of Translation I: Initiation



Cryo-electron microscopy model of the eIF3-mRNA-40S ribosomal particle complex. Yellow-green, ribosomal particle; magenta, eIF3; red, mRNA, with purple internal ribosomal entry site (IRES); e1, site of attachment of eIF1. *(© Tripos Associates/Peter Arnold/ PhotoLibrary Group)*

Translation is the process by which ribosomes read the genetic message in mRNA and produce a protein product according to the message's instructions. Ribosomes therefore serve as protein factories. Transfer RNAs (tRNAs) play an equally important role as adapters that can bind an amino acid at one end and interact with the mRNA at the other. Chapter 3 presented an outline of the translation process. In this chapter we will begin to fill in some of the details.

We can conveniently divide the mechanism of translation into three phases: initiation, elongation, and termination. In the initiation phase, the ribosome binds to the mRNA, and the first amino acid, attached to its tRNA, also binds. During the elongation phase, the ribosome adds one amino acid at a time to the growing polypeptide chain. Finally, in the termination phase, the ribosome releases the mRNA and the finished

polypeptide. The overall scheme is similar in bacteria and eukaryotes, but there are significant differences, especially in the added complexity of the eukaryotic translation initiation system.

This chapter concerns the initiation of translation in eukaryotes and bacteria. Because the nomenclatures of the two systems are different, it is easier to consider them separately. Therefore, let us begin with a discussion of the simpler system, initiation in bacteria. Then we will move on to the more complex eukaryotic scheme.

17.1 Initiation of Translation in Bacteria

Two important events must occur even before translation initiation can take place. One of these prerequisites is to generate a supply of **aminoacyl-tRNAs** (tRNAs with their cognate amino acids attached). In other words, amino acids must be covalently bound to tRNAs. This process is called **tRNA charging;** the tRNA is said to be "charged" with an amino acid. Another preinitiation event is the dissociation of ribosomes into their two subunits. This is necessary because the cell assembles the initiation complex on the small ribosomal subunit, so the two subunits must separate to make this assembly possible.

tRNA Charging

All tRNAs have the same three bases (CCA) at their 3′-ends, and the terminal adenosine is the target for charging. An amino acid is attached by an ester bond between its carboxyl group and the 2′- or 3′-hydroxyl group of the terminal adenosine of the tRNA, as shown in Figure 17.1. Charging takes place in two steps (Figure 17.2), both catalyzed by the enzyme **aminoacyl-tRNA synthetase.** In the first reaction (1), the amino acid is activated, using energy from ATP; the product of the reaction is aminoacyl-AMP. The pyrophosphate by-product is simply the two end phosphate groups (the β- and γ-phosphates), which the ATP lost in forming AMP.

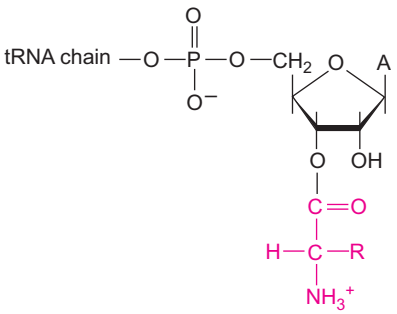


Figure 17.1 Linkage between tRNA and an amino acid. Some amino acids are bound initially by an ester linkage to the 3′-hydroxyl group of the terminal adenosine of the tRNA as shown, but some bind initially to the 2′-hydroxyl group. In any event, the amino acid is transferred to the 3′-hydroxyl group before it is incorporated into a protein.

(1) amino acid + ATP → aminoacyl-AMP + pyrophosphate (PP_i)

The bonds between phosphate groups in ATP (and the other nucleoside triphosphates) are high-energy bonds. When they are broken, this energy is released. In this case, the energy is trapped in the **aminoacyl-AMP,** which is why we call this an *activated amino acid.* In the second reaction of charging, the energy in the aminoacyl-AMP is used to transfer the amino acid to a tRNA, forming *aminoacyl-tRNA*.

(2) aminoacyl-AMP + tRNA → aminoacyl-tRNA + AMP

The sum of reactions 1 and 2 is this:

(3) amino acid + ATP + tRNA → aminoacyl-tRNA + AMP + PP_i

Just like other enzymes, an aminoacyl-tRNA synthetase plays a dual role. Not only does it catalyze the reaction leading to an aminoacyl-tRNA, but it determines the specificity of this reaction. Only 20 synthetases exist, one for each amino acid, and they are very specific. Each will almost always place an amino acid on the right kind of tRNA. This is essential to life: If the aminoacyl-tRNA synthetases made many mistakes, proteins would be put together with a correspondingly large number of incorrect amino acids and could not function properly. We will return to this theme and see how the synthetases select the proper tRNAs and amino acids in Chapter 19.

SUMMARY Aminoacyl-tRNA synthetases join amino acids to their cognate tRNAs. They do this very specifically in a two-step reaction that begins with activation of the amino acid with AMP, derived from ATP.

Dissociation of Ribosomes

We learned in Chapter 3 that ribosomes consist of two subunits. The 70S ribosomes of *E. coli,* for example, contain one 30S and one 50S subunit. Each subunit has one or two ribosomal RNAs and a large collection of ribosomal proteins. The 30S subunit binds the mRNA and the anticodon ends of the tRNAs. Thus, it is the decoding agent of the ribosome that reads the genetic code in the mRNA and allows binding with the appropriate aminoacyl-tRNAs. The 50S subunit binds the ends of the tRNAs that are charged with amino acids and has the peptidyl transferase activity that links amino acids together through peptide bonds.

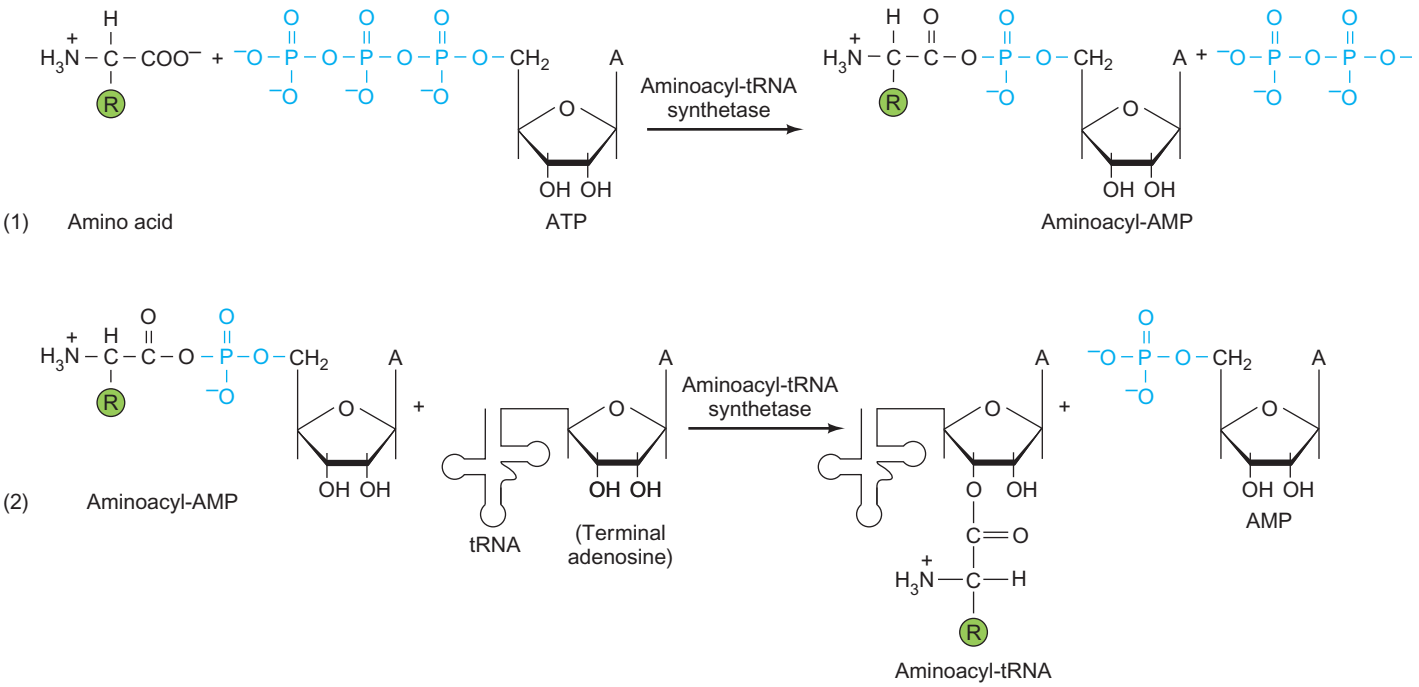


Figure 17.2 Aminoacyl-tRNA synthetase activity. Reaction 1: The aminoacyl-tRNA synthetase couples an amino acid to AMP, derived from ATP, to form an aminoacyl-AMP, with pyrophosphate (P-P) as a by-product. Reaction 2: The synthetase replaces the AMP in the aminoacyl-AMP with tRNA, to form an aminoacyl-tRNA, with AMP as a by-product. The amino acid is joined to the 3′-hydroxyl group of the terminal adenosine of the tRNA.

We will see shortly that both bacterial and eukaryotic cells build translation initiation complexes on the small ribosomal subunit. This implies that the two ribosomal subunits must dissociate after each round of translation for a new initiation complex to form. And as early as 1968, Matthew Meselson and colleagues provided direct evidence for the dissociation of ribosomes, using an experiment outlined in Figure 17.3. These workers labeled *E. coli* ribosomes with heavy isotopes of nitrogen (^{15}N), carbon (^{13}C), and hydrogen (^{2}H, deuterium), plus a little ^{3}H

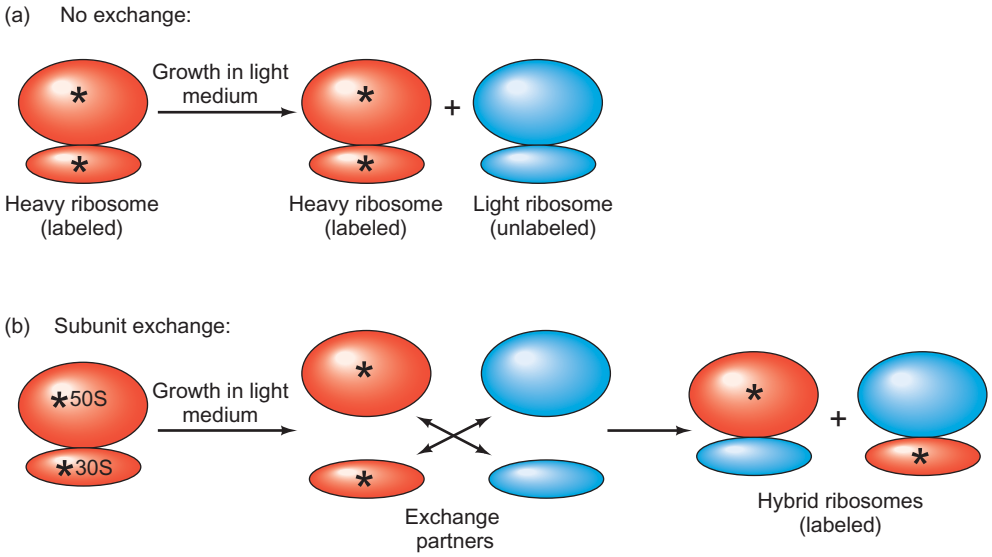


Figure 17.3 Experimental plan to demonstrate ribosomal subunit exchange. Meselson and colleagues made ribosomes heavy (red) by growing *E. coli* in the presence of heavy isotopes of nitrogen, carbon, and hydrogen, and made them radioactive (asterisks) by including some ^{3}H. Then they shifted the cells with labeled, heavy ribosomes to light medium containing the standard isotopes of nitrogen, carbon, and hydrogen. **(a)** No exchange. If no ribosome subunit exchange occurs, the heavy ribosomal subunits will stay together, and the only labeled ribosomes observed will be heavy. The light ribosomes made in the light medium will not be detected because they are not radioactive. **(b)** Subunit exchange. If the ribosomes dissociate into 50S and 30S subunits, heavy subunits can associate with light ones to form labeled hybrid ribosomes.

as a radioactive tracer. The ribosomes so labeled became much denser than their normal counterparts grown in ^{14}N, ^{12}C, and hydrogen, as illustrated in Figure 17.4a. Next, the investigators placed cells with labeled, heavy ribosomes in medium with ordinary light isotopes of nitrogen, carbon, and hydrogen. After 3.5 generations, they isolated the ribosomes and measured their masses by sucrose density gradient centrifugation with ^{14}C-labeled light ribosomes for comparison. Figure 17.4b shows the results. As expected, they observed heavy radioactively labeled ribosomal subunits (38S and 61S instead of the standard 30S and 50S). But the labeled whole ribosomes had a hybrid sedimentation coefficient, in between the standard 70S and the 86S they would have had if both subunits were heavy. This indicated that subunit exchange had occurred. Heavy ribosomes had dissociated into subunits and taken new, light partners.

More precise resolution of the ribosomes on CsCl gradients demonstrated two species: one with a heavy large subunit and a light small subunit, and one with a light large subunit and a heavy small subunit, as predicted in Figure 17.3. Meselson and colleagues performed the same experiments on yeast cells and obtained the same results, so eukaryotic ribosomes also cycle between intact ribosomes (80S) and ribosomal subunits (40S and 60S). What causes the ribosomal subunits to dissociate? We will learn in Chapter 18 that bacteria have a ribosome release factor (RRF) that acts in conjunction with an elongation factor (EF-G) to separate the subunits. In addition, an initiation factor, IF3 binds to the small subunit and keeps it from reassociating with the large subunit.

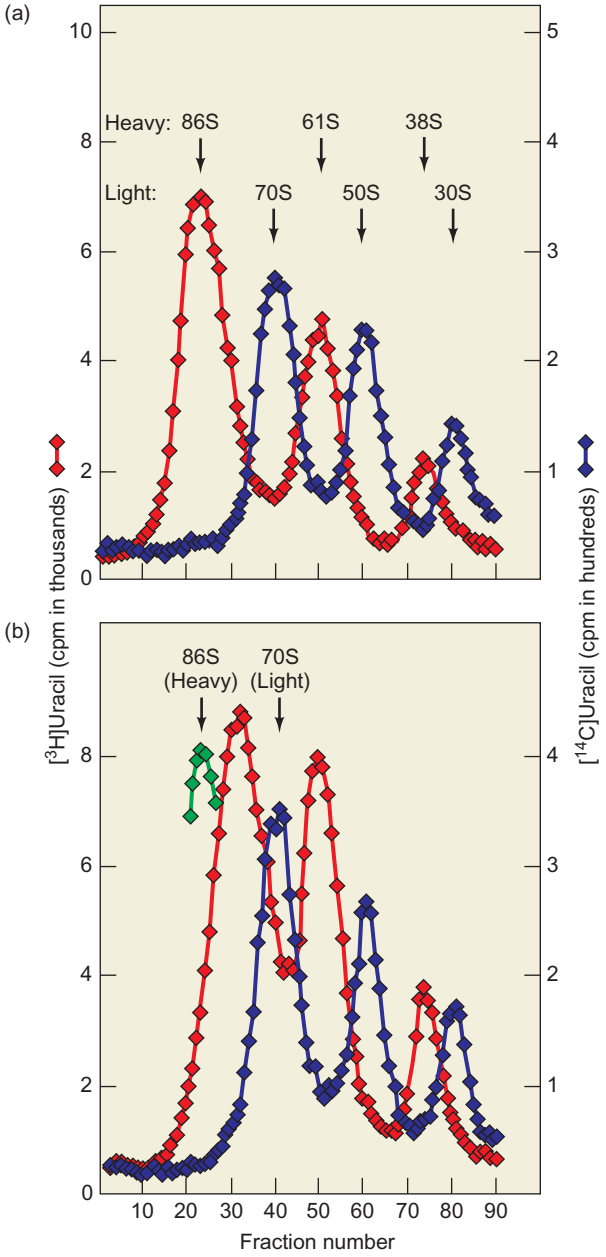


Figure 17.4 Demonstration of ribosomal subunit exchange. **(a)** Sedimentation behavior of heavy and light ribosomes. Meselson and coworkers made heavy ribosomes labeled with [^{3}H]uracil as described in Figure 17.3, and light (ordinary) ribosomes labeled with [^{14}C]uracil. Then they subjected these ribosomes to sucrose gradient centrifugation, collected fractions from the gradient, and detected the two radioisotopes by liquid scintillation counting. The positions of the light ribosomes and subunits (70S, 50S, and 30S; blue) and of the heavy ribosomes and subunits (86S, 61S, and 38S; red) are indicated at top. **(b)** Experimental results. Meselson and colleagues cultured *E. coli* cells with ^{3}H-labeled heavy ribosomes as in panel (a) and shifted these cells to light medium for 3.5 generations. Then they extracted the ribosomes, added ^{14}C-labeled light ribosomes as a reference, and subjected the mixture of ribosomes to sucrose gradient ultracentrifugation. They collected fractions and determined their radioactivity as in panel (a): ^{3}H, red; ^{14}C, blue. The position of the 86S heavy ribosomes (green) was determined from heavy ribosomes centrifuged in a parallel tube. The ^{3}H-labeled ribosomes (leftmost red peak) were hybrids that sedimented midway between the light (70S) and heavy (86S) ribosomes. (*Source:* Adapted from Kaempfer, R.O.R., M. Meselson, and H.J. Raskas, Cyclic dissociation into stable subunits and reformation of ribosomes during bacterial growth, *Journal of Molecular Biology* 31:277–89, 1968.)

SUMMARY *E. coli* ribosomes dissociate into subunits at the end of each round of translation. RRF and EF-G actively promote this dissociation, and IF3 binds to the free 30S subunit and prevents its reassociation with a 50S subunit to form a whole ribosome.

Formation of the 30S Initiation Complex

Once the ribosomal subunits have dissociated, the cell builds a complex on the 30S ribosomal subunit, including mRNA, aminoacyl-tRNA, and **initiation factors.** This is known as the **30S initiation complex.** The three initiation factors are **IF1**, **IF2**, and **IF3**. IF3 is capable of binding by itself to 30S subunits, and IF1 and IF2 stabilize this binding.

Similarly, IF2 can bind to 30S particles, but achieves much more stable binding with the help of IF1 and IF3. IF1 does not bind by itself, but does so with the assistance of the other two factors. In other words, the three initiation factors bind cooperatively to the 30S ribosomal subunit. Therefore, it is not surprising that all three factors bind close together at a site on the 30S subunit near the 3′-end of the 16S rRNA. Once the three initiation factors have bound, they attract two other key players to the complex: mRNA and the first aminoacyl-tRNA. The order of binding of these two substances appears to be random. We will return to the roles of the initiation factors later in this section. First, let us consider the initiation codon and the aminoacyl-tRNA that responds to it.

The First Codon and the First Aminoacyl-tRNA In 1964, Fritz Lipmann showed that digestion of leucyl-tRNA from *E. coli* with RNase yielded the adenosyl ester of leucine (Figure 17.5a). This is what we expect, because we know that the amino acid is bound to the 3′-hydroxyl group of the terminal adenosine of the tRNA. However, when K.A. Marcker and Frederick Sanger tried the same procedure with methionyl-tRNA from *E. coli,* they found not only the expected adenosyl-methionine ester, but also an adenosyl-*N*-formyl-methionine ester (Figure 17.5b). This demonstrated that the tRNA with which they started was esterified, not only to methionine, but also to a methionine derivative, ***N*-formyl-methionine,** which is abbreviated **fMet.** Figure 17.5c compares the structures of methionine and *N*-formyl-methionine.

Next, B.F.C. Clark and Marcker showed that *E. coli* cells contain two different tRNAs that can be charged with methionine. They separated these two tRNAs by an old purification method called countercurrent distribution. The faster moving tRNA, now called **$tRNA_m^{Met}$** could be charged with methionine, but the methionine could not be formylated. That is, it could not accept a formyl group onto its amino group. The slower moving tRNA was called **$tRNA_f^{Met}$,** to denote the fact that the methionine attached to it could be formylated. Notice that the methionine formylation takes place *on* the tRNA. The tRNA cannot be charged directly with formyl-methionine. Clark and Marcker went on to test the two tRNAs for two properties: (1) the codons they respond to, and (2) the positions within the protein into which they placed methionine.

The assay for codon specificity used a method introduced by Marshall Nirenberg, which we will describe more fully in Chapter 18. The strategy is to make a labeled aminoacyl-tRNA, mix it with ribosomes and a variety of trinucleotides, such as AUG. A trinucleotide that codes for a

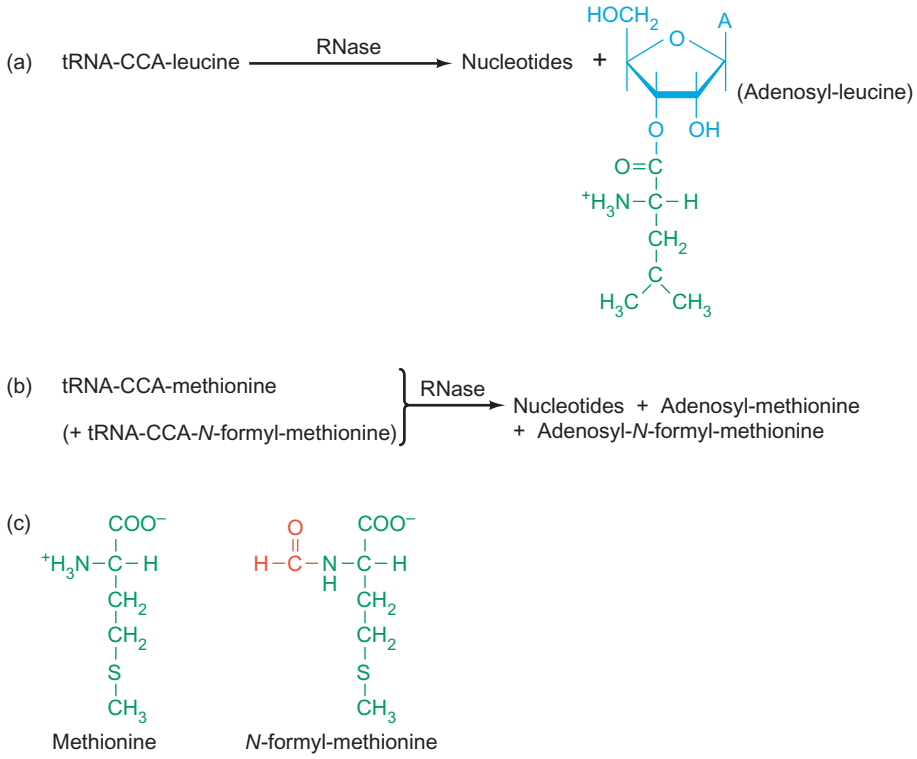


Figure 17.5 Discovery of *N*-formyl-methionine. (a) Lipmann and colleagues degraded leucyl-tRNA with RNase to yield nucleotides plus adenosyl-leucine. The leucine was attached to the terminal A of the ubiquitous CCA sequence at the 3′-end of the tRNA. **(b)** Marcker and Sanger performed the same experiment with what they assumed was pure methionyl-tRNA. However, they obtained a mixture of adenosyl-amino acids: adenosyl-methionine and adenosyl-*N*-formyl-methionine, demonstrating that the aminoacyl-tRNA with which they started was a mixture of methionyl-tRNA and *N*-formyl-methionyl-tRNA. **(c)** Structures of methionine and *N*-formyl-methionine, with the formyl group of fMet highlighted in red.

given amino acid will usually cause the appropriate aminoacyl-tRNA to bind to the ribosomes. In the case at hand, $tRNA_m^{Met}$ responded to the codon AUG, whereas $tRNA_f^{Met}$ responded to AUG, GUG, and UUG. As we have already indicated, $tRNA_f^{Met}$ is involved in initiation, which suggests that all three of these codons, AUG, GUG, and UUG, can serve as initiation codons. Indeed, sequencing of many *E. coli* genes has confirmed that AUG is the initiating codon in about 83% of the genes, whereas GUG and UUG are initiating codons in about 14% and 3% of the genes, respectively.

By the way, in addition to the three well-recognized initiation codons (AUG, GUG, and UUG), AUU can serve as an initiation codon, but only two genes in *E. coli* use it. One of these genes encodes a toxic protein, which makes sense because AUU is an inefficient start codon and it would be dangerous to translate this gene too actively. The other gene encodes IF3, which is interesting because one of the roles of IF3 is to help ribosomes bind to the standard initiation codons and avoid the inefficient nonstandard initiation codons such as AUU. In other words, IF3 works against recognition of its own start codon. This provides a neat autoregulation mechanism: When the level of IF3 is high and there is little need for more, this protein inhibits translation of the IF3 mRNA. But when the level of IF3 drops and more IF3 is needed, there is little IF3 to prevent access to the AUU initiation codon, so more IF3 is produced.

Next, Clark and Marcker determined the positions in the protein chain in which the two tRNAs placed methionines. To do this, they used an in vitro translation system with a synthetic mRNA that had AUG codons scattered throughout it. When they used $tRNA_m^{Met}$, methionines were incorporated primarily into the interior of the protein product. By contrast, when they used $tRNA_f^{Met}$, methionines (actually, formyl-methionines) went only into the first position of the polypeptide. Thus, $tRNA_f^{Met}$ appears to serve as the initiating aminoacyl-tRNA. Is this due to the formylation of the amino acid, or to some characteristic of the tRNA? To find out, Clark and Marcker tried their experiment with formylated and unformylatated methionyl-$tRNA_f^{Met}$. They found that formylation made no difference; in both cases, this tRNA directed incorporation of the first amino acid. Thus, the tRNA part of formyl-methionyl-$tRNA_f^{Met}$ is what makes it the initiating aminoacyl-tRNA.

Martin Weigert and Alan Garen reinforced the conclusion that $tRNA_f^{Met}$ is the initiating aminoacyl-tRNA with an in vivo experiment. When they infected *E. coli* with R17 phage and isolated newly synthesized phage coat protein, they found fMet in the N-terminal position, as it should be if it is the initiating amino acid. Alanine was the second amino acid in this new coat protein. On the other hand, mature phage R17 coat protein has alanine in the N-terminal position, so maturation of this protein must involve removal of the N-terminal fMet. Examination of many different bacterial and phage proteins has shown that the fMet is frequently removed. In some cases the methionine remains, but the formyl group is always removed.

SUMMARY The initiation codon in bacteria is usually AUG, but it can also be GUG, or more rarely, UUG. The initiating aminoacyl-tRNA in bacteria is *N*-formyl-methionyl-$tRNA_f^{Met}$. *N*-formyl-methionine (fMet) is therefore the first amino acid incorporated into a polypeptide, but it is frequently removed from the protein during maturation.

Binding mRNA to the 30S Ribosomal Subunit We have seen that the initiating codon is AUG, or sometimes GUG or UUG. But these codons also occur in the interior of a message. An interior AUG codes for ordinary methionine, and GUG and UUG code for valine and leucine, respectively. How does the cell detect the difference between an initiation codon and an ordinary codon with the same sequence? Two explanations come readily to mind: Either a special primary structure (RNA sequence) or a special secondary RNA structure (e.g., a base-paired stem-loop) occurs near the initiation codon that identifies it as an initiation codon and allows the ribosome to bind there. In 1969, Joan Steitz searched for such distinguishing characteristics in the mRNA from an *E. coli* phage called R17. This phage belongs to a group of small spherical RNA phages, which also includes phages f2 and MS2. These are **positive strand phages,** which means that their genomes are also their mRNAs. Thus, these phages provide a convenient source of pure mRNA. These phages are also very simple; for example, each has only three genes, which encode the A protein (or maturation protein), the coat protein, and the replicase. Steitz searched the neighborhoods of the three initiation codons in phage R17 mRNA for distinguishing primary or secondary structures. She began by binding ribosomes to R17 mRNA under conditions in which the ribosomes would remain at the initiation sites. Then she used RNase A to digest the RNA not protected by ribosomes. Finally, she sequenced the initiation regions protected by the ribosomes. She found no obvious sequence or secondary structure similarities around the start sites.

In fact, subsequent work on phage MS2 has shown that the secondary structures at all three start sites are inhibitory; relaxing these secondary structures actually enhances initiation. This is particularly true of the A protein gene, where the base-pairing around the initiation codon is so strong that the gene can be translated only in a short period just after the RNA has replicated. This brief window of opportunity occurs because the RNA has not yet had a chance to form the base pairs that hide the initiation codon. In the replicase gene, the initiation codon is buried in a

double-stranded structure that also involves part of the coat gene, as illustrated in Figure 17.6a. This base-pairing is not strong enough on its own to block translation, but a repressor protein stabilizes the base-paired stem enough that translation of the replicase gene cannot occur. This explains why the replicase gene of these phages cannot be translated until the coat gene is translated: The ribosomes moving through the coat gene open up the secondary structure hiding the initiation codon of the replicase gene (Figure 17.6b).

We have seen that secondary structure does not identify the start codons, and the first start site sequences did not reveal any obvious similarities, so what does constitute a ribosome binding site? The answer is that it *is* a special sequence, but sometimes, as in the case of the R17 coat protein gene, it diverges so far from the consensus sequence that it is hard to recognize. Richard Lodish and his colleagues laid some of the groundwork for the discovery of this sequence in their work on the translation of the f2 coat mRNA by ribosomes from different bacteria. They found that *E. coli* ribosomes could translate all three f2 genes in vitro, but that ribosomes from the bacterium *Bacillus stearothermophilus* could translate only the A protein gene. The real problem was in translating the coat gene; as we have seen, the translation of the replicase gene depends on translating the coat gene, so the inability of *B. stearothermophilus* ribosomes to translate the f2 replicase gene was simply an indirect effect of their inability to translate the coat gene. With mixing experiments, Lodish and coworkers demonstrated that the *B. stearothermophilus* ribosomes, not the initiation factors, were at fault.

Next, Nomura and his colleagues performed more detailed mixing experiments using R17 phage RNA. They found that the important element lay in the 30S ribosomal subunit. If the 30S subunit came from *E. coli,* the R17 coat gene could be translated. If it came from *B. stearothermophilus,* this gene could not be translated. Finally, they dissociated the 30S subunit into its RNA and protein components and tried them in mixing experiments. This time, two components stood out: one of the ribosomal proteins, called S12, and the 16S ribosomal RNA. If either of these components came from *E. coli,* translation of the coat gene was active. If either came from *B. stearothermophilus,* translation was depressed (though not as much as if the whole ribosomal subunit came from *B. stearothermophilus*).

These findings stimulated John Shine and Lynn Dalgarno to look for possible interactions between the 16S rRNA and sequences around the start sites of the R17 genes. They noted that all binding sites contained, just upstream of the initiation codon, all or part of this sequence: AGGAGGU, which is complementary to the underlined part of the following sequence, found at the very 3′-end of *E. coli* 16S rRNA: 3′HO-AUUCCUCCAC5′. Note that the hydroxyl group denotes the 3′-end of the 16S rRNA, and that this sequence is written 3′→5′, so its complementarity to the AGGAGGU sequence is obvious. This relationship is very

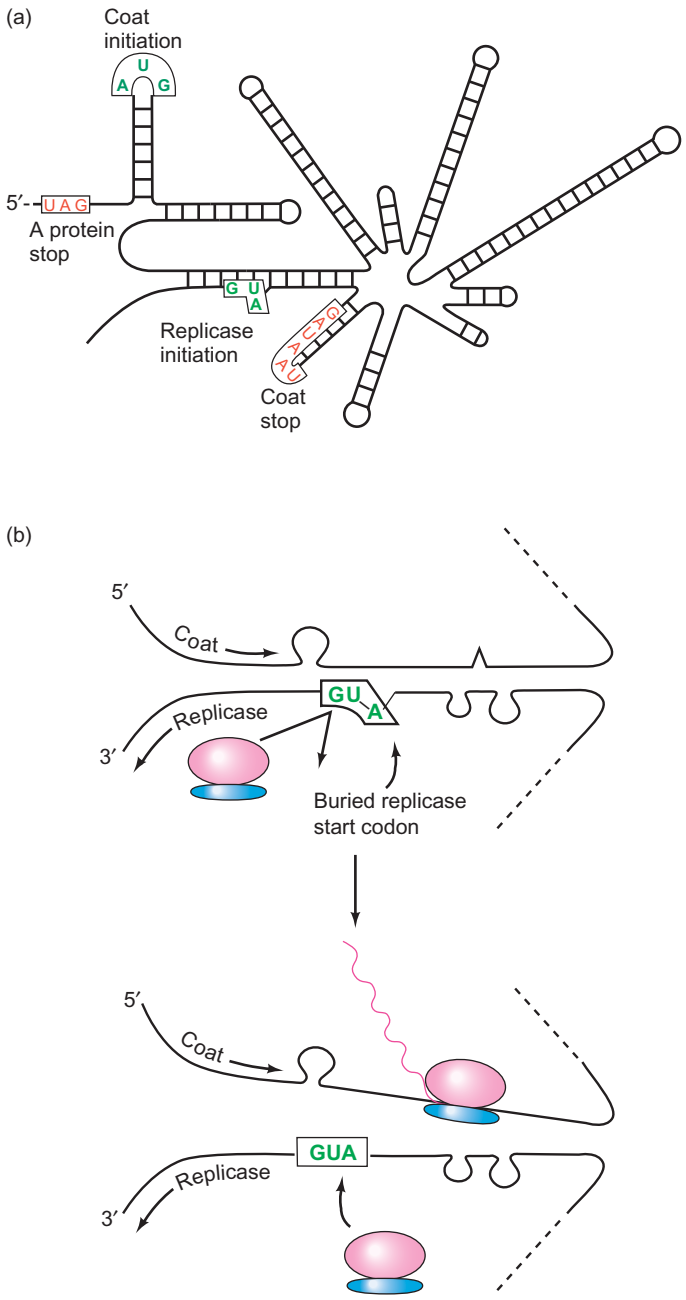


Figure 17.6 Potential secondary structure in MS2 phage RNA and its effect on translation. (a) The simplified secondary structure of the coat gene and surrounding regions in the MS2 RNA. Initiation and termination codons are boxed and labeled. **(b)** Effect of translation of coat gene on replicase translation. At top, the coat gene is not being translated, and the replicase initiation codon (AUG, green, written right to left here) is buried in a stem that is base-paired to part of the coat gene. Thus, the replicase gene cannot be translated. At bottom, a ribosome is translating the coat gene. This disrupts the base pairing around the replicase initiation codon and opens it up to ribosomes that can now translate the replicase gene. (*Source:* (a) Adapted from Min Jou, W., G. Haegeman, M. Ysebaert, and W. Fiers, Nucleotide sequence of the gene coding for the bacteriophage MS2 coat protein. *Nature* 237:84, 1972.)

suggestive, especially considering that the complementarity between the coat protein sequence and the 16S rRNA is the weakest of the three genes, and therefore would be likely to be the most sensitive to alterations in the sequence of the 16S rRNA.

The story gets even more intriguing when we compare the sequences of the *E. coli* and *B. stearothermophilus* 16S rRNAs and find an even poorer match between the R17 coat ribosome binding site and the *Bacillus* 16S rRNA. The *Bacillus* 16S rRNA can make four Watson–Crick base pairs with the A protein and replicase ribosome-binding sites, but only two such base pairs with the coat protein gene. The *E. coli* 16S rRNA can make at least three base pairs with the ribosome-binding sites of all three genes. Could the base pairing between 16S rRNA and the region upstream of the translation initiation site be vital to ribosome binding? If so, it would explain the inability of the *Bacillus* ribosomes to bind to the R17 coat protein initiation site, and it would also identify the AGGAGGU sequence as the ribosome-binding site. As we will see, other evidence shows that this really is the ribosome-binding site, and it has come to be called the **Shine–Dalgarno sequence,** or **SD sequence,** in honor of its discoverers.

To bolster their hypothesis, Shine and Dalgarno isolated ribosomes from two other bacterial species, *Pseudomonas aeruginosa* and *Caulobacter crescentus,* sequenced the 3′-ends of their 16S rRNAs, and tested the ribosomes for the ability to bind to the three R17 initiation sites. In accord with their other results, they found that whenever three or more contiguous base pairs were possible between the 16S rRNA and the sequence upstream of the initiation codon, ribosome binding occurred. Whenever fewer than 3 bp were possible, no ribosome binding occurred. It has since been shown that SD sequences as short as 3 nt must allow at least two G-C pairs with the 16S rRNA in order to support ribosome binding.

Steitz and Karen Jakes added strong evidence in favor of the Shine–Dalgarno hypothesis. They bound *E. coli* ribosomes to the R17 A protein gene's initiation region, then treated the complexes with a sequence-specific RNase called colicin E3, which cuts near the 3′-end of the 16S rRNA of *E. coli.* Next, they fingerprinted the RNA and found a double-stranded RNA fragment, as pictured in Figure 17.7. One strand of this RNA was an oligonucleotide from the A protein gene initiation site, including the Shine–Dalgarno sequence. Base-paired to it was an oligonucleotide from the 3′-end of the 16S rRNA. This demonstrated directly that the Shine–Dalgarno sequence base-paired to the 3′-end of the 16S rRNA and left little doubt that this was indeed the ribosome binding site. It is also important to remember that prokaryotic mRNAs are usually polycistronic. That is, they contain information from more than one cistron, or gene. Each cistron represented in the mRNA has its own initiation codon and its own ribosome-binding site. Thus, ribosomes bind independently to each initiation site, and this provides a means for controlling gene expression, by making some initiation sites more attractive to ribosomes than others.

Anna Hui and Herman De Boer produced excellent evidence for the importance of base pairing between the Shine–Dalgarno sequence and the 3′-end of the 16S rRNA in 1987. They cloned a mutant human growth hormone gene into an *E. coli* expression vector bearing a wild-type Shine–Dalgarno (SD) sequence (GGAGG), which is

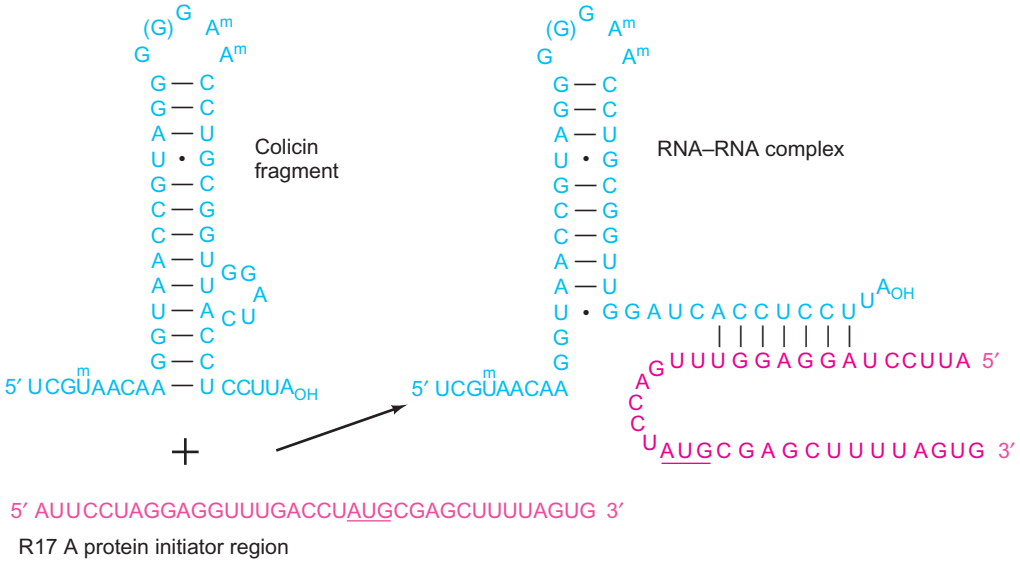


Figure 17.7 Potential structure of the colicin fragment from the 3′-end of *E. coli* 16S rRNA and the initiator region of the R17 phage A protein cistron. The initiation codon (AUG) is underlined. An "m" on the colicin fragment denotes a methylated base. G • U wobble base pairs are denoted by dots. (*Source:* Adapted from Steitz, J.A. and K. Jakes, How ribosomes select initiator regions in mRNA, *Proceedings of the National Academy of Sciences USA* 72(12):4734–38, December 1975.)

Table 17.1 Roles of Initiation Factors in Formation of the 30S Initiation Complex with Natural mRNAs

				Ribosomal binding (pmol)	
Experiment	**Ribosomes**	**mRNA**	**Factor additions**	**mRNA**	**fMet-tRNA$_f^{Met}$**
1	30S + 50S	R17	IF1 + IF2	0.4	0.4
			IF2	0.3	0.3
			IF3	2.7	0.1
			IF1 + IF3	4.8	0.2
			IF2 + IF3	2.5	1.3
			IF1 + IF2 + IF3	6.2	6.6
2	30S	MS2	IF1 + IF3		0.0
			IF2		1.8
			IF1 + IF2		3.7
			IF2 + IF3		2.7
			IF1 + IF2 + IF3		7.3
3	30S + 50S	TMV	IF1 + IF3		0.5
			IF2		1.7
			IF1 + IF2		3.1
			IF2 + IF3		8.3
			IF1 + IF2 + IF3		16.9

Source: Role of Initiation Factors in Formation of the 30S Initiation Complex with Natural mRNA from A.J. Wahba, K. Iwasaki, M.J. Miller, S. Sabol, M.A.G. Sillero, & C. Vasquez, "Initiation of Protein Synthesis in *Escherichia Coli* II," *Cold Spring Harbor Symposia in Quantitative Biology,* 34:292. Copyright © 1969, Cold Spring Harbor Laboratory Press. Reprinted with permission.

complementary to the wild-type 16S rRNA anti-SD sequence (CCUCC). This gave high levels of human growth hormone protein. Then they mutated the SD sequence to either CCUCC or GUGUG, which would not base-pair with the anti-SD sequence on the 16S rRNA. Neither of these constructs produced very much human growth hormone. But the clincher came when they mutated the anti-SD sequence in a 16S rRNA gene (on the same vector) to either GGAGG or CACAC, which restored the base pairing with CCUCC and GUGUG, respectively. Now the mRNA with the mutant CCUCC SD sequence was translated very well by the mutant cells with the 16S rRNA having the GGAGG anti-SD sequence, and the mRNA with the mutant GUGUG SD sequence was translated very well in cells with the 16S rRNA having the CACAC anti-SD sequence. This kind of intergenic suppression is strong evidence that important base-pairing occurs between these sequences.

What factors are involved in binding mRNA to the 30S ribosomal subunit? In 1969, Albert Wahba and colleagues showed that all three initiation factors are required for optimum binding, but that IF3 is the most important of the three. They mixed ^{32}P-labeled mRNAs from two *E. coli* phages, R17 and MS2, and from tobacco mosaic virus (TMV), with ribosomal subunits and initiation factors, either singly or in combinations. These viruses all have RNA genomes that serve as mRNAs, so they are convenient sources of mRNAs for experiments like this. Table 17.1, experiment 1, shows the results. IF2 or IF2 + IF1 showed little ability to cause R17 mRNA to bind to ribosomes, but IF3 by itself could cause significant binding. IF1 stimulated this binding further, and all three factors worked best of all. Thus, IF3 seems to be the primary factor involved in mRNA binding to ribosomes, but the other two factors also assist in this task. We have seen that IF3 is already bound to the 30S subunit, by virtue of its role in keeping 50S subunits from associating with the free 30S particles. The other two initiation factors also bind near the IF3 binding site on the 30S subunit, where they can participate in assembling the 30S initiation complex.

SUMMARY The 30S initiation complex is formed from a free 30S ribosomal subunit plus mRNA and fMet-tRNA$_f^{Met}$. Binding between the 30S prokaryotic ribosomal subunit and the initiation site of a message depends on base pairing between a short RNA sequence called the Shine–Dalgarno sequence just upstream of the initiation codon, and a complementary sequence at the 3′-end of the 16S rRNA. This binding is mediated by IF3, with help from IF1 and IF2. All three initiation factors have bound to the 30S subunit by this time.

Binding fMet-tRNA$_f^{Met}$ to the 30S Initiation Complex If IF3 bears the primary responsibility for binding mRNA to the 30S ribosome, which initiation factor plays this role for fMet-tRNA$_f^{Met}$? Table 17.1 shows that the answer is IF2. IF1 and IF3 together yielded little or no fMet-tRNA$_f^{Met}$ binding, whereas IF2 by itself could cause significant binding. Again, as is the case with mRNA binding, all three factors together yielded optimum fMet-tRNA$_f^{Met}$ binding.

In 1971, Sigrid and Robert Thach showed that one mole of GTP binds to the 30S ribosomal subunit along with every mole of fMet-tRNA$_f^{Met}$, but the GTP is not hydrolyzed until the 50S ribosomal subunit joins the complex and IF2 departs. We will discuss this matter further later in this chapter.

In 1973, John Fakunding and John Hershey performed in vitro experiments with labeled IF2 and fMet-tRNA$_f^{Met}$ to show the binding of both to the 30S ribosomal subunit, and the lack of necessity for GTP hydrolysis for such binding to occur. They labeled fMet-tRNA$_f^{Met}$ with ^{3}H, and IF2 by phosphorylating it with [^{32}P]ATP. This phosphorylated IF2 retained full activity. Then they mixed these components with 30S ribosomal subunits in the presence of either GTP or an unhydrolyzable analog of GTP, **GDPCP.** This analog has a methylene linkage (-CH_2-) between the β- and γ-phosphates where ordinary GTP would have an oxygen atom, which explains why it cannot be hydrolyzed to GDP and phosphate. After mixing all these components together, Fakunding and Hershey displayed the initiation complexes by sucrose gradient ultracentrifugation. Figure 17.8 shows the results. All of the labeled IF2 and a significant amount of the fMet-tRNA$_f^{Met}$ comigrated with the 30S ribosomal subunit, indicating the formation of an initiation complex. The same results were seen in the presence of either authentic GTP or GDPCP, demonstrating that GTP hydrolysis is not required for binding of either IF2 or fMet-tRNA$_f^{Met}$ to the complex. Indeed, IF2 can bind to 30S subunits in the absence of GTP, but only at unnaturally high concentrations of IF2.

This kind of experiment also allowed Fakunding and Hershey to estimate the stoichiometry of binding between the 30S subunit, IF2, and fMet-tRNA$_f^{Met}$. They added more and more IF2 to generate a saturation curve. The curve leveled off at 0.7 molecule of IF2 bound per 30S subunit. Because some of the 30S subunits were probably not competent to bind IF2, this number seems close enough to 1.0 to conclude that the real stoichiometry is 1:1. Furthermore, at saturating IF2 concentration, 0.69 molecule of fMet-tRNA$_f^{Met}$ bound to the 30S subunits. This is almost exactly the amount of IF2 that bound, so the stoichiometry of fMet-tRNA$_f^{Met}$ also appears to be 1:1. However, as we will see, IF2 is ultimately released from the initiation complex, so it can recycle and bind another fMet-tRNA$_f^{Met}$ to another complex. In this way, it really acts catalytically.

As we learned earlier in this chapter, all three factors can bind cooperatively to the 30S subunit. Indeed, the binding of all three factors seems to be the first step in formation of the 30S initiation complex. Once bound, the factors can direct the binding of mRNA and fMet-tRNA$_f^{Met}$, yielding a complete 30S initiation complex, which consists of a 30S ribosomal subunit plus one molecule each of mRNA, fMet-tRNA$_f^{Met}$, GTP, IF1, IF2, and IF3.

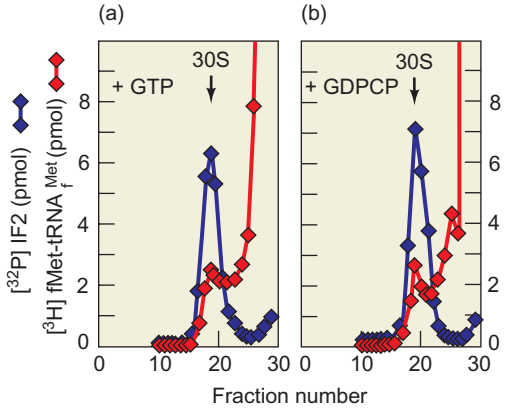


Figure 17.8 Formation of 30S initiation complex with GTP or GDPCP. Fakunding and Hershey mixed [^{32}P]IF2, [^{3}H]fMet-tRNA$_f^{Met}$ and AUG, an mRNA substitute, with 30S ribosomal subunits and either **(a)** GTP or **(b)** the unhydrolyzable GTP analog GDPCP. Then they centrifuged the mixtures in sucrose gradients and assayed each gradient fraction for radioactive IF2 (blue) and fMet-tRNA$_f^{Met}$ (red). Both substances bound to 30S ribosomes equally well with GTP and GDPCP. (*Source:* Adapted from Fakunding, J.L. and J.W.B., Hershey, The interaction of radioactive initiation factor IF2 with ribosomes during initiation of protein synthesis. *Journal of Biological Chemistry* 248:4208, 1973.)

SUMMARY IF2 is the major factor promoting binding of fMet-tRNA$_f^{Met}$ to the 30S initiation complex. The other two initiation factors play important supporting roles. GTP is also required for IF2 binding at physiological IF2 concentrations, but it is not hydrolyzed in the process. The complete 30S initiation complex contains one 30S ribosomal subunit plus one molecule each of mRNA, fMet-tRNA$_f^{Met}$, GTP, IF1, IF2, and IF3.

Formation of the 70S Initiation Complex

For elongation to occur, the 50S ribosomal subunit must join the 30S initiation complex to form the **70S initiation complex.** In this process, IF1 and IF3 dissociate from the complex. Then GTP is hydrolyzed to GDP and inorganic phosphate, as IF2 leaves the complex. We will see that GTP hydrolysis does not drive the binding of the 50S ribosomal subunit. Instead, it drives the release of IF2, which would otherwise interfere with formation of an active 70S initiation complex.

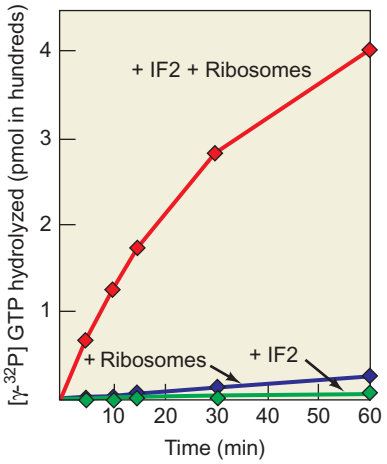


Figure 17.9 Ribosome-dependent GTPase activity of IF2. Dubnoff and Maitra measured the release of labeled inorganic phosphate from [γ-^{32}P]GTP in the presence of IF2 (green), ribosomes (blue), and IF2 plus ribosomes (red). Together, ribosomes and IF2 could hydrolyze the GTP. (*Source:* Adapted from Dubhoff, J.S., A.H. Lockwood, and U. Maitra, Studies on the role of guanosine triphosphate in polypeptide chain initiation in *Escherichia coli*. *Journal of Biological Chemistry* 247:2878, 1972.)

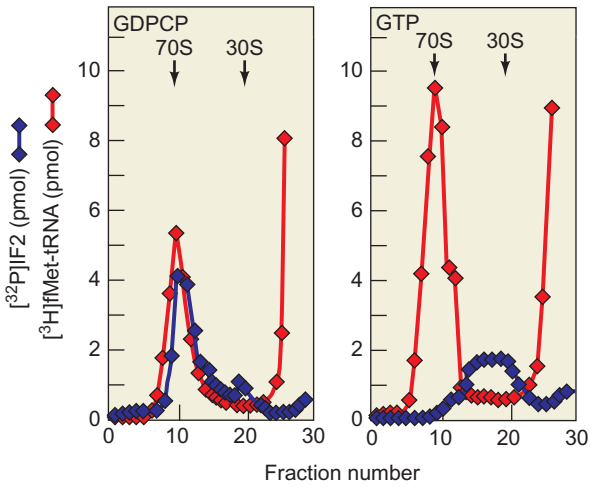


Figure 17.10 Effect of GTP hydrolysis on release of IF2 from the ribosome. Fakunding and Hershey mixed [^{32}P]IF2 (blue) and [^{3}H] fMet-tRNA$_f^{Met}$ (red) with 30S ribosomal subunits to form 30S initiation complexes. Then they added 50S ribosomal subunits in the presence of either **(a)** GDPCP, or **(b)** GTP, and then analyzed the complexes by sucrose gradient ultracentrifugation as in Figure 17.8. (*Source:* Adapted from Fakunding, J.L. and J.W.B. Hershey, The interaction of radioactive initiation factor IF2 with ribosomes during initiation of protein synthesis. *Journal of Biological Chemistry* 248:4210, 1973.)

We have already seen that GTP is part of the 30S initiation complex, and that it is removed when the 50S ribosomal subunit joins the complex. But how is it removed? Jerry Dubnoff and Umadas Maitra demonstrated in 1972 that IF2 contains a ribosome-dependent GTPase activity that hydrolyzes the GTP to GDP and inorganic phosphate (P_i). They mixed [γ-^{32}P]GTP with salt-washed ribosomes (devoid of initiation factors), or with IF2, or with both, and plotted the $^{32}P_i$ released. Figure 17.9 shows that ribosomes or IF2 separately could not hydrolyze the GTP, but together they could. Thus, IF2 and ribosomes together constitute a GTPase. Our examination of the 30S initiation complex in the previous section showed that the 30S ribosomal subunit cannot complement IF2 this way because GTP is not hydrolyzed until the 50S particle joins the complex.

What is the function of GTP hydrolysis? Fakunding and Hershey's experiments with labeled IF2 also shed light on this question: They showed that GTP hydrolysis is necessary for removal of IF2 from the ribosome. These workers formed 30S initiation complexes with labeled IF2 and fMet-tRNA$_f^{Met}$ and either GDPCP or GTP, added 50S subunits and then ultracentrifuged the mixtures to see which components remained associated with the 70S initiation complexes. Figure 17.10 shows the results. With GDPCP, both IF2 and fMet-tRNA$_f^{Met}$ remained associated with the 70S complex. By contrast, GTP allowed IF2 to dissociate, while fMet-tRNA$_f^{Met}$ remained with the 70S complex. This demonstrated that GTP hydrolysis is required for IF2 to leave the ribosome.

Another feature of Figure 17.10 is that much *more* fMet-tRNA$_f^{Met}$ bound to the 70S initiation complex in the presence of GTP than in the presence of GDPCP. This hints at the catalytic function of IF2: Hydrolysis of GTP is necessary to release IF2 from the 70S initiation complex so it can bind another molecule of fMet-tRNA$_f^{Met}$ to another 30S initiation complex. This recycling constitutes catalytic activity. However, if the factor remains stuck to the 70S complex because of failure of GTP to be hydrolyzed, it cannot recycle and therefore acts only stoichiometrically.

Is GTP hydrolysis also required to prime the ribosome for translation? Apparently not, since Maitra and colleagues removed GTP from 30S initiation complexes by gel filtration and found that these complexes were competent to accept 50S subunits and then carry out peptide bond formation. The GTP was not hydrolyzed in this procedure, and a similar procedure with GDPCP gave the same results, so GTP hydrolysis is not a prerequisite for an active 70S initiation complex, at least under these experimental conditions. This reinforces the notion that the real function of GTP hydrolysis is to remove IF2 (and GTP itself) from the 70S initiation complex so it can go about its business of linking together amino acids to make proteins.

SUMMARY GTP is hydrolyzed after the 50S subunit joins the 30S complex to form the 70S initiation complex. This GTP hydrolysis is carried out by IF2 in conjunction with the 50S ribosomal subunit. The purpose of this hydrolysis is to release IF2 and GTP from the complex so polypeptide chain elongation can begin.

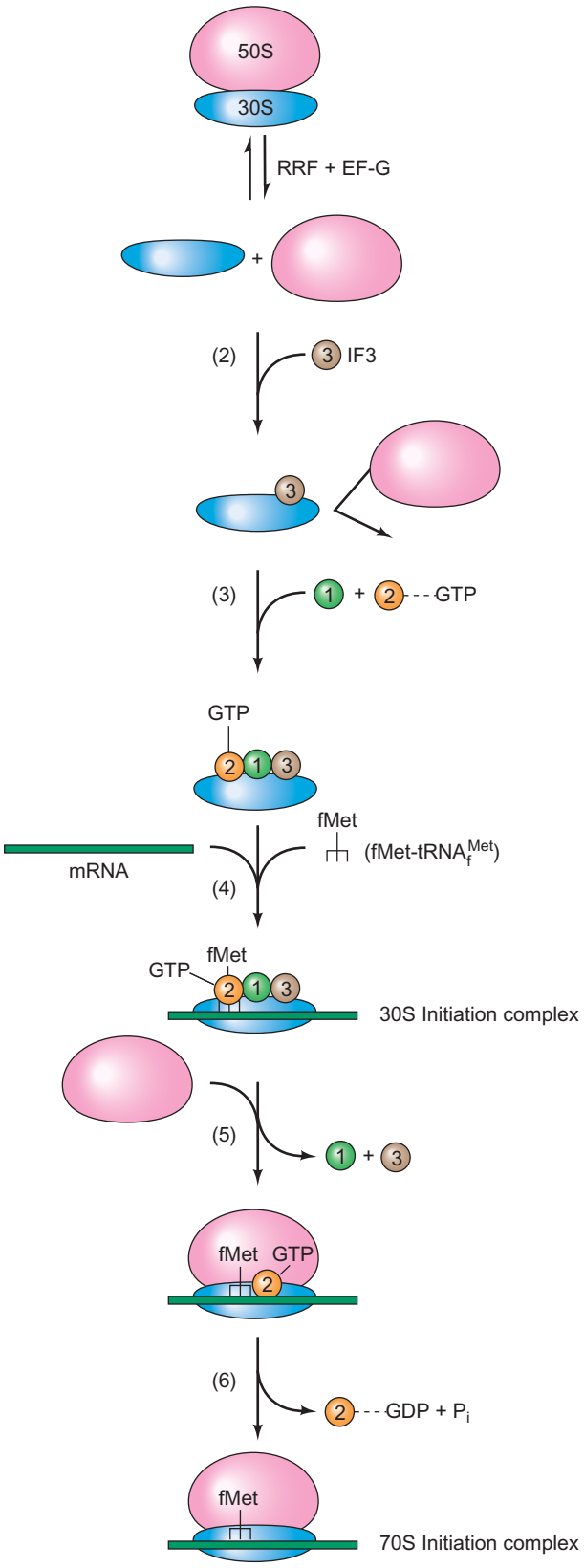


Figure 17.11 Summary of bacterial translation initiation. See the text for a description of steps 1–6. Steps 2 and 3 may be combined in vivo.

Summary of Initiation in Bacteria

Figure 17.11 summarizes what we have learned about translation initiation in bacteria. It includes the following features:

1. Dissociation of the 70S ribosome into 50S and 30S subunits, under the influence of RRF and EF-G.
2. Binding of IF3 to the 30S subunit, which prevents reassociation between the ribosomal subunits.
3. Binding of IF1 and IF2–GTP alongside IF3. This step probably occurs simultaneously with step 2.
4. Binding of mRNA and fMet-tRNA$_f^{Met}$ to form the 30S initiation complex. These two components can apparently bind in either order, but IF2 sponsors fMet-tRNA$_f^{Met}$ binding, and IF3 sponsors mRNA binding. In each case, the other initiation factors also help.
5. Binding of the 50S subunit, with loss of IF1 and IF3.
6. Dissociation of IF2 from the complex, with simultaneous hydrolysis of GTP. The product is the 70S initiation complex, ready to begin elongation.

17.2 Initiation in Eukaryotes

Several features distinguish eukaryotic translation initiation from bacterial. First, eukaryotic initiation begins with methionine, not *N*-formyl-methionine. But the initiating tRNA is different from the one that adds methionines to the interiors of polypeptides (tRNA$_m^{Met}$). The initiating tRNA bears an unformylated methionine, so it seems improper to call it tRNA$_f^{Met}$. Accordingly, it is frequently called **tRNA$_i^{Met}$**, or just **tRNA$_i$**. A second major difference distinguishing eukaryotic translation initiation from bacterial is that eukaryotic mRNAs contain no Shine–Dalgarno sequence to show the ribosomes where to start translating. Instead, most eukaryotic mRNAs have caps (Chapter 15) at their 5′-ends, which direct initiation factors to bind and begin searching for an initiation codon. This less direct recognition of the proper translation start site requires at least 12 factors, in contrast to the three that bacteria use. The eukaryotic mechanism of initiation and the initiation factors it requires will be our topics in this section.

The Scanning Model of Initiation

Most bacterial mRNAs are polycistronic. They contain information from multiple genes, or cistrons, and each cistron has its own initiation codon and ribosome-binding site. But polycistronic mRNAs that are translated intact are rare in eukaryotes, except for the transcripts of certain viruses. Thus, eukaryotic cells are usually faced with the task of finding a start codon near the 5′-end of a transcript. They accomplish this task by recognizing the cap at the 5′-end,

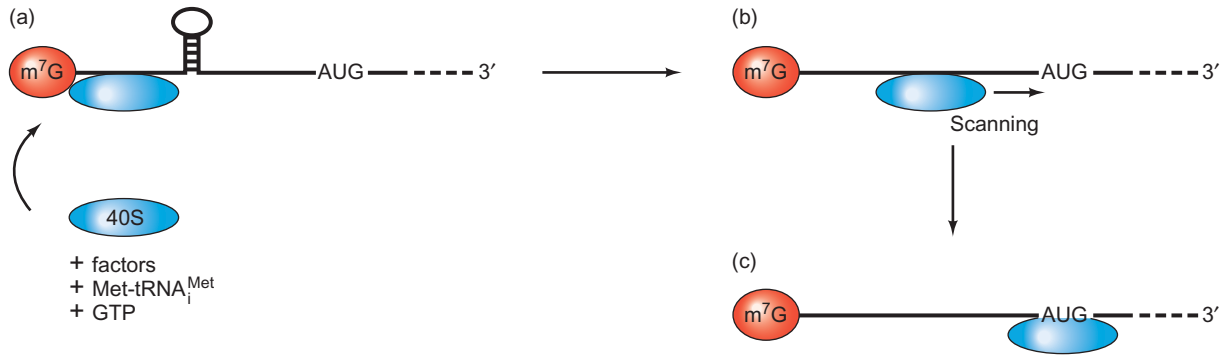


Figure 17.12 A simplified version of the scanning model for translation initiation. (a) The 40S ribosomal subunit, along with initiation factors, Met-tRNA$_i^{Met}$, and GTP, recognize the m^{7}G cap (red) at the 5′-end of an mRNA and allow the ribosomal subunit to bind at the end of the mRNA. All the other components (factors, etc.) are omitted for simplicity. **(b)** The 40S subunit is scanning the mRNA toward the 3′-end, searching for an initiation codon. It has melted a stem-loop structure in its way. **(c)** The ribosomal subunit has located an AUG initiation codon and has stopped scanning. Now the 60S ribosomal subunit can join the complex and initiation can occur.

then **scanning** the mRNA in the 5′→3′ direction until they encounter a start codon, as illustrated in Figure 17.12.

Marilyn Kozak first developed this scanning model in 1978, based on four considerations: (1) In no known instance was eukaryotic translation initiated at an internal AUG, as in a polycistronic mRNA. (2) Initiation did not occur at a fixed distance from the 5′-end of an mRNA. (3) In all of the first 22 eukaryotic mRNAs examined, the first AUG downstream of the cap was used for initiation. (4) As we saw in Chapter 15, the cap at the 5′-end of the mRNA facilitates initiation. We will see more definitive evidence for the scanning model later in this chapter.

The simplest version of the scanning model has the ribosome recognizing the first AUG it encounters and initiating translation there. However, a survey of 699 eukaryotic mRNAs revealed that the first AUG is not the primary initiation site in 5–10% of the cases. Instead, in those cases, most ribosomes skip over one or more AUGs before encountering the right one and initiating translation, a process Kozak called "leaky scanning." This raises the question: What sets the right AUG apart from the wrong ones? To find out, Kozak examined the sequences surrounding initiating AUGs and found that the consensus sequence in mammals was CCRCCAUGG, where R is a purine (A or G), and the initiation codon is underlined.

If this is really the optimum sequence, then mutations should reduce its efficiency. To check this hypothesis, Kozak systematically mutated nucleotides around the initiation codon in a cloned rat preproinsulin gene. She substituted a synthetic ATG-containing oligonucleotide for the normal initiating ATG, then introduced mutations into this initiation region, placed the mutated genes under control of the SV40 virus promoter, introduced them into monkey (COS) cells, then labeled newly synthesized proteins with [^{35}S]methionine, immunoprecipitated the proinsulin, electrophoresed it, and detected it by fluorography, a technique akin to autoradiography (Chapter 5). Finally, she scanned the fluorograph with a densitometer to quantify the production of proinsulin. The better the translation initiation, the more proinsulin was made. Throughout this discussion we will refer to the initiation codon as AUG, even though the mutations were done at the DNA level.

Figure 17.13 shows some of the results, which include alterations in positions −3 and +4, where the A in AUG is position +1. The best initiation occurred with a G or an A in position −3 and a G in position +4. Similar experiments showed that the best initiation of all occurred with the sequence ACCAUGG, and the −3 and +4 positions are the most important. These requirements are sometimes called **Kozak's rules.**

If this really is the optimum sequence for translation initiation, introducing it out of frame and upstream of the normal initiation codon should provide a barrier to scanning ribosomes and force them to initiate out of frame. The more this occurs, the less proinsulin should be produced. Kozak performed this experiment with the A's of the two AUGs 8 nt apart as follows: AUGNCACCAUGG. Note that the downstream AUG is in an optimal neighborhood, so initiation should start there readily if the ribosome can reach it without initiating upstream first. Figure 17.14 shows the results. Mutant F10 had no upstream AUG, and initiation from the normal AUG was predictably strong. Mutant F9 had the upstream AUG in a very weak context, with U's in both −3 and +4 positions. Again, this did not interfere much with initiation at the downstream AUG. But all the other mutants exhibited strong interference with normal initiation, and the strength of this interference was related to the context of the upstream AUG. The closer it resembled the optimal sequence, the more it interfered with initiation at the downstream AUG. This is just what the scanning model predicts.

What about natural mRNAs that have an upstream AUG in a favorable context, yet still manage to initiate from a downstream AUG? Kozak noted that these mRNAs

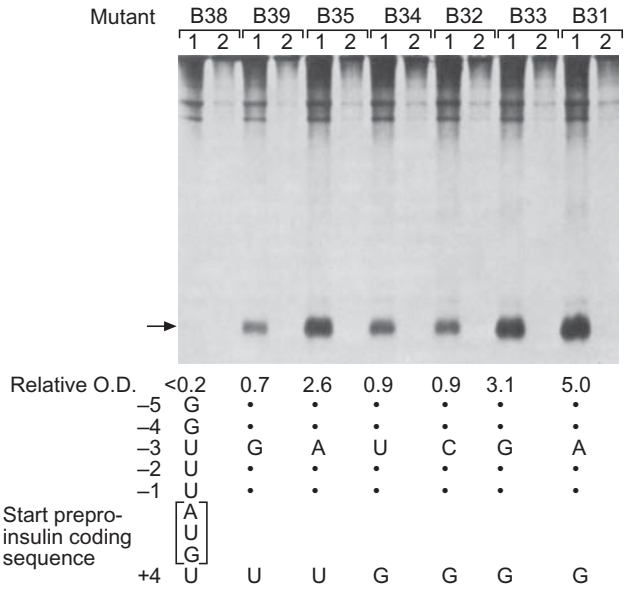


Figure 17.13 Effects of single base changes in positions −3 and +4 surrounding the initiating AUG. Starting with a cloned rat preproinsulin gene under the control of an SV40 viral promoter, Kozak replaced the natural initiation codon with a synthetic oligonucleotide containing an ATG, which was transcribed to AUG in the mRNA. She then mutagenized the nucleotides at positions −3 and +4 as shown at bottom, introduced the manipulated genes into COS cells growing in medium containing [^{35}S]methionine to label any proinsulin produced. She purified the proinsulin by immunoprecipitation, then electrophoresed it and detected the labeled protein by fluorography. This is a technique similar to autoradiography in which the electrophoresis gel is impregnated with a fluorescent compound to amplify the relatively weak radioactive emissions from an isotope such as ^{35}S. The arrow at left indicates the position of the proinsulin product. Kozak subjected the proinsulin bands in the fluorograph to densitometry to quantify their intensities. These are listed as relative O. D., or optical density, beneath each band. Optimal initiation occurred with a purine in position −3 and a G in position +4. Proinsulin is the product of the preproinsulin gene because the "signal peptide" at the amino terminus of preproinsulin is removed during translation, yielding proinsulin. The signal peptide directs the growing polypeptide, along with the ribosome and mRNA, to the endoplasmic reticulum (ER). This ensures that the polypeptide enters the ER and can therefore be secreted from the cell. All sequences are shown as they appear in mRNA. (*Source*: Kozak, M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44 (31 Jan 1986) p. 286, f. 2. Reprinted by permission of Elsevier Science.)

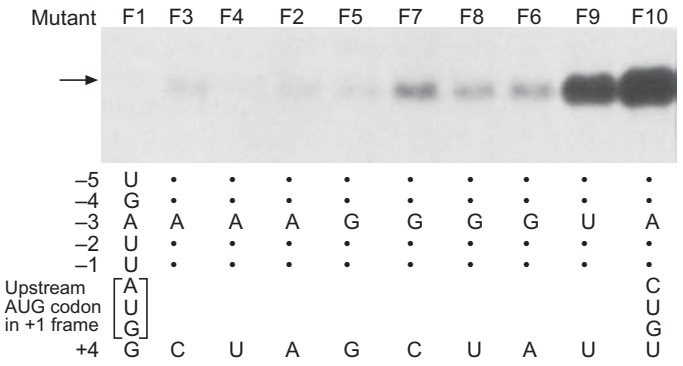


Figure 17.14 Influence of the context of an upstream "barrier" AUG. Kozak made a construct having the normal AUG initiation codon of the rat preproinsulin transcript preceded by an out-of-frame AUG, then made mutations in the −3 and +4 positions surrounding the upstream AUG (shown at bottom) and assayed the effect on proinsulin synthesis as in Figure 17.13. The arrow at left indicates the position of correctly initiated proinsulin. The more favorable the context of the upstream AUG, the better it serves as a barrier to correct downstream initiation. All sequences are presented as they appear in mRNA. (*Source:* Kozak, M., Point mutations define a sequence flanking the AUG initiation codon that modulates translation by eukaryotic ribosomes. *Cell* 44 (31 Jan 1986) p. 288, f. 6. Reprinted by permission of Elsevier Science.)

have in-frame stop codons between the two AUGs, and she argued that initiation at the downstream AUG actually represents reinitiation by ribosomes that have initiated at the upstream start codon, terminated at the stop codon, then continued scanning for another start codon. To illustrate the effect of a stop codon between the two AUGs, Kozak made another set of constructs with such a stop codon and tested them by the same assay. Abundant initiation occurred at the downstream AUG in this case, as long as the downstream AUG was in a good environment.

Note that an initiation codon and a downstream termination codon in the same reading frame define the boundaries of an **open reading frame (ORF).** Such an ORF potentially encodes a protein; whether it is actually translated in vivo is another matter. Further experiments have revealed another requirement for efficient reinitiation at a downstream ORF: The upstream ORF must be short. In every case in which a dicistronic mRNA with a full-sized upstream ORF has been examined, reinitiation at the downstream ORF has been extremely inefficient. Perhaps by the time a ribosome finishes translating a long ORF, the initiation factors needed for reinitiation have diffused away, so it ignores the second ORF.

To check rigorously the hypothesis that an upstream AUG is favored over downstream AUGs, Kozak created mRNAs with exact repeats of the initiation region of the rat preproinsulin cistron. She then tested these for the actual translation initiation site by isolating the resulting proteins and electrophoresing them to determine their sizes, which tell us which initiation site the ribosomes used in making them. In each case, the farthest upstream AUG was used, which is again consistent with the scanning model.

What is the effect of mRNA secondary structure on efficiency of initiation? Hairpins in the mRNA can affect initiation both positively and negatively. Kozak showed that a stem loop 12–15 nt downstream of an AUG in a weak context could act positively by preventing 40S ribosomal subunits from skipping that initiation site. The hairpin presumably stalled the ribosomal subunit at the AUG long enough for initiation to occur. Secondary structure can also have a negative effect. Kozak tested the effects of two different stem-loop structures in the leader of an

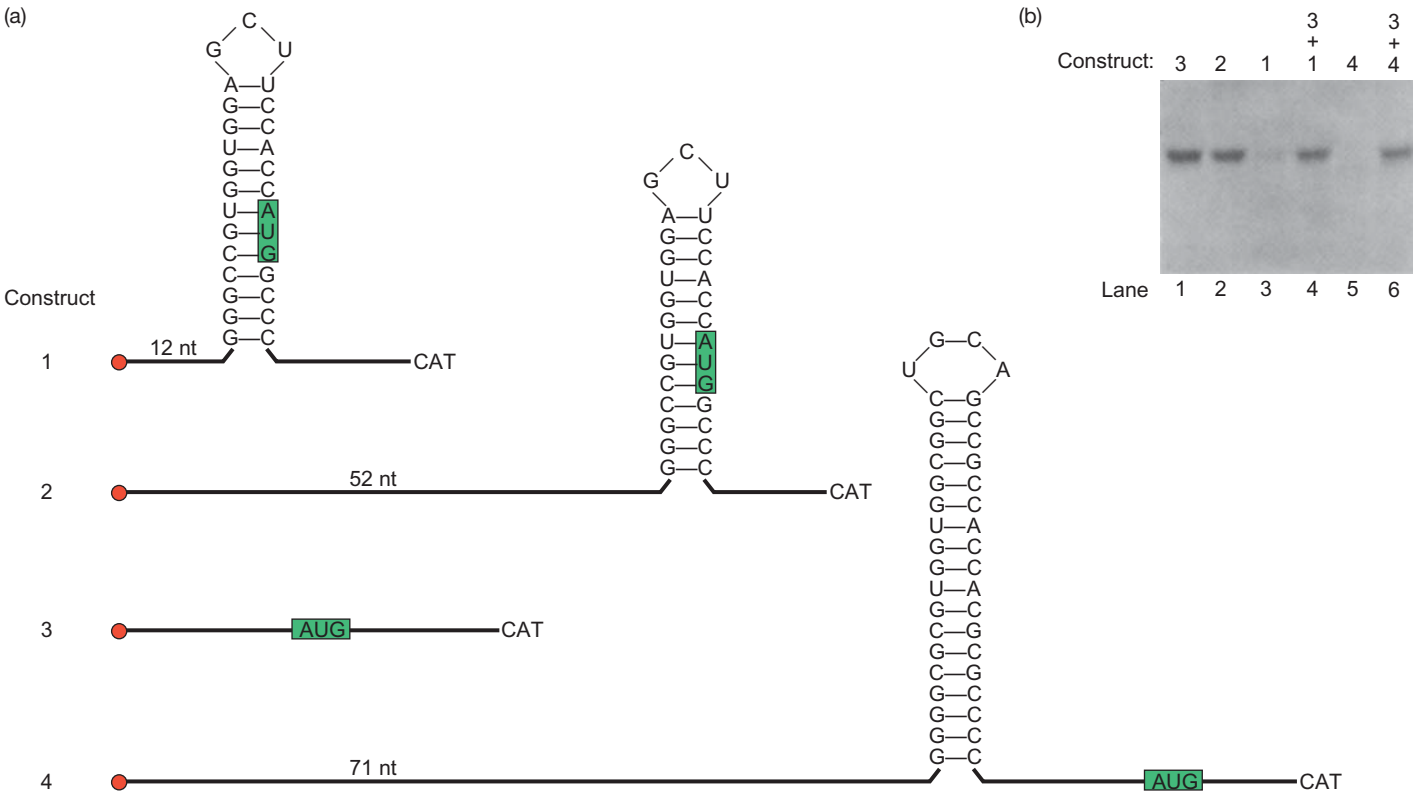


Figure 17.15 Effect of secondary structure in an mRNA leader on translation efficiency. (a) mRNA constructs. Kozak made the synthetic leader constructs pictured here, with the cap in red and the initiation codon highlighted in green, with the CAT ORF attached to the 3′-end of each. **(b)** Results of in vitro translation. Kozak translated each mRNA in vitro in a rabbit reticulocyte extract with [^{35}S]methionine. She electrophoresed the labeled proteins and detected them by fluorography. The short hairpin near the cap (construct 1) interfered, as did the long hairpin between the cap and the initiation codon (construct 4). (*Source:* Kozak, M., Circumstances and mechanisms of inhibition of translation by secondary structure in eukaryotic mRNAs. *Molecular and Cellular Biology* 9 (1989) p. 5136, f. 3. American Society for Microbiology.)

mRNA (Figure 17.15a). One was relatively short and had a free energy of formation (or stability) of −30 kcal/mol; the other was much longer, with a higher stability of −62 kcal/mol. She introduced these stem loops into various positions in the leader of the chloramphenicol acetyl transferase (CAT) gene, then transcribed the altered genes and translated their transcripts in vitro in the presence of [^{35}S] methionine. Finally, she electrophoresed the CAT proteins and detected them by fluorography. The results in Figure 17.15b show that a −30-kcal stem loop 52 nt downstream of the cap does not interfere with translation, even if it includes the initiating AUG. However, a −30-kcal stem loop only 12 nt downstream of the cap strongly inhibits translation, presumably because it interferes with binding of the 40S ribosomal subunit and factors at the cap. Furthermore, a −62-kcal stem loop placed 71 nt downstream of the cap completely blocked appearance of the CAT protein.

Why was the construct with the stable hairpin not translated? The simplest explanation is that the very stable stem loop blocked the scanning 40S ribosomal subunit and would not let it through to the initiation codon. This effect was observed only *in cis* (on the same molecule). When construct 3 and 4 (or 3 and 1) were tested together, translation occurred on the linear mRNA made from construct 3 (lanes 4 and 6). This indicates that the untranslatable constructs were not poisoning the translation system somehow.

The fact that construct 2 is translated well, even though its initiation codon lies buried in a hairpin, suggests that the scanning ribosomal subunit and initiation factors can unwind a certain amount of double-stranded RNA, as predicted by Kozak in her original scanning model (see Figure 17.12). However, as we have just seen, this unwinding ability has limits; the long hairpin in construct 4 effectively blocks the ribosomal subunits from reaching the initiation codon.

How do 40S ribosomal subunits recognize an AUG start codon? Thomas Donahue and colleagues have shown that the initiator tRNA ($tRNA_i^{Met}$) plays a critical role. They changed the anticodon of one of the four yeast $tRNA_i^{Met}$s to 3′-UCC-5′ so it would recognize the codon AGG instead of AUG. Then they placed *his4* genes with various mutant initiation codons into a *his4*$^-$ yeast strain. Figure 17.16a shows that the *his4* gene bearing an AGG

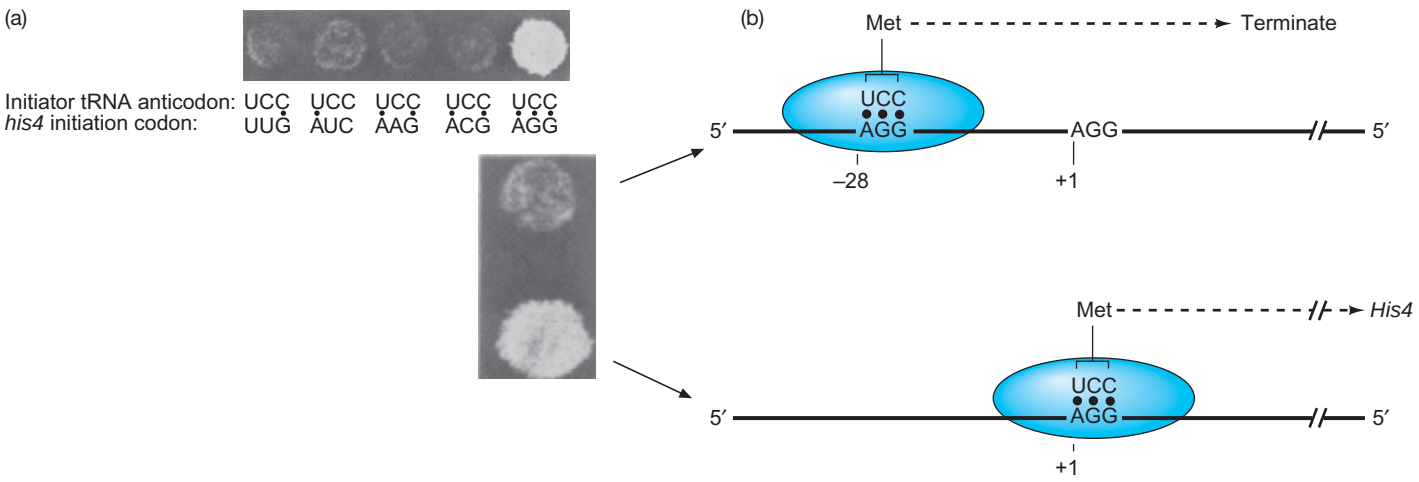


Figure 17.16 Role of initiator tRNA in scanning. (a) An initiator tRNA with an altered anticodon can recognize a complementary initiation codon. Donahue and colleagues mutated the anticodon of one of the initiator tRNAs in the yeast *Saccharomyces cerevisiae* to 3′-UCC-5′- and introduced the gene encoding this altered tRNA into *his4*$^{-}$ cells, using a high-copy yeast vector. Then they changed the initiation codon of the *his4* gene to any of the five versions listed at the bottom and tested the mutant yeast cells for growth in the absence of histidine. When the initiation codon was AGG, it could base-pair with the UCC anticodon on the initiator tRNA, so the mutant mRNA could be translated and growth occurred. **(b)** Effect of an extra AGG upstream and out of frame. Donahue and colleagues made a *his4* construct with an extra AGG in good context beginning at position −28 (top), placed it in cells bearing the initiator tRNA with the UCC anticodon, and tested these cells for ability to grow in the absence of histidine. Growth was much reduced compared with cells with no upstream AGG (bottom). The scanning 40S ribosomal subunit, together with the mutant $tRNA_i^{Met}$, apparently encountered the first AGG and initiated there, producing a shortened *his4* product. (*Source:* (a) Cigan, A.M., L. Feng, and T.F. Donohne, $tRNA_i^{Met}$ functions in directing the scanning ribosomes to the start site of translation. *Science* 242 (7 Oct 1988) p. 94, f. 1B & C (left). Copyright © AAAS.)

codon in place of the initiation codon could support yeast growth. None of the other substitute initiation codons worked, presumably because they could not pair with the UCC anticodon in the altered initiator tRNA. In another experiment, these workers placed a second AGG 28 nt upstream of the AGG in the initiation site and out of frame with it. This construct could not support growth. This result supports the scanning model, as illustrated in Figure 17.16b. The initiator tRNA, with a UCC anticodon in this case, binds to the 40S ribosomal subunit and the complex scans the mRNA searching for the first initiation codon (AGG in this case). Since the first AGG is out-of-frame with the *his4* coding region, translation will occur in the wrong reading frame and will soon encounter a stop codon and terminate prematurely.

The scanning model has some apparent exceptions. The best documented of these concern the polycistronic mRNAs of the picornaviruses such as poliovirus, which lack caps. In these cases, ribosomes can apparently enter at internal initiation codons using **internal ribosome entry sequences** (**IRESs**) that can attract ribosomes directly without help from the cap. We will discuss this phenomenon in more detail later in this chapter.

SUMMARY Eukaryotic 40S ribosomal subunits, together with the initiator Met-tRNA (Met-$tRNA_i^{Met}$), generally locate the appropriate start codon by binding to the 5′-cap of an mRNA and scanning downstream until they find the first AUG in a favorable context. The best context is a purine in the –3 position and a G in the +4 position where the A of the AUG is +1. In 5–10% of genes, most ribosomal subunits will bypass the first AUG and continue to scan for a more favorable one. Sometimes ribosomes apparently initiate at an upstream AUG, translate a short ORF, then continue scanning and reinitiate at a downstream AUG. This mechanism works only with short upstream ORFs. Secondary structure near the 5′-end of an mRNA can have positive or negative effects. A hairpin just past an AUG can force a ribosomal subunit to pause at the AUG and thus stimulate initiation. A very stable stem loop between the cap and an initiation site can block ribosomal subunit scanning and thus inhibit initiation. Some viral mRNAs that lack caps contain IRESs that attract ribosomes directly to the mRNAs.

Eukaryotic Initiation Factors

We have seen that bacterial translation initiation requires initiation factors and so does initiation in eukaryotes. As you might expect, though, the eukaryotic system is more complex than the bacterial. One level of extra complexity

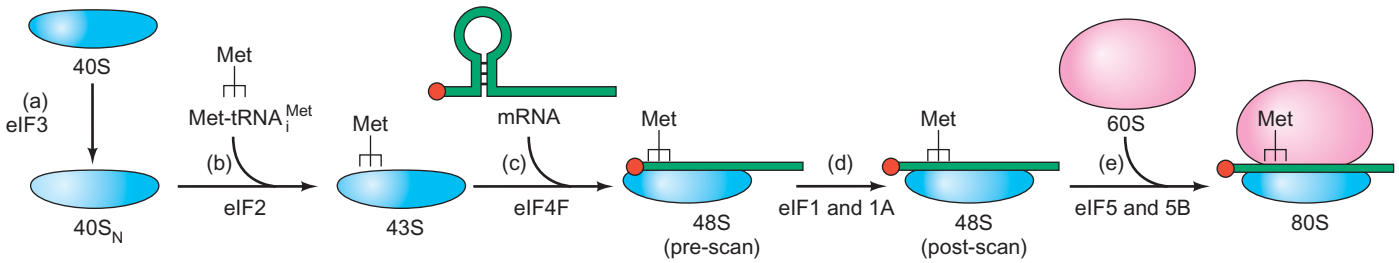


Figure 17.17 Summary of translation initiation in eukaryotes. **(a)** The eIF3 factor converts the 40S ribosomal subunit to 40S$_N$, which resists association with the 60S ribosomal particle and is ready to accept the initiator aminoacyl-tRNA. **(b)** With the help of eIF2, Met-tRNA$_i^{Met}$ binds to the 40S$_N$ particle, forming the 43S complex. **(c)** Aided by eIF4F the mRNA binds to the 43S complex, forming the 48S complex. **(d)** The eIF1 and 1A factors promote scanning to the initiation codon. **(e)** The eIF5 factor promotes hydrolysis of eIF2-bound GTP, which is a precondition for ribosomal subunit joining. eIF5B has a ribosome-dependent GTPase activity that helps the 60S ribosomal particle bind to the 48S complex, yielding the 80S complex that is ready to begin translating the mRNA.

we have already seen is the scanning process. Factors are needed to recognize the cap at the 5′-end of an mRNA and bind the 40S ribosomal subunit nearby. In this section we will examine the factors involved at the various stages of initiation in eukaryotes. We will also see that some of these steps are natural sites for regulation of the translation process.

Overview of Translation Initiation in Eukaryotes Figure 17.17 provides an outline of the initiation process in eukaryotes, showing the major classes of initiation factors involved. Notice that the eukaryotic initiation factor names all begin with e, which stands for "eukaryotic." An example is **eIF2,** which, like bacterial IF2 is responsible for binding the initiating aminoacyl-tRNA (Met-tRNA$_i^{Met}$) to the ribosome.

Another way in which eIF2 resembles IF2 is that it requires GTP to do its job, and this GTP is hydrolyzed to GDP when the factor dissociates from the ribosome. Then GTP must replace GDP on the factor for it to function again. This requires an exchange factor, **eIF2B,** which exchanges GTP for GDP on eIF2. This factor is also called **GEF,** for guanine nucleotide exchange factor. Notice that all of the factors acting at a given step are given the same number. For example, we have seen that at least two factors (eIF2 and eIF2B) are required for initiator aminoacyl-tRNA binding, and both of these share the number 2. Despite all the functional similarities between IF2 and eIF2, the two proteins are not homologous. Instead, IF2 is homologous to eIF5B, which we will discuss later in this chapter.

Another eukaryotic factor whose function bears at least some resemblance to that of a bacterial factor is **eIF3,** which binds to the 40S (small) ribosomal subunit and discourages its reassociation with the 60S (large) subunit. In this way, it resembles IF3. **eIF4F** is a complex cap-binding protein that allows the 40S ribosomal particle to bind to the 5′-end of an mRNA. This binding is mediated by eIF3, which binds to both eIF4F and the 40S ribosomal particle. Once the 40S particle has bound at the cap, it requires **eIF1** (and **eIF1A**) to scan to the initiation codon. **eIF5** is a factor with no known bacterial counterpart. It stimulates association between the 60S ribosomal subunit and the 40S initiation complex, which is actually called the 48S complex because it includes mRNA and many factors in addition to the 40S ribosomal subunit, and these raise the sedimentation coefficient. **eIF6** is another antiassociation factor, like eIF3. It binds to the 60S ribosomal subunit and discourages premature association with the 40S subunit.

SUMMARY The eukaryotic initiation factors have the following general functions: eIF2 is involved in binding Met-tRNA$_i^{Met}$ to the ribosome. eIF2B activates eIF2 by replacing its GDP with GTP. eIF1 and eIF1A aid in scanning to the initiation codon. eIF3 binds to the 40S ribosomal subunit and inhibits its reassociation with the 60S subunit. eIF4F is a cap-binding protein that allows the 40S ribosomal subunit to bind (through eIF3) to the 5′-end of an mRNA. eIF5 encourages association between the 60S ribosomal subunit and the 48S complex (40S subunit plus mRNA and Met-tRNA$_i^{Met}$). eIF6 binds to the 60S subunit and blocks its reassociation with the 40S subunit.

Function of eIF4F Now we come to a major novelty of eukaryotic translation initiation: the role of the cap. We have seen in Chapter 15 that the cap greatly stimulates the efficiency of translation of an mRNA. That implies that some factor can recognize the cap at the 5′-end of an mRNA and aid in the translation of that mRNA. Nahum Sonenberg, William Merrick, Aaron Shatkin, and colleagues identified a cap-binding protein in 1978 by cross-linking it to a modified cap as follows: First they oxidized the ribose of the capping nucleotide on a ^{3}H-reovirus mRNA to convert its 2′- and 3′-hydroxyl groups to a reactive dialdehyde. Then they incubated this altered mRNA with initiation factors. Free amino groups of any factor that binds to the modified cap should bind covalently to

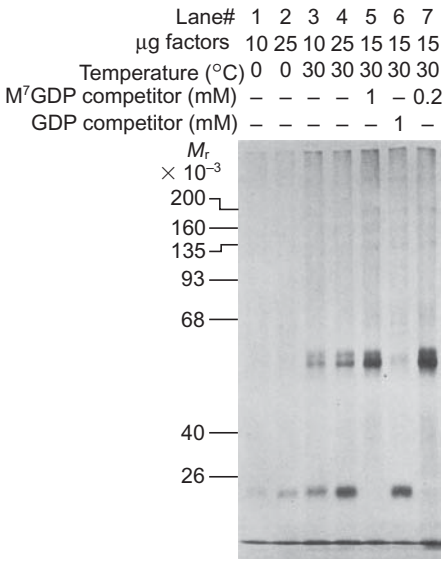


Figure 17.18 Identifying a cap-binding protein by chemical cross-linking. Sonenberg and colleagues placed a reactive dialdehyde in the ribose of the capping nucleotide of a ^{3}H-reovirus mRNA. Then they mixed initiation factors with this mRNA to cross-link any cap-binding protein via a Schiff base between an aldehyde on the cap and a free amino group on the protein. They made this covalent bond permanent by reduction with $NaBH_3CN$. Then they digested these complexes with RNase to remove everything but the cap, and electrophoresed the labeled cap–protein complexes to detect the sizes of any polypeptides that bound to the cap. The conditions in each lane were as listed at top. Note that m^7GDP competed with the 24-kD band for binding, but that the 50–55-kD bands did not. (*Source:* Sonenberg, N., M.A., Morgan, W.C. Merrick, and A.J. Shatkin, A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 59-terminal cap in mRNA. *Proceedings of the National Academy of Science USA* 75 (1978) p. 4844, f. 1.)

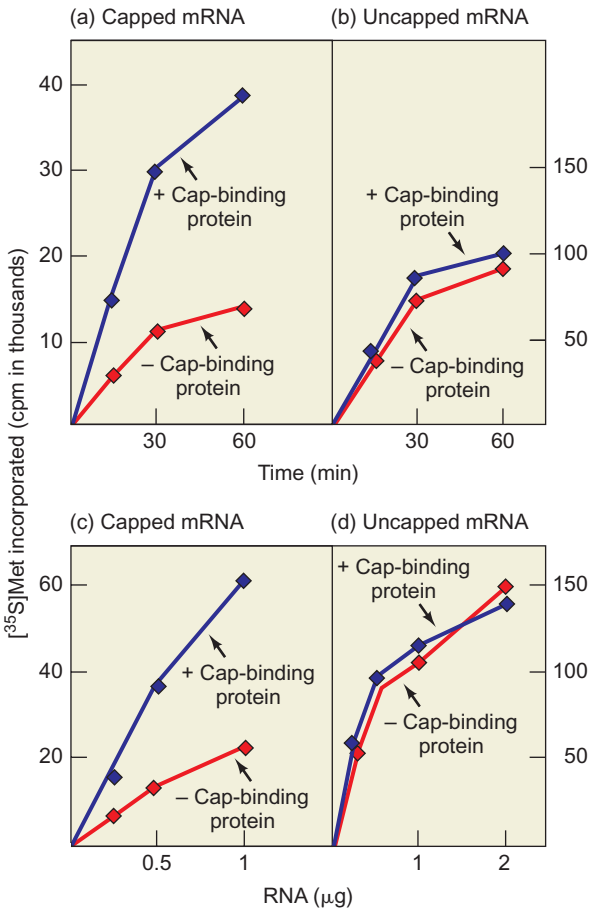


Figure 17.19 Cap-binding protein stimulates translation of capped, but not uncapped, mRNA. Shatkin and collaborators used HeLa cell-free extracts to translate capped and uncapped mRNAs in the presence of [^{35}S]methionine. Panels **(a)** and **(c):** translation of capped Sindbis virus mRNA with (blue) or without (red) cap-binding protein. Panels **(b)** and **(d):** translation of uncapped encephalomyocarditis virus (EMC) with (blue) or without (red) cap-binding protein. (*Source:* Adapted from Sonenberg, N., H. Trachsel, S. Hecht, and A.J. Shatkin, Differential stimulation of capped mRNA translation in vitro by cap-binding protein. *Nature* 285:331, 1980.)

one of the reactive aldehydes. This bond can be made permanent by reduction. After cross-linking, the investigators digested all of the RNA but the cap with RNase, then electrophoresed the products to measure the sizes of any proteins cross-linked to the labeled cap. Figure 17.18 shows that a polypeptide with a M_r of about 24 kD bound, even at low temperature. At higher temperature, another pair of polypeptides of higher molecular mass (50–55 kD) bound. However, unlabeled m^7GDP did not compete with these high M_r polypeptides for binding to the mRNA, whereas the unlabeled cap analog did compete with the 24-kD polypeptide for binding. This suggested that the 24-kD polypeptide bound specifically to the cap, but the 50–55-kD-polypeptides did not. On the other hand, GDP competed with the 50–55-kD polypeptides for binding to the mRNA, but it did not compete with the 24-kD polypeptide. This may mean that the larger polypeptides are GDP-binding proteins, rather than cap-binding proteins.

Sonnenberg, Shatkin, and colleagues followed up their discovery of the cap-binding protein by purifying it by affinity chromatography on an m^7GDP-Sepharose column. Then they added this protein to HeLa cell-free extracts and demonstrated that it stimulated transcription of capped, but not uncapped, mRNAs (Figure 17.19). They used viral mRNAs in both experiments: Sindbis virus mRNA for capped mRNA, and encephalomyocarditis virus mRNA for uncapped mRNA. (Encephalomyocarditis virus is a picornavirus similar to poliovirus.)

As we have seen, picornavirus mRNAs are not capped. Nevertheless, these viruses have mechanisms for ensuring that their mRNAs are translated. In fact, they take advantage of the cap-free nature of their mRNAs to eliminate competition from capped host mRNAs. They do this by inactivating the host cap-binding protein, thus blocking translation of capped host mRNAs, at least in certain cells. Molecular biologists have taken advantage of this situation by using poliovirus-infected cell extracts as an assay system for the cap-binding protein. Any protein that can restore

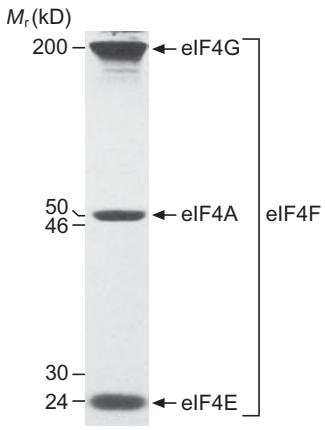


Figure 17.20 Components of eIF4F (complete cap-binding protein). Sonenberg and colleagues purified the cap-binding protein using a series of steps, including m^7GTP affinity chromatography. Then they displayed the subunits of the purified protein by SDS-PAGE. The relative molecular masses (in kilodaltons) of the subunits and markers (200, 46, and 30 kD) are given at left. The whole complex, composed of three polypeptides, is called eIF4F. (*Source:* Edery, I., M. Hümbelin, A. Darveau, K.A.W. Lee, S. Milburn, J.W.B. Hershey, H. Trachsel, and N. Sonenberg, Involvement of eukaryotic initiation factor 4A in the cap recognition process. *Journal of Biological Chemistry* 258 (25 Sept 1983) p. 11400, f. 2. American Society for Biochemistry and Molecular Biology.)

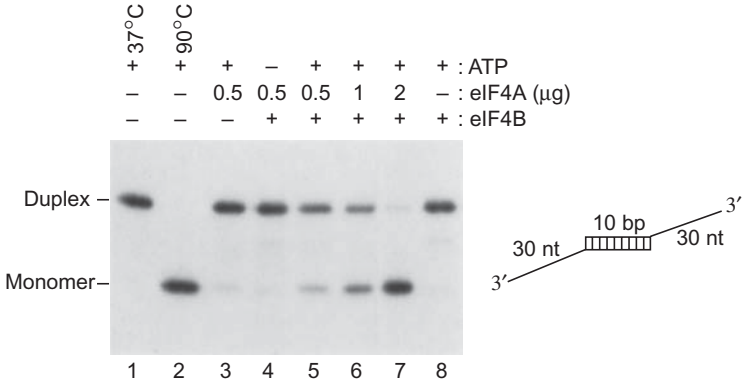


Figure 17.21 RNA helicase activity of eIF4A. Pause and Sonenberg tested combinations of ATP, eIF4A, and eIF4B (as indicated at top) on the radioactive helicase substrate shown at right. RNA helicase unwinds the 10-bp double-stranded region of the substrate, converting the dimer to two monomers. The dimer and monomers are then easily separated by gel electrophoresis, as indicated at left, and detected by autoradiography. The first two lanes are just substrate at low and high temperatures. The high temperature melts the double-stranded region of the substrate, yielding monomers. Lanes 3–8 show that ATP and eIF4A are required for helicase activity, and eIF4B stimulates this activity. (*Source:* Pause A. and N. Sonenberg, Mutational analysis of a DEAD box RNA helicase: The mammalian initiation translation factor eIF-4A. *EMBO Journal* 11 (1992) p. 2644, f. 1.)

translation of capped mRNAs to such extracts must contain the cap-binding protein. This assay revealed that the 24-kD protein by itself was quite labile, but a higher molecular mass complex was much more stable. Sonenberg and collaborators have refined this analysis to demonstrate that the active purified complex contains three polypeptides: the original 24-kD cap-binding protein, and two other polypeptides with M_rs of 50 kD and 220 kD (Figure 17.20). These polypeptides were then given new names: The 24-kD cap-binding protein is **eIF4E;** the 50-kD polypeptide is **eIF4A,** and the 220-kDa polypeptide is **eIF4G.** The whole three-polypeptide complex is called **eIF4F.**

SUMMARY eIF4F is a cap-binding protein composed of three parts: eIF4E has the actual cap-binding activity; it is accompanied by the two other subunits: eIF4A and eIF4G.

Functions of eIF4A and elF4B The eIF4A polypeptide is a subunit of eIF4F, but it also has an independent function: It is a member of the so-called **DEAD protein** family, which has the consensus amino acid sequence Asp (D), Glu (E), Ala (A), Asp (D), and has **RNA helicase** activity. It can therefore unwind the hairpins that are frequently found in the 5′-leaders of eukaryotic mRNAs. To do this job effectively, eIF4A needs the help of **eIF4B,** which has an RNA-binding domain and can stimulate the binding of eIF4A to mRNA. Arnim Pause and Sonenberg used a well-defined in vitro system to demonstrate the activities of both eIF4A and 4B. They started with the products of the eIF4A and 4B genes cloned in bacteria, so there was no possibility of contamination by other eukaryotic proteins. Then they added the labeled RNA helicase substrate pictured on the right in Figure 17.21. This is actually two 40-nt RNAs with complementary 5′-ends, which form a 10-bp RNA double helix. If an RNA helicase unwinds this 10-bp structure, it separates the two 40-nt monomers. Electrophoresis then easily discriminates between monomers and dimer. The more monomers form, the greater is the RNA helicase activity.

Figure 17.21 depicts the results. A small amount of eIF4A (with ATP) caused a very modest amount of unwinding (lane 3), suggesting that this factor has some RNA helicase activity of its own. However, this helicase activity was stimulated by eIF4B (lane 5), and this activity depended on ATP (compare lanes 4 and 5). Greater amounts of eIF4A produced even greater RNA helicase activity (lanes 6 and 7). To show that eIF4B has no helicase activity of its own, Pause and Sonenberg added eIF4B and ATP without eIF4A and observed no helicase activity (lane 8). Thus, these two factors cooperate to unwind RNA helices, including hairpins, and this activity depends on ATP.

SUMMARY eIF4A has RNA helicase activity that can unwind hairpins found in the 5′-leaders of eukaryotic mRNAs. It is aided in this task by another factor, eIF4B, and requires ATP for activity.

Functions of eIF4G We have seen that most eukaryotic mRNAs are capped, and the cap serves to help the ribosome bind. But some viral mRNAs are uncapped; these mRNAs, and perhaps a few cellular mRNAs, have IRESs that can help ribosomes bind. Furthermore, we know that the poly(A) tail at the 3′-end of mRNAs stimulates translation. This latter process involves recruitment of ribosomes to the mRNA via a poly(A)-binding protein called **Pab1p** (yeast) or **PABP1** (human). The eIF4G protein participates in all of these kinds of initiations by serving as an adapter, or "scaffold" protein, that can interact with a variety of different proteins.

Figure 17.22 illustrates three different ways in which eIF4G can participate in translation initiation. In panel (a) we see the function eIF4G performs in initiating on ordinary, capped mRNAs. The amino terminus of eIF4G binds to eIF4E, which in turn binds to the cap. The central portion of eIF4G binds to eIF3, which in turn binds to the 40S ribosomal particle. Thus, by tethering together eIF4E and eIF3, eIF4G can bring the 40S subunit close to the 5′-end of the mRNA, where it can begin scanning.

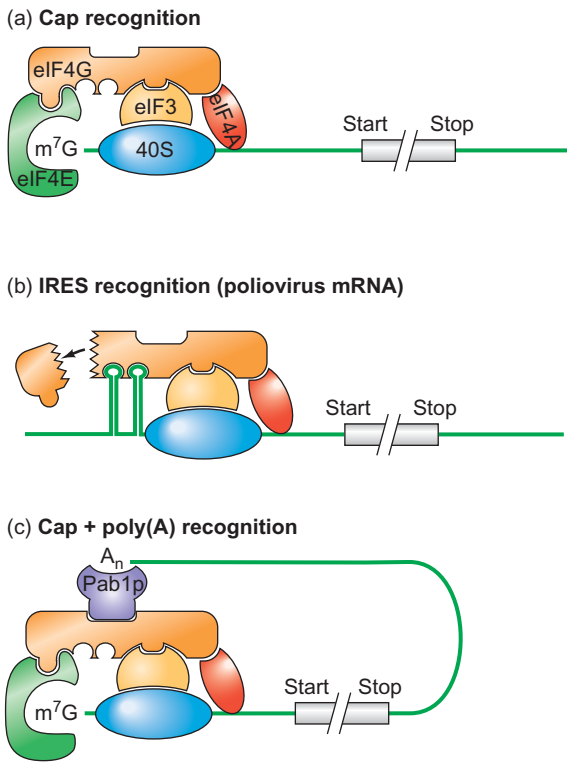


Figure 17.22 The adapter role of eIF4G in recruiting the 40S ribosomal particle in four different situations. (a) Capped mRNA. eIF4G (orange) serves as an adapter between eIF4E (green), bound to the cap, and eIF3 (yellow), bound to the 40S ribosomal particle (blue). The formation of this chain of molecules recruits the 40S particle to a site on the mRNA (dark green) near the cap, where it can begin scanning. eIF4A (red) is also bound to eIF4G, but does not play a role in the interactions illustrated here. **(b)** An mRNA, such as poliovirus mRNA, with an IRES. The IRES interacts directly with the remnant (p100) of eIF4G after a viral protease has cleaved it, ensuring recruitment of the 40S particle. This interaction happens even after removal of the N-terminal part of eIF4G, which blocks binding to capped cellular mRNAs, at least in certain cells. **(c)** Synergism between cap and poly(A). eIF4E bound to the cap and Pab1p (purple) bound to the poly(A) both bind to eIF4G and act synergistically in recruiting the 40S particle. (*Source:* Adapted from Hentze, M.W., eIF4: A multipurpose ribosome adapter? *Science* 275:501, 1997.)

Panel (b) depicts the corruption of translation initiation by a picornavirus such as poliovirus. A viral protease cleaves off the amino terminal domain from eIF4G, impairing its ability to interact with eIF4E in recognizing caps. Thus, capped cellular mRNAs go untranslated. However, the remaining part of eIF4G is still capable of binding to the poliovirus IRES, so 40S subunits are still recruited to the viral mRNA. In fact, the famous Sabin vaccine, which has helped in the ongoing effort to eradicate polio, contains three attenuated strains of the virus. In each strain, an important attenuating event was an alteration in the viral IRES that reduced the affinity for eIF4G, thus impairing translation of the viral mRNA.

When the viral protease cleaves off the N-terminal domain of eIF4G, it leaves a C-terminal domain called p100. Although the poliovirus IRES binds directly to p100, it depends on several cellular proteins (not pictured in Figure 17.22b) for optimum binding. Other viruses, including hepatitis C virus (HCV, another picornavirus), contain IRESs that bind directly to eIF3, without any need for p100 or intact eIF4G. Still other viruses, including hepatitis A virus (HVA, a flavivirus), have IRESs that bind directly to the 40S ribosomal subunit, bypassing the need for all the subunits of eIF4F, and even for eIF3.

It has been commonly assumed that p100 is ineffective in binding to eIF4E, and therefore that cleavage of eIF4G blocks cap-dependent host protein synthesis. On the other hand, Richard Jackson and colleagues demonstrated in 2001 that p100 can stimulate translation of capped mRNAs in a cell-free reticulocyte extract depleted of its own eIF4G, suggesting that p100 is indeed capable of supporting cap-dependent translation. However, maximum levels of cap-dependent translation required a concentration of p100 that is about four times higher than the natural concentration of eIF4G in reticulocyte lysates, leading Jackson and colleagues to suggest the following hypothesis: The loss of cap-dependent host protein synthesis in poliovirus-infected cells is due to competition by viral RNA for the limiting amount of p100, not to an inherent inability of p100 to support the translation of host mRNAs.

A further qualification of the model in Figure 17.22b is also necessary. Although the model appears to describe the situation in HeLa cells accurately, it should not be taken to imply that cleavage of eIF4G blocks host protein synthesis in all kinds of cells. Indeed, Akio Nomoto and colleagues have shown that, although eIF4G cleavage appears to be complete by about 5 h post-infection in human neural cells, host protein synthesis continues unabated. These

workers suggested that another factor in neural cells can compensate for the loss of eIF4G, but no direct evidence for such a factor has been presented.

Finally, panel (c) illustrates the simultaneous interactions between eIF4G and eIF4E bound to the cap and between eIF4G and Pab1p bound to the poly(A) tail of the mRNA. This dual binding of eIF4G to proteins at both ends of the mRNA effectively circularizes the mRNA, which appears to aid translation in at least three ways: First, regulatory proteins and miRNAs bound to the 3′-UTR are close to the cap, which could help them influence initiation of translation. Second, ribosomes completing one round of translation are close to the cap, which may facilitate re-initiation. Finally, the two ends of the mRNA are sequestered and therefore relatively unavailable to RNases that would otherwise degrade the mRNA.

It is important to note that the cap-binding initiation factors we have just studied are the ones used *after* the so-called **pioneer round** of translation, in which the first ribosome binds to the mRNA and translates it. For the pioneer round, the ribosome uses a different set of proteins known as the **cap-binding complex** (**CBC**), which binds to the cap in the nucleus and is exported to the cytoplasm along with the mRNA, as part of an mRNA–protein complex known as the **mRNP** (messenger ribonucleoprotein). The cap-binding protein within the CBC in humans is a heterodimeric cap-binding protein, **CBP80/20**, named for the molecular masses (in kD) of its two subunits. After the pioneer round, the cytoplasmic eIF4F complex replaces the nuclear CBC.

CBP80 is important not only in cap binding, but also in the export of the mRNP out of the nucleus. This export requires a complex of proteins called the **TREX** (transcription export) complex. Mammalian TREX is composed of a seven-subunit complex known as THO, and two other proteins, UAP56 and Aly. Robin Reed and colleagues showed in 2006 that the CBP80 subunit of the cap-binding complex associates with Aly, recruiting TREX to a position near the cap of the growing mRNA. This association with TREX will allow the mature mRNP to be exported 5′-end first, from the nucleus to the cytoplasm, where it can be translated.

TREX is not recruited to pre-mRNAs before they are spliced, nor to the transcripts of synthetic cDNAs, which lack introns, leading to the hypothesis that splicing is necessary for recruitment of TREX to an mRNP. However, TREX does appear to be involved in the export of mRNPs derived from natural genes that lack introns, suggesting that splicing is not always required to attract TREX.

SUMMARY eIF4G is a scaffold protein that is capable of binding to a variety of other proteins, including eIF4E (the cap-binding protein), eIF3 (the 40S ribosomal subunit-binding protein), and Pab1p (a poly[A]-binding protein). By interacting with these proteins, eIF4G can recruit 40S ribosomal subunits to the mRNA and thereby stimulate translation initiation. In the pioneer round of translation, the cap-binding role of eIF4F is played by the CBC, which binds to the cap before export of the mRNP out of the nucleus. A subunit of the CBC also attracts TREX, which guides the mRNP, 5′-end first, out of the nucleus.

Functions of eIF1 and eIF1A eIF1 causes only a modest (about 20%) stimulation of translation activity in vitro. Thus, it was long thought to be dispensable. However, the genes encoding both eIF1 and eIF1A are essential for yeast viability, so their products are hardly dispensable. But what roles do they play? In 1998, Tatyana Pestova and colleagues found the answer: Without eIF1 and eIF1A, the 40S subunit scans only a few nucleotides, if at all, and remains only loosely bound to the mRNA. With these factors, the 40S particle scans to the initiation codon and forms a stable 48S complex.

Pestova and coworkers used a **toeprint assay** based on the primer extension technique (Chapter 5) to locate the leading edge of the 40S ribosomal subunit as it bound to an mRNA. They isolated complexes between the 40S subunit and a mammalian β-globin mRNA, then mixed them with a primer that binds downstream of the initiation codon on the mRNA. Then they extended the primer with nucleotides and reverse transcriptase. When the reverse transcriptase hits the leading edge of the 40S subunit, it stops, so the length of the extended primer shows where that leading edge lies. If you think of the 40S subunit as a foot, its leading edge would be the toe, which is why we call this a toeprint assay. Finally, Pestova and colleagues electrophoresed the primer extension products to measure their sizes. Figure 17.23 presents a schematic view of this procedure.

The actual results are presented in Figure 17.24. Lanes 1 and 2 contained only mRNA or mRNA and 40S subunits, with no factors, so it is not surprising that no complex formed. Lane 3 contained mRNA, 40S subunits, and eIF2, 3, 4A, 4B, and 4F. These factors promoted formation of complex I (the pre-scan complex) only, with no trace of complex II (the post-scan complex). The leading edge of the 40S particle under these circumstances was between positions +21 and +24 relative to the cap of the mRNA, about where we would expect it if the 40S subunit bound at the cap and did not begin scanning or scanned at most a short distance. Lane 4 contains all the factors in lane 3, plus a mixture of initiation factors obtained by washing ribosomes with a saline solution, then

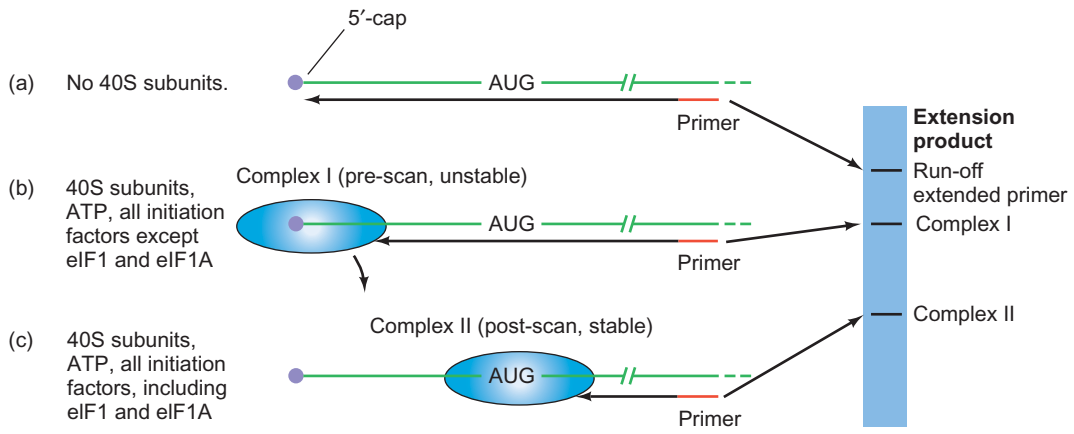


Figure 17.23 Principle of toeprint assay. (a) Negative control. Leave out an essential ingredient, such as 40S subunits, so no complex can form between 40S ribosomal subunits and mRNA. With no 40S particle to block the reverse transcriptase, the primer is extended to the 5′-end of the mRNA. This yields a run-off extended primer corresponding to naked mRNA. **(b)** Complex formed in the absence of eIF1 and eIF1A. Add all the components listed at left, but omit eIF1 and 1A. Complex I forms at the cap, but does not progress far, if at all. Thus, the primer is extended a long distance to the leading edge of the 40S particle. **(c)** Complex formed in the presence of eIF1 and eIF1A. The 40S ribosomal particle has scanned downstream to the initiation codon (AUG) and formed a stable complex (complex II). Thus, the primer is extended only a short distance before it is blocked by the leading edge of the 40S particle in the 48S complex. (*Source:* Adapted from Jackson, R.J., Cinderella factors have a ball. *Nature* 394:830, 1998.)

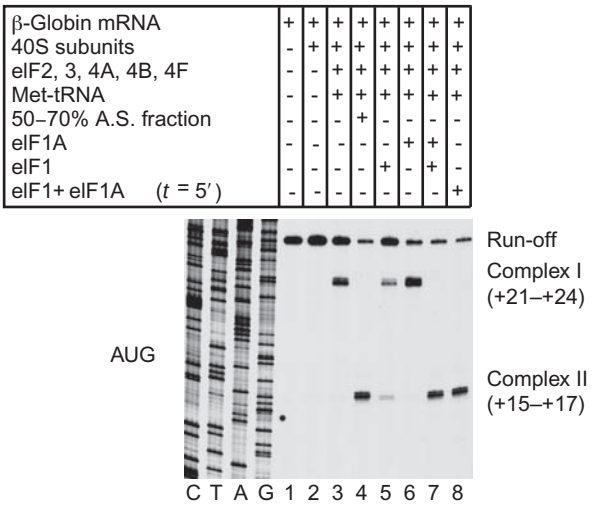


Figure 17.24 Results of toeprint assay. Pestova and colleagues carried out a toeprint assay as described in Figure 17.23, using mammalian β-globin mRNA. The components added to each assay are listed at the top of lanes 1–8. "50–70% A.S. fraction" (lane 4) refers to the factors obtained by precipitating proteins from a ribosome salt wash with ammonium sulfate concentrations between 50 and 70% saturated. "eIF1 + eIF1A (t = 5′)" refers to eIF1 and eIF1A added 5 min after adding the other components of the assay. Lanes C, T, A, and G were the results of sequencing a DNA corresponding to the β-globin mRNA. These sequencing lanes were included as markers to determine the exact positions of the leading edges (toeprints) of the 40S ribosomal particle in the complexes. The position of the initiation codon (AUG) is given at left. The bands corresponding to full-length run-off extended primer and complexes I and II are given at right, with the leading edge of the 40S particle relative to the cap and the initiation codon, respectively. eIF1 and eIF1A were required for complex II formation. (*Source:* Pestova, T.V., S.I. Borukhov, and C.V.T. Hellen, Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature* 394 (27 Aug 1998) f. 2, p. 855. Copyright © Macmillan Magazines Ltd.)

collecting those proteins that could be precipitated by ammonium sulfate concentrations between 50 and 70%. Clearly, this mixture of factors, along with others, could promote the formation of complex II, whose leading edge was between positions +15 and +17 relative to the A of the AUG initiation codon, about where we would expect it if the 40S particle was centered on the initiation codon.

Next, Pestova and colleagues purified the important proteins in the 50–70% ammonium sulfate fraction to homogeneity and obtained partial amino acid sequences to identify them. They turned out to be eIF1 and eIF1A. Figure 17.24, lanes 5 and 6 show that each of these factors individually had little or no ability to stimulate complex II formation. On the other hand, lane 7 demonstrates that these two factors together caused complex II to be formed almost exclusively. Thus, these two factors act synergistically to promote complex II formation. In lane 8, complex I was allowed to form for 5 min, then eIF1 and eIF1A were added. Under these conditions, only complex II formed. Thus, complex I was not a dead end; initiation factors could convert it to complex II.

Did eIF1 and eIF1A convert complex I to complex II by simply causing the 40S subunit to scan farther on the same mRNA, or did these factors cause the 40S particle to dissociate from the mRNA and bind again to scan to the initiation codon? To find out, Pestova and colleagues formed complex I on a radiolabeled mRNA, then added eIF1 and eIF1A with and without a 15-fold excess of unlabeled competitor mRNA. They purified 48S complexes (presumably equivalent to complex II) by sucrose gradient ultracentrifugation and checked these complexes for radioactivity by scintillation counting (Chapter 5).

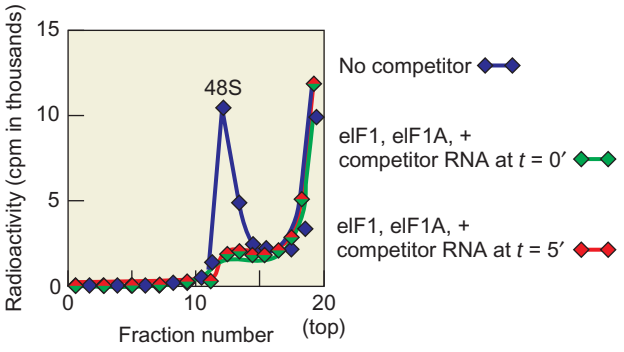


Figure 17.25 Effect of competitor RNA on formation of 48S complex. Pestova and colleagues incubated [^{32}P]β-globin mRNA with 40S ribosomal particles plus the initiation factors and unlabeled competitor RNA combinations indicated at right: blue, no competitor; green, competitor, along with eIF1 and eIF1A, added at time zero; red, competitor, along with eIF1 and eIF1A, added after 5 min of incubation (by which time complex I had formed). After the incubations, the investigators subjected the mixtures to sucrose gradient ultracentrifugation to detect the formation of stable 48S complexes involving 40S particles, [^{32}P]mRNA, and Met-tRNA$_i^{Met}$. They plotted the radioactivity in counts per minute (cpm) detected in each fraction by scintillation counting. The top of the gradient was in fraction 19, as indicated at bottom right. (*Source:* Adapted from Pestova, T.V., S.I. Borukhov, and C.V.T. Hellen, Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature* 394:856, 1998.)

As expected (Figure 17.25), they found a clear radioactive peak of 48S complexes in the absence of competitor mRNA. However, they found no radioactive peak of 48S complexes when they added the competitor mRNA at the beginning of the incubation *or* when they added the competitor mRNA after complex I had formed for 5 min. Thus, eIF1 and eIF1A did not simply allow 40S subunits in complex I to scan downstream and form complex II on the same, labeled mRNA. If they did, labeled 48S complexes would have been seen when these factors and the competitor mRNA were added after 5 min, when complex I had already formed on the labeled mRNA. Instead, these factors disrupted complex I on the labeled mRNA and forced a new complex to form on the excess, unlabeled mRNA. Presumably, the 40S subunits abandoned the labeled mRNA, bound to the caps of (mostly) unlabeled mRNAs, and scanned to the initiation codons of these unlabeled mRNAs, forming complex II.

Thus, eIF1 and eIF1A are not only essential for proper 48S complex formation, they also appear to disrupt improper complexes between 40S ribosomal subunits and mRNA.

In fact, later work has shown that the interaction between eIF1 and eIF1A is antagonistic: eIF1 tends to prevent the scanning 40S subunit from committing to initiate at a given start codon, and this helps to ensure that the wrong codon will not be chosen. In other words, eIF1 promotes scanning. On the other hand, eIF1A slows scanning down. It helps the scanning complex pause long enough at the right start codon to facilitate commitment to initiate there.

SUMMARY eIF1 and eIF1A act synergistically to promote formation of a stable 48S complex, involving initiation factors, Met-tRNA$_i^{Met}$, and 40S ribosomal subunits bound at the initiation codon of an mRNA. eIF1 and eIF1A appear to act by dissociating improper complexes between 40S subunits and mRNA and encouraging the formation of stable 48S complexes. They do this by antagonizing each other: eIF1 promotes scanning, while eIF1A causes the scanning 40S subunit to pause long enough to commit to initiating at the correct start codon.

Functions of eIF5 and eIF5B Once eIF2 has delivered Met-tRNA to the 40S ribosomal subunit and mRNA has also bound to complete the 48S initiation complex, eIF2 needs to dissociate from the complex. To accomplish this dissociation, GTP hydrolysis is required. However, unlike IF2, eIF2 needs the help of another factor—eIF5—to hydrolyze its bound GTP. Even after the eIF5-induced hydrolysis of the GTP bound to eIF2, the 48S complex is not ready to accept the 60S ribosomal subunit to finish the initiation process. Instead, an additional factor, **eIF5B,** is required.

Christopher Hellen and colleagues discovered eIF5B in 2000 when they tested recombinant eIF5 for the ability to induce 60S ribosomal subunits to bind to 48S complexes after dissociation of eIF2. They found that eIF5 alone was not sufficient, but a mixture of proteins released from ribosomes by washing with a high-ionic-strength buffer could complement eIF5 and cause joining of the ribosomal subunits. From this "salt wash," these investigators purified eIF5B, which had the joining-inducing activity. The purified eIF5B (or a modified eIF5B obtained by cloning its gene) could not induce subunit joining on its own. However, it could stimulate subunit joining in a reaction containing other factors, including eIF1, eIF2, eIF3, and eIF5.

Hellen and colleagues next asked whether GTP hydrolysis is required for the subunit-joining reaction. For this experiment, they mixed preformed 48S complexes with eIF5, eIF5B, 60S subunits, and either GTP or the unhydrolyzable analog, GDPNP. No subunit joining took place without either GTP or GDPNP. Thus, we know that GTP is required. Furthermore, GDPNP could support subunit joining, but it required stoichiomentric quantities of eIF5B. On the other hand, eIF5B acted catalytically with GTP in stimulating subunit joining. Thus, because GDPNP will suffice, GTP hydrolysis is not required for subunit joining.

Hellen and colleagues also showed that eIF5B was not released from 80S complexes formed in the presence of GDPNP, but it was released from complexes formed with GTP. Thus, GTP hydrolysis appears to be required for release of eIF5B from the ribosome. In this respect, eIF5B resembles bacterial IF2, which also requires GTP hydrolysis

in order to be released from the ribosome. The two factors are also similar in having a ribosome-stimulated GTPase, and they both play a similar role in ribosomal subunit joining. In fact, the two factors are homologous, so their similarity of functions is not surprising. On the other hand, eIF5B is quite different from IF2 in that it cannot stimulate binding of Met-tRNA$_i^{Met}$, whereas IF2 can carry out the equivalent reaction in bacteria. Instead of eIF5B, eIF2 is responsible for this reaction in eukaryotes.

SUMMARY eIF5B is homologous to the prokaryotic factor IF2. It resembles IF2 in binding GTP and stimulating association of the two ribosomal subunits. eIF5B works with eIF5 in this reaction. eIF5B also resembles IF2 in using GTP hydrolysis to promote its own dissociation from the ribosome so protein synthesis can begin. But it differs from IF2 in that it cannot stimulate the binding of the initiating aminoacyl-tRNA to the small ribosomal subunit. That task is performed by eIF2 in eukaryotes.

17.3 Control of Initiation

We have already examined control of gene expression at the transcriptional and post-transcriptional levels. But control also occurs at the translational level. Given the extensive control we see at the transcriptional and post-transcriptional levels, it is fair to ask why organisms have also evolved mechanisms to control gene expression at the translational level. The major advantage of translational control is speed. New gene products can be produced quickly, simply by turning on translation of preexisting mRNAs. This is especially valuable in eukaryotes, where transcripts are relatively long and take a correspondingly long time to make. Naturally enough, most of this translational control happens at the initiation step.

Bacterial Translational Control

We have learned that most of the control of bacterial gene expression occurs at the transcription level. The very short lifetime (only 1–3 min) of the great majority of bacterial mRNAs is consistent with this scheme, because it allows bacteria to respond quickly to changing circumstances. It is true that different cistrons on a polycistronic transcript can be translated better than others. For example, the *lacZ, Y,* and *A* cistrons yield protein products in a molar ratio of 10:5:2. However, this ratio is constant under a variety of conditions, so it seems to reflect the relative efficiencies of the ribosome-binding sites of the three cistrons as well as differential degradation of parts of the polycistronic mRNA. However, some examples of real control of bacterial translation do occur. Let us consider several of them.

Shifts in mRNA Secondary Structure RNA secondary structure can play a role in translation efficiency, as we observed in Figure 17.6 earlier in this chapter. We learned that the initiation codon of the replicase cistron of the MS2 family of RNA phages is buried in a double-stranded structure that also involves part of the coat gene. This explains why the replicase gene of these phages cannot be translated until the coat protein is translated: The ribosomes moving through the coat gene open up the secondary structure that hides the initiation codon of the replicase gene.

Another example of control via mRNA structure comes from the induction of σ^{32} synthesis during heat shock in *E. coli,* which we mentioned in Chapter 8. When *E. coli* cells experience a rise in temperature from the normal 37°C to 42°C, they switch on a set of heat shock genes that help them cope with the higher temperature. These new, heat shock genes respond to σ^{32}, rather than the normal σ^{70}. But σ^{32} begins accumulating in less than a minute after heat shock, which is too little time for transcription of the σ^{32} gene *(rpoH)* and translation of the corresponding mRNA. So how can we account for such rapid accumulation of σ^{32}?

The data support two answers. First, preexisting σ^{32}, which is normally unstable, becomes stabilized. Second, and more relevant to our discussion here, the σ^{32} gene is controlled at the level of translation initiation. The mRNA encoding σ^{32} is normally folded in such a way that its initiation codon is hidden in secondary structure. That is, the initiation codon is base-paired to another, downstream region of the mRNA. But when the temperature rises, the base pairs causing this secondary structure melt, unmasking the initiation codon so the mRNA can be translated. Thus, there is always plenty of mRNA for this special σ-factor, but it is untranslatable until the temperature rises to dangerous levels. In other words, the built-in thermosensor in the mRNA allows for heating to stimulate gene expression at the translation level.

Takashi Yura and colleagues provided strong support for this hypothesis in 1999 using a derivative of the *rpoH* gene that produced an mRNA with the secondary structure shown in Figure 17.26. This mRNA showed the same regulation characteristics as the wild-type mRNA. Note the base pairing between the initiation codon (boxed) and a region near the 3′-end of the mRNA, forming "stem I," which would presumably prevent translation of this mRNA under physiological conditions. Next, Yura and colleagues made mutations in the stem I region that made the base pairing either stronger or weaker and measured the effects of these mutations on induction by heat.

When the mutations made the base-pairing in stem I stronger, induction was weakened. For example, the C in position +5 with respect to the A of the AUG codon is normally not paired with the U in the opposite strand.

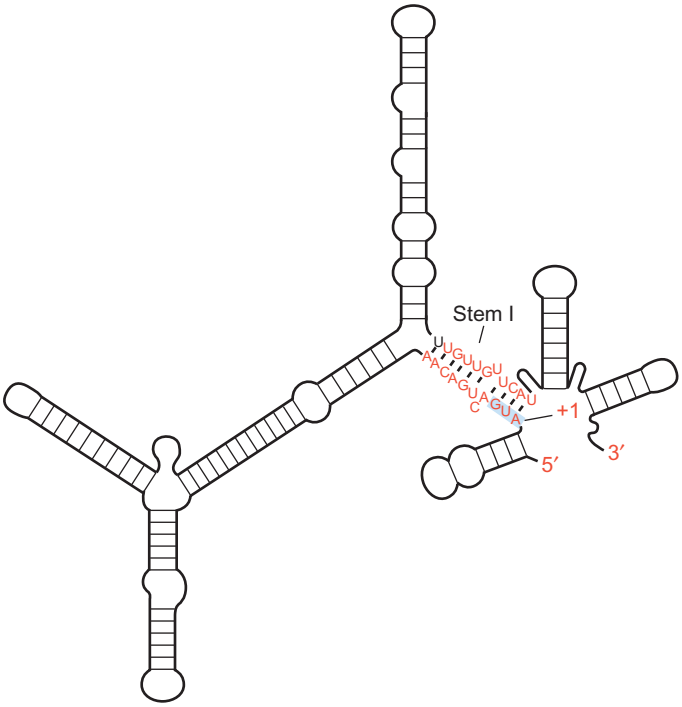


Figure 17.26 Secondary structure of a portion of the *rpoH* mRNA. The sequence in the base-paired region of stem I is shown, including the AUG initiation codon, which is shaded gray. (*Source:* Adapted from Morita, M.T., Y. Tanaka, T.S. Kodama, Y. Kyogoku, K. Yanagi, and T. Yura, Translational induction of heat shock transcription Factor σ^{32}. Evidence for a built-in RNA thermosensor. *Genes and Development* 13 [1999] p. 656, f. 1b.)

However, when this C was changed to A, it could pair to the U and increase the stability of stem I by 2.9 kcal/mol. This reduced induction from the normal 3.5-fold to only 1.4-fold. This makes sense because stronger base pairing is more difficult to disrupt by heating. On the other hand, most mutations that weakened base pairing also increased gene expression at both high and low temperatures. Again, this makes sense because weaker base pairing would be easier to disrupt even at lower temperatures.

SUMMARY The fact that bacterial mRNAs are very short-lived means that transcriptional control is a very efficient way to control gene expression in these organisms. However, translational control also occurs. Messenger RNA secondary structure can govern translation initiation, as in the replicase gene of the MS2 class of phages, whose initiation codon is buried in secondary structure until ribosomes translating the coat gene open up this structure. In another example, the initiation codon in the mRNA for the *E. coli* heat shock σ-factor, σ^{32}, is repressed by secondary structure that is relaxed by heating. Thus, heat can cause an immediate unmasking of σ^{32} mRNA initiation codons, and a burst of σ^{32} synthesis.

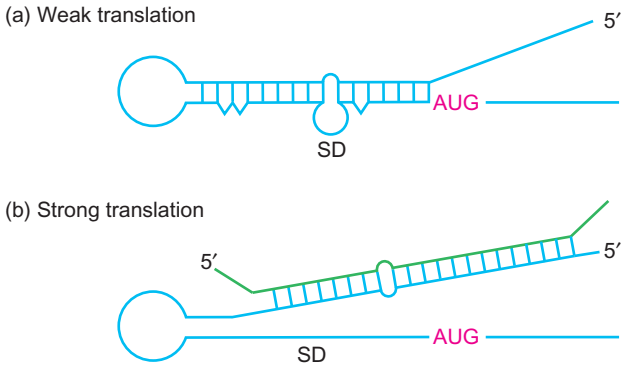


Figure 17.27 Model for activation of *rpoS* mRNA translation by an sRNA. (a) Base-pairing within the 5′-UTR of the *rpoS* mRNA creates a stem loop that hides the Shine–Dalgarno sequence (SD) and the initiation codon (AUG, pink). **(b)** The DsrA sRNA binds to the RNA-binding protein Hfq and base-pairs with part of the 5′-UTR, opening up the SD sequence and initiation codon for binding to the ribosome.

Shifts in mRNA Secondary Structure Induced by Proteins and RNAs In Chapter 16, we learned that small RNAs called microRNAs can control mRNA stability and translation in eukaryotes. Translation in bacteria can also be controlled by a class of short RNAs known simply as **small RNAs (sRNAs),** and these can act on mRNA secondary structure. For example, the initiation codon of the mRNA *(rpoS)* for the stress sigma factor (σ^S, or σ^{38}) is normally buried in secondary structure, so little if any protein is made. However, as shown in Figure 17.27, the DsrA sRNA, in concert with the chaperone protein Hfq, can base-pair with the upstream region of the mRNA, unmasking the *rpoS* initiation codon, and allowing translation to occur.

As we learned in Chapter 7, **riboswitches** are regions within mRNAs that can bind to small molecules, change conformation, and thereby switch gene expression on or off—for example, by shifting from an antiterminator to a terminator to cause attenuation of transcription. The region of the RNA that binds to the small molecule is known as an **aptamer.**

One of the first examples of a riboswitch was discovered by Ronald Breaker and colleagues in 2002. They showed that the *E. coli* mRNAs that encode the enzymes required to synthesize thiamine (vitamin B_1) can assume at least two different conformations. When thiamine or thiamine pyrophosphate binds to an aptamer in the mRNA, the mRNA assumes a conformation that hides the ribosome binding site, so the mRNA cannot be translated. Of course, this is helpful because the presence of thiamine indicates that the cell does not need to waste energy making more enzymes to make this vitamin. Notice that no proteins are involved in this riboswitch. The small molecule thiamine can change the conformation of the mRNA by itself.

Breaker and colleagues had already demonstrated that the leader of the mRNA encoding one of the enzymes in

coenzyme B_{12} synthesis could bind to the coenzyme, and this caused a structural change in the mRNA that was important in control of coenzyme synthesis. They wondered if a similar mechanism applied to the thiamine biosynthesis pathway because two of the genes (*thiM* and *thiC*) encoding enzymes in this pathway contained *thi boxes* with conserved sequences and secondary structures.

Accordingly, they linked the thi boxes to a *lacZ* reporter gene, and tested these constructs for ability to produce β-galactosidase in the presence and absence of thiamine. They found that thiamine suppressed the production of β-galactosidase by 18- and 110-fold, respectively. Thus, the thi boxes were indeed involved in suppression of gene activity. Much of the suppression by the thi box in the *thiC* construct turned out to be at the transcriptional level, whereas all of the suppression by the *thiM* thi box was at the translational level. Since we are concerned with translational control in this chapter, let us focus on the *thiM* gene.

Breaker and colleagues next applied an **in-line probing** technique (Chapter 7) to see if thiamine or its derivatives could cause a structural change in the mRNA leader. This strategy is based on the fact that an unstructured RNA is more susceptible to spontaneous cleavage than one with lots of secondary structure (intramolecular base pairs) or tertiary structure (three-dimensional structure). So the investigators incubated a 165-nt fragment of the mRNA containing the thi box (165 *thiM* RNA) for 40 h in the presence or absence of thiamine pyrophosphate (TPP) and then electrophoresed the products to see where cleavage had occurred. Figure 17.28a reveals that plenty of cleavage occurred with or without TPP, but there were significant

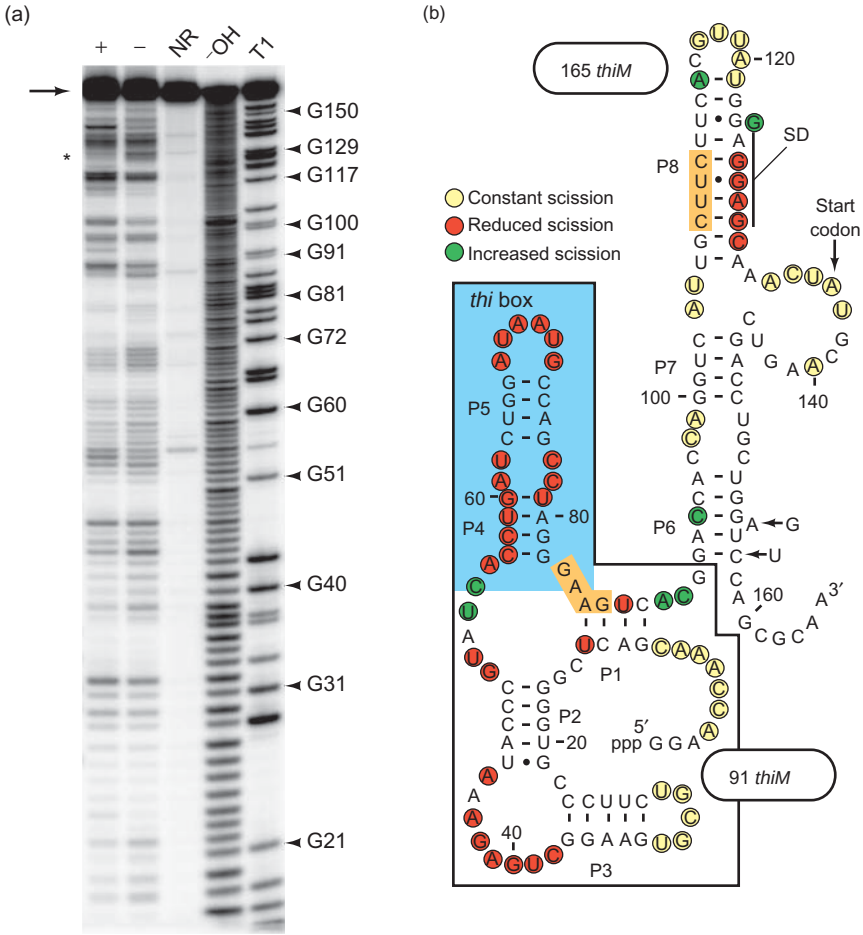


Figure 17.28 TPP binding by *thiM* mRNA. (a) In-line probing of 165 *thiM* mRNA. Breaker and colleagues incubated labeled 165 *thiM* mRNA for 40 h at 25°C in the presence (+) or absence (–) of TPP, then electrophoresed the products. NR is a lane containing RNA that was not incubated, and ⁻OH and T1 denote lanes containing RNAs incubated with base and RNase T1, respectively. **(b)** Predicted secondary structure of the 165 *thiM* RNA in the presence of TPP. The thi box is highlighted in blue. Bases in red experienced reduced cleavage in the presence of TPP, while those in green experienced increased cleavage. Unpaired bases in yellow experienced no change in cleavage. The bases in orange are the CUUC that is shown here paired with GGAG in the Shine–Dalgarno sequence (SG), and an AGGA that is another potential partner for the CUUC. (*Source: Nature*, 419, Wade Winkler, Ali Nahvi, Ronald R. Breaker, "Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression," fig. 1 a&b, p. 953, Copyright 2002, reprinted by permission from Macmillan Publishers Ltd.)

differences. In particular, less cleavage in the region spanning positions 39–80 (including the thi box) occurred in the presence of TPP.

Notice also the region (bases 126–130) denoted by the asterisk. This is the only region that is more ordered (less cleavage) in the presence of TPP, aside from the thi box and nucleotides on the immediate 5′-side of the thi box. And this region encompasses the Shine–Dalgarno sequence, where the ribosome binds. Thus, these results suggest that TPP causes a shift in conformation of the *thiM* mRNA that hides the Shine–Dalgarno sequence in a base-paired stem. This would impede ribosome binding and lower the efficiency of translation of the mRNA.

Breaker and colleagues identified a GAAG sequence, highlighted in orange in Figure 17.28b just at the end of the thi box, that could base-pair with the CUUC at position 108–111 (also highlighted in orange) across from the Shine–Dalgarno sequence in stem P8. This suggested a model in which the CUUC (positions 108–111) normally base-pairs with the GAAG at the end of the thi box, leaving the Shine–Dalgarno sequence available for ribosome binding. This mRNA structure allows active translation. However, TPP, by binding to an aptamer in the thi box, changes the mRNA secondary structure such that the CUUC at position 108–111 base-pairs to the GGAG in the Shine–Dalgarno sequence, hiding it from the ribosomes, and slowing down translation.

This hypothesis makes several predictions. First, a piece of the mRNA containing the thi box should respond to low concentrations of TPP. Indeed, Breaker and colleagues showed that the structural modification of 165 *thiM* RNA was half-complete at a TPP concentration of only 600 nM. Second, TPP should be able to bind tightly to 165 *thiM* RNA, and Breaker and colleagues used a technique called equilibrium dialysis to demonstrate that it does indeed bind tightly. Equilibrium dialysis uses a labeled ligand (tritium-labeled TPP in this case) placed in one chamber, and a large molecule (a *thiM* RNA fragment) in a second chamber, separated from the first by a dialysis membrane which allows small molecules like TPP to pass through, but retains large molecules like RNA. After equilibrium between the two chambers is established, the experimenter measures the amount of label in each chamber and thereby derives a dissociation constant. In this case, the chamber containing the RNA had much more label than the other, reflecting a low dissociation constant (tight binding between TPP and the RNA).

A third prediction is that the binding between thiamine family members and *thiM* mRNA should be specific. Indeed, thiamine, thiamine phosphate (TP), and TPP bound well to the RNA, but oxythiamine and other thiamine derivatives did not. Finally, RNAs with alterations that would disrupt the important structural elements of the *thiM* leader sequence should block both TPP binding and control of *thiM* expression. Breaker and colleagues tested this prediction by making alterations in bases that participate in the predicted stems P3, P5, and P8. These mutant RNAs all failed to bind TPP, and failed to show reduced *thiM* expression in the presence of TPP. However, compensating mutations that restored base-pairing in stems P3, P5, and P8, all restored TPP binding and *thiM* control. For example, changing bases 106 and 107 from U and G, respectively, to A and C, respectively, blocked base-pairing with A and C, respectively at positions 130 and 131. This weakened stem P8, and blocked TPP binding and control. However, if the A and C at positions 130 and 131 were changed to G and U, respectively, TPP binding and control were restored. Thus, base-pairing in all three of these stems appears to be essential for control, as the hypothesis predicts.

SUMMARY Small RNAs, in concert with proteins, can affect mRNA secondary structure to control translation initiation. Riboswitches can also be used to control translation initiation via mRNA secondary structure. The 5′-untranslated region of the *E. coli thiM* mRNA contains a riboswitch, including an aptamer that binds thiamine and its metabolites, thiamine phosphate and, especially, thiamine pyrophosphate (TPP). When TPP is abundant, it binds to this aptamer, causing a conformational shift in the mRNA that ties up the Shine–Dalgarno sequence in secondary structure. This shift hides the SD sequence from ribosomes, and inhibits translation of the mRNA. This saves energy because the *thiM* mRNA encodes an enzyme that is needed to produce more thiamine and, thus, TPP.

Eukaryotic Translational Control

Eukaryotic mRNAs are much longer-lived than bacterial ones, so there is more opportunity for translational control. The rate-limiting factor in translation is usually initiation, so we would expect to find most control exerted at this level. In fact, the most common mechanism of such control is phosphorylation of initiation factors, and we know of cases where such phosphorylation can be inhibitory, and others where it can be stimulatory. Finally, there is an example of a protein binding directly to the 5′-untranslated region of an mRNA and preventing its translation. Removal of this protein activates translation.

Phosphorylation of Initiation Factor eIF2α The best known example of inhibitory phosphorylation occurs in reticulocytes, which make one protein, hemoglobin, to the exclusion of almost everything else. But sometimes reticulocytes are starved for heme, the iron-containing part of hemoglobin, so it would be wasteful to go on producing

α- and β-globins, the protein parts. Instead of stopping the production of the globin mRNAs, reticulocytes block their translation as follows (Figure 17.29): The absence of heme unmasks the activity of a protein kinase called the **heme-controlled repressor,** or **HCR.** This enzyme phosphorylates one of the subunits of eIF2, known as **eIF2α.** The phosphorylated form of eIF2 binds more tightly than usual to eIF2B, which is an initiation factor whose job is to exchange GTP for GDP on eIF2. When eIF2B is stuck fast to phosphorylated eIF2, it cannot get free to exchange GTP for GDP on other molecules of eIF2, so eIF2 remains in the inactive GDP-bound form and cannot attach Met-tRNA$_i^{Met}$ to 40S ribosomes. Thus, translation initiation grinds to a halt.

The antiviral proteins known as **interferons** follow this same pathway. In the presence of interferon and double-stranded RNA, which appears in many viral infections, but not in normal cellular life, another eIF2α kinase is activated. This one is called **DAI,** for **double-stranded RNA-activated inhibitor of protein synthesis.** The effect of DAI is the same as that of HCR—blocking translation initiation. This is useful in a virus-infected cell because the virus has taken over the cell, and blocking translation will block production of progeny viruses, thus short-circuiting the infection.

SUMMARY Eukaryotic mRNA lifetimes are relatively long, so there is more opportunity for translation control than in bacteria. The α-subunit of eIF2 is a favorite target for translation control. In heme-starved reticulocytes, HCR is activated, so it can phosphorylate eIF2α and inhibit initiation. In virus-infected cells, another kinase, DAI, is activated; it also phosphorylates eIF2α and inhibits translation initiation.

Phosphorylation of an eIF4E-Binding Protein The rate-limiting step in translation initiation is cap binding by the cap-binding factor eIF4E. Thus, it is intriguing that eIF4E is also subject to phosphorylation, which stimulates, rather than represses, translation initiation. Phosphorylated eIF4E binds the cap with about four times the affinity of unphosphorylated eIF4E, which explains the stimulation of translation. We saw that the conditions that favor eIF2α phosphorylation and translation repression are unfavorable for cell growth, (e.g., heme starvation and virus infection). This suggests that the conditions that favor eIF4E phosphorylation and translation stimulation should be favorable for cell growth, and this is generally true. Indeed, stimulation of cell division with insulin or mitogens leads to an increase in eIF4E phosphorylation.

Insulin and various growth factors, such as platelet-derived growth factor (PDGF), also stimulate translation in

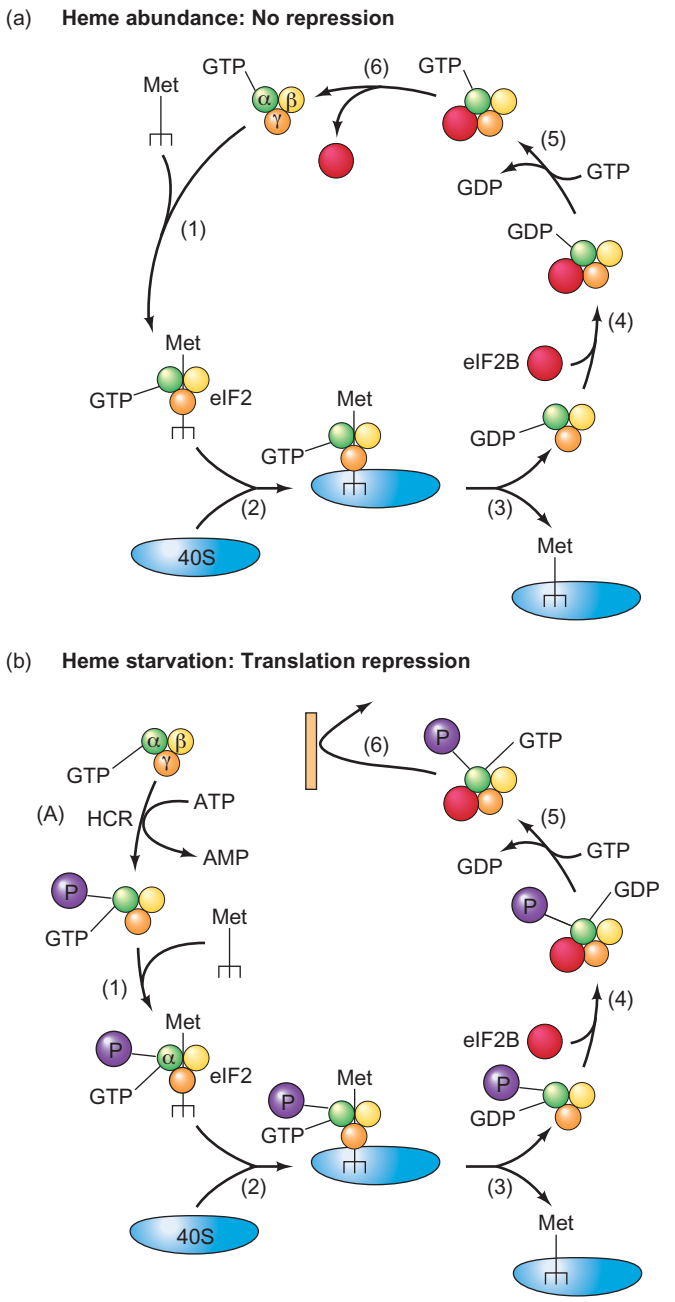


Figure 17.29 Repression of translation by phosphorylation of eIF2α (a) Heme abundance, no repression. Step 1, Met-tRNA$_i^{Met}$ binds to the eIF2-GTP complex, forming the ternary Met-tRNA$_i^{Met}$ GTP-eIF2 complex. The eIF2 factor is a trimer of nonidentical subunits (α [green], β [yellow], and γ [orange]). Step 2, the ternary complex binds to the 40S ribosomal subunit (blue). Step 3, GTP is hydrolyzed to GDP and phosphate, allowing the GDP–eIF2 complex to dissociate from the 40S ribosome, leaving Met-tRNA$_i^{Met}$ attached. Step 4, eIF2B (red) binds to the eIF2–GDP complex. Step 5, eIF2B exchanges GTP for GDP on the complex. Step 6, eIF2B dissociates from the complex. Now eIF2–GTP and Met-tRNA$_i^{Met}$ can get together to form a new complex to start a new round of initiation. **(b)** Heme starvation leads to translational repression. Step A, HCR (activated by heme starvation) attaches a phosphate group (purple) to the α-subunit of eIF2. Then, steps 1–5 are identical to those in panel (a), but step 6 is blocked because the high affinity of eIF2B for the phosphorylated eIF2α prevents its dissociation. Now eIF2B will be tied up in such complexes, and translation initiation will be repressed.

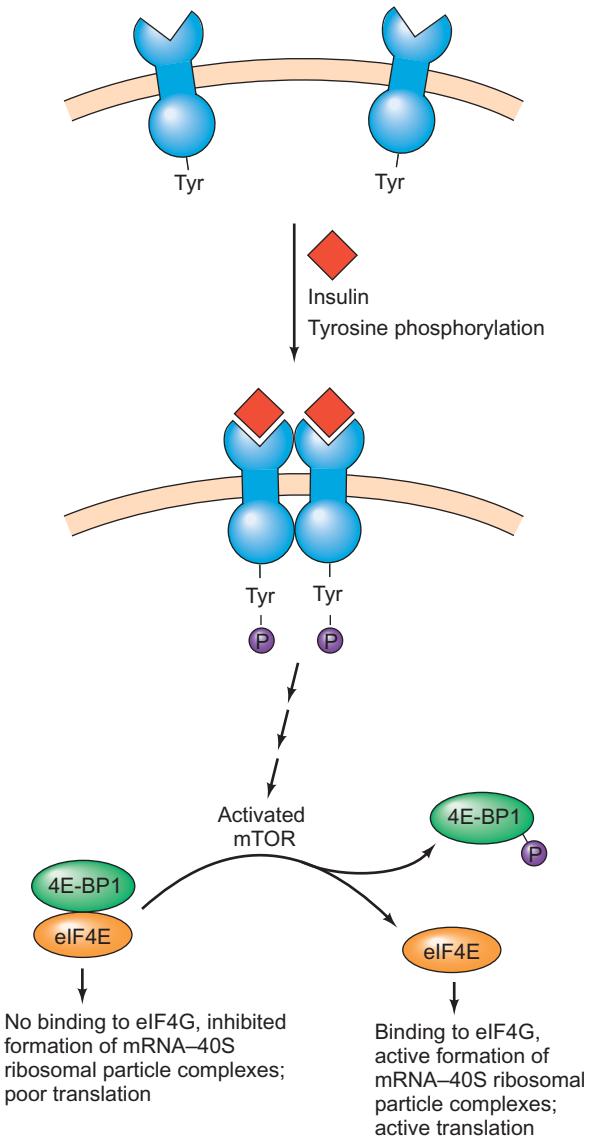


Figure 17.30 Stimulation of translation by phosphorylation of PHAS-I. Insulin, or a growth factor such as EGF, binds to its receptor at the cell surface. Through a series of steps, this activates the protein kinase mTOR. One of the targets of mTOR is 4E-BP1. When 4E-BP1 is phosphorylated by mTOR, it dissociates from eIF4E, releasing it to bind to eIF4G and therefore to participate in active translation initiation.

mammals by an alternative signal transduction pathway that involves eIF4E. We have known for many years that insulin and many growth factors interact with specific receptors at the cell surface (Figure 17.30). These receptors have intracellular domains with protein tyrosine kinase activity. When they interact with their ligands, these receptors can dimerize and autophosphorylate. In other words, the tyrosine kinase domain of one monomer phosphorylates a tyrosine on the other monomer. This triggers several signal transduction pathways (Chapter 12). One of these activates a protein called **mTOR** (**target of rapamycin**, where rapamycin is an antibiotic that inhibits translation initiation). mTor is a protein kinase, and is part of a complex called **mTOR complex 1** (**mTORC1**), which binds to eIF3 in the translation preinitiation complex. From that vantage point, mTOR can stimulate translation initiation by phosphorylating at least two other proteins in the preinitiation complex.

One of the targets of mTORC1 is a protein called **4E-BP1** (**eIF4E-binding protein**). In rats, the same protein is called PHAS-1. 4E-BP1 binds to eIF4E and inhibits its activity. In particular, 4E-BP1 inhibits binding between eIF4E and eIF4G. But once phosphorylated by mTOR, 4E-BP1 dissociates from eIF4E, which is then free to bind eIF4G and promote formation of active complexes between mRNA and 40S ribosomal subunits (Figures 17.30 and 17.22). Thus, translation is stimulated.

Sonenberg and John Lawrence and colleagues discovered human 4E-BP1 in 1994 in a Far Western screen for proteins that bind to eIF4E. A **Far Western screen** is similar to a screen of an expression library with an antibody (Chapter 4), except that the probe is a labeled ordinary protein instead of an antibody. Thus, one is looking for the interaction between two non-antibody proteins instead of the recognition of a protein by an antibody. In this case, the investigators probed a human expression library (in λgt11) with a derivative of eIF4E, looking for eIF4E-binding proteins. The probe was eIF4E, coupled to the phosphorylation site of heart muscle kinase (HMK), which was then phosphorylated with [γ-^{32}P]ATP to label it. Of about one million plaques screened, nine contained genes encoding proteins that bound the eIF4E probe. Three of these contained at least part of the gene that codes for the eIF4G subunit of eIF4F, so it is not surprising that these bound to eIF4E. The other six positive clones coded for two related proteins, 4E-BP1 and 4E-BP2.

The binding of mTORC1 to eIF3 activates translation in other ways besides removing 4E-BP1. It also causes phosphorylation of another eIF3-bound protein, **S6K1** (**S6 kinase-1**), one of whose functions is to phosphorylate the ribosomal protein S6 (Chapter 19). But S6K1 has two more important roles in the present context. First, once phosphorylated and dissociated from the eIF3 complex, S6K1 phosphorylates eIF4B, which facilitates its association with eIF4A. Second, S6K1 phosphorylates an inhibitor of eIF4A known as PDCD4. This phosphorylation leads to ubiquitylation and destruction of PDCD4, which relieves the inhibition of eIF4A. As we learned earlier in this chapter, eIF4A and eIF4B collaborate to unwind mRNA leaders and expedite scanning for the initiation codon. By encouraging the association between eIF4A and eIF4B, and removing an inhibitor of eIF4A, S6K1 stimulates scanning, thereby accelerating translation.

We have seen that mTORC1 responds to insulin and growth factors by stimulating translation. We also know from Chapter 14 that splicing stimulates translation. John

Blenis and colleagues proposed that there was a connection between these two phenomena, and this hypothesis gained support from their finding that rapamycin, which inhibits mTOR, blocks the stimulation of translation by splicing. In 2008, Blenis and colleagues showed that the connection between splicing and mTOR is mediated by a protein known as **SKAR** (**S6K1 Aly/REF-like substrate**). SKAR is recruited to the **exon junction complex** (**EJC**), a collection of proteins placed on mRNAs as they are spliced. Once in the cytoplasm, SKAR, now a part of the messenger ribonucleoprotein (mRNP), can recruit S6K1, activated by mTOR, to the mRNA. And activated S6K1, as we have seen, stimulates translation.

It is important to note that this model of translation stimulation can apply only to the first ribosome translating the newly made mRNA—the so-called pioneer round of translation. That is so because the first ribosome to translate an mRNA removes the EJC, including SKAR, so it can no longer recruit S6K1. We can only speculate about how splicing stimulates the overall rate of translation. Perhaps the efficiency of the pioneer round of translation somehow affects the efficiency of subsequent rounds. Another possibility is based on the fact that recruitment of eIF4E to the cap is rate limiting in translation. Blenis and colleagues speculated that, during remodeling of the mRNP during the pioneer round, mTOR and S6K1 help with the replacement of CBP80/20 by eIF4E and thereby enhance the efficiency of translation.

SUMMARY Insulin and a number of growth factors stimulate a pathway involving a protein kinase complex known as mTORC1, which binds to eIF3 and then phosphorylates its target proteins in the preinitiation complex. One of the targets for mTOR kinase is a protein called 4E-BP1. Upon phosphorylation by mTOR, this protein dissociates from eIF4E and releases it to participate in more active translation initiation. Another target of mTOR is S6K1. Once phosphorylated, activated S6K1, itself a protein kinase, phosphorylates eIF4B, which facilitates that protein's association with eIF4A, stimulating translation initiation. It also phosphorylates PDCD4, which leads to that protein's destruction. Because PDCD4 is an eIF4A inhibitor, its removal also stimulates initiation. Splicing stimulates translation via SKAR, a component of the EJC. SKAR recruits activated S6K1 for the pioneering round of translation.

Control of Translation Initiation via Maskin, an eIF4E-Binding Protein Eukaryotic cells can also use other proteins to target eIF4E, thereby inhibiting translation initiation. One of these proteins, discovered in the frog *Xenopus laevis,* is called **Maskin.** Figure 17.31 illustrates the current hypothesis for how Maskin acts to inhibit translation of the cyclin B mRNA in *Xenopus* oocytes. As we learned in Chapter 15, many mRNAs in *Xenopus* oocytes have very short poly(A) tails and are not well translated. One reason for this situation may be that the cytoplasmic polyadenylation element (CPE) is occupied by a binding protein, **CPEB.** This protein in turn binds to Maskin, which binds to eIF4E. In this interaction, Maskin behaves like 4E-BP1 in blocking the interaction between eIF4E and eIF4G, thereby inhibiting initiation of translation.

When the *Xenopus* oocyte is activated, CPEB is phosphorylated by an enzyme called Eg2. This phosphorylation appears to have two major effects. First, it attracts the cleavage and polyadenylation specificity factor (CPSF) to the polyadenylation signal in the mRNA (AAUAAA), and this stimulates polyadenylation of the dormant mRNA.

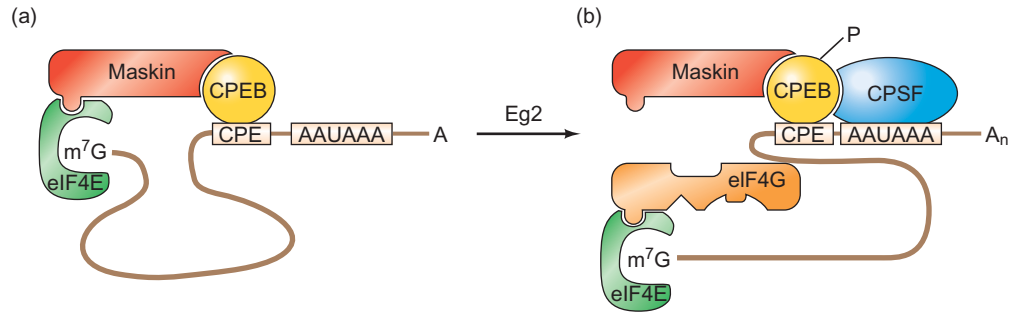


Figure 17.31 Model for control of translation initiation by Maskin. **(a)** In dormant *Xenopus* oocytes, CPEB is bound to CPE on cyclin B mRNA, Maskin is bound to CPEB, and eIF4E is bound to Maskin. The last interaction interferes with the ability of eIF4E to bind to eIF4G, which is necessary for translation initiation. As a result, the cyclin B mRNAs are dormant. **(b)** Upon activation, Eg2 phosphorylates CPEB, allowing recruitment of CPSF and polyadenylation of the mRNA. This event also apparently causes Maskin to dissociate from eIF4E, which enables eIF4E to bind to eIF4G, stimulating translation initiation. (*Source:* Adapted from Richter, J.D. and W.E. Theurkauf, The message is in the translation. *Science* 293 [2001] p. 61, f. 1.)

Second, phosphorylation of CPEB (or perhaps the polyadenylation resulting from this phosphorylation) apparently causes Maskin to lose its grip on eIF4E, allowing eIF4E to bind to eIF4G, stimulating initiation of translation.

It is important to note that cyclin B, one of the genes controlled by Maskin, is a key activator of the cell cycle. Thus, a process as fundamental as cell division is subject to control at the level of translation.

SUMMARY In *Xenopus* oocytes, Maskin binds to eIF4E and to CPEB bound to dormant cyclin B mRNAs. With Maskin bound to it, eIF4E cannot bind to eIF4G, so translation is inhibited. Upon activation of the oocytes, CPEB is phosphorylated, which stimulates polyadenylation and causes Maskin to dissociate from eIF4E. With Maskin no longer attached, eIF4E is free to associate with eIF4G, and translation can initiate.

Repression by an mRNA-Binding Protein We have seen that mRNA secondary structure can influence translation of bacterial genes. This is also true in eukaryotes. Let us consider a well-studied example of repression of translation of an mRNA by interaction between an RNA secondary structure element (a stem loop) and an RNA-binding protein. In Chapter 16 we learned that the concentrations of two iron-associated proteins, the transferrin receptor and ferritin, are regulated by iron concentration. When the serum concentration of iron is high, the synthesis of the transferrin receptor slows down due to destabilization of the mRNA encoding this protein. At the same time, the synthesis of **ferritin,** an intracellular iron storage protein, increases. Ferritin consists of two polypeptide chains, L and H. Iron causes an increased level of translation of the mRNAs encoding both ferritin chains.

What causes this increased efficiency of translation? Two groups arrived at the same conclusion almost simultaneously. The first, led by Hamish Munro, examined translation of the rat ferritin mRNAs; the second, led by Richard Klausner, studied translation of the human ferritin mRNAs. Recall from Chapter 16 that the 3′-untranslated region (3′-UTR) of the transferrin receptor mRNA contains several stem-loop structures called iron response elements (IREs) that can bind proteins. We also saw that the ferritin mRNAs have a very similar IRE in their 5′-UTRs. Furthermore, the ferritin IREs are highly conserved among vertebrates, much more so than the coding regions of the genes themselves. These observations strongly suggest that the ferritin IREs play a role in ferritin mRNA translation.

To test this prediction, Munro and colleagues made DNA constructs containing the CAT reporter gene flanked by the 5′- and 3′-UTRs from the rat ferritin L gene. In one construct (pLJ5CAT3), CAT transcription was driven by a very strong retroviral promoter–enhancer. In the other (pWE5CAT3), CAT transcription was under the control of the weak β-actin promoter. Next, they introduced these DNAs into mammalian cells and tested for CAT production in the presence of an iron source (hemin), an iron chelator (desferal), or no additions. Figure 17.32 shows the results. When cells carried the CAT gene in the pWE5CAT3 plasmid, CAT mRNA was relatively scarce. Under these circumstances, CAT production was low, but inducible by iron (compare left-hand lanes C and H) and inhibited by the iron chelator (compare left-hand lanes C and D). By contrast, when cells carried the pLJ5CAT3 plasmid, the CAT mRNA was relatively abundant, and CAT production was high and noninducible. The simplest explanation for these results is that a repressor binds to the IRE in the ferritin 5′-UTR and blocks translation of the associated CAT cistron. Iron somehow removes the repressor and allows translation to occur. CAT production was not inducible when the CAT mRNA was abundant because the

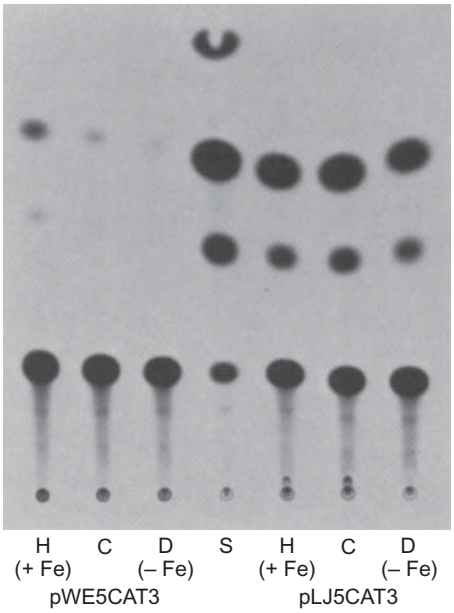


Figure 17.32 Relief of repression of recombinant 5CAT3 translation by iron. Munro and colleagues prepared two recombinant genes with the CAT reporter gene flanked by the 5′-and 3′-UTRs of the rat ferritin L gene. They introduced this construct into cells under control of a weak promoter (the β-actin promoter in the plasmid pWE5CAT3) or a strong promoter (a retrovirus promoter–enhancer in the plasmid pLJ5CAT3). They treated the cells in lanes H with hemin, and those in lanes D with the iron chelator desferal to remove iron. The cells in lanes C were untreated. They assayed CAT activity in each group of cells as described in Chapter 5. Lane S was a standard CAT reaction showing the positions of the chloramphenicol substrate and the acetylated forms of the antibiotic. The lanes on the left show that when the CAT mRNA is not abundant, its translation is inducible by iron. By contrast, the lanes on the right show that when the mRNA is abundant, its translation is not inducible by iron. (*Source:* Adapted from Aziz, N. and H.N. Munro, Iron regulates ferritin mRNA translation through a segment of its 5′ untranslated region. *Proceedings of the National Academy of Sciences USA* 84 (1997) p. 8481, f. 6.)

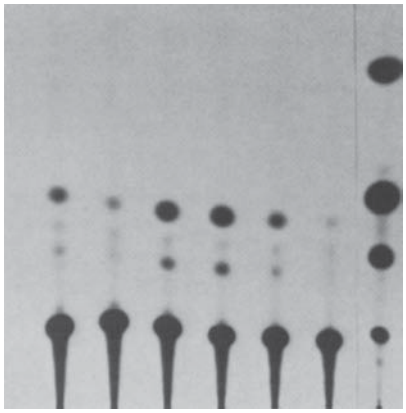


Figure 17.33 Importance of the IRE in the 5′-UTR of pWE5CAT3 for iron inducibility. Munro and colleagues transfected cells with the parent plasmid pWE5CAT3, as described in Figure 17.32, and with two derivatives: pWE5sCAT3, which lacked the first 67 nt of the ferritin 5′-UTR, including the IRE; and pWE5CAT, which lacked the ferritin 3′-UTR. These cells were either treated (H) or not treated (C) with hemin. Then the experimenters assayed each batch of cells for CAT activity. Loss of the IRE caused a loss of iron inducibility. (*Source:* Adapted from Aziz, N. and H.N. Munro, Iron regulates ferritin mRNA translation through a segment of its 5′-untranslated region. *Proceedings of the National Academy of Sciences USA* 84 (1987) p. 8482, f. 7.)

mRNA molecules greatly outnumbered the repressor molecules. With little repression happening, induction cannot be observed.

How do we know that the IRE is involved in repression? In fact, how do we even know that the 5′-UTR, and not the 3′-UTR, is important? Munro and colleagues answered these questions by preparing two new constructs, one containing the 5′-UTR, but lacking the 3′-UTR, and one containing both UTRs, but lacking the first 67 nt, including the IRE in the 5′-UTR. Figure 17.33 shows that pWE5CAT, the plasmid lacking the ferritin mRNA's 3′-UTR, still supported iron induction of CAT. On the other hand, pWE5sCAT3, which lacked the IRE, was expressed at a high level with or without added iron. This result not only indicates that the IRE is responsible for induction, it also reinforces the conclusion that the IRE mediates repression because loss of the IRE leads to high CAT production even without iron.

We can conclude that some repressor protein(s) must bind to the IRE in the ferritin mRNA 5′-UTR and cause repression until removed somehow by iron. Because such great conservation of the IREs occurs in the ferritin mRNAs and the transferrin receptor mRNAs, we suspect that at least some of these proteins might operate in both cases. In fact, as we learned in Chapter 16, the aconitase apoprotein is the IRE-binding protein. When it binds to iron, it dissociates from the IRE. In this case, that would relieve repression.

SUMMARY Ferritin mRNA translation is subject to induction by iron. This induction seems to work as follows: A repressor protein (aconitase apoprotein), binds to a stem-loop iron response element (IRE) near the 5′-end of the 5′-UTR of the ferritin mRNA. Iron removes this repressor and allows translation of the mRNA to proceed.

Blockage of Translation Initiation by an miRNA We have seen in Chapter 16 that miRNAs can control gene expression in two ways: They can cause degradation of mRNAs when base-paired perfectly to their target mRNAs, or, if base-pairing is not perfect, they can inhibit protein production by an unexplained mechanism. Witold Filipowicz and colleagues set out to elucidate that mysterious mechanism, and presented results in 2005 that indicated that imperfectly-paired mammalian *let-7* miRNA can inhibit initiation of translation, probably by interfering with cap recognition.

These workers used reporter genes as probes. In particular, they used the *Renilla reniformis* (sea pansy) luciferase (RL) and firefly luciferase (FL) genes, because the gene products (luciferase) are easily assayed: When mixed with luciferin and ATP, they generate light. The 3′-UTRs of these reporter genes were engineered to have a region that aligns perfectly with *let-7* miRNA (Perf), or to have one or three mismatched regions of complementarity that cause bulges in the miRNA–mRNA duplex. These altered genes were named 1xBulge and 3xBulge, respectively. The wild-type control gene (Con) had no complementarity to *let-7* miRNA.

When they transfected human cells with the reporter genes, Filipowicz and colleagues found that the expression of the RL-Perf and the RL-3xBulge genes decreased dramatically (up to 10-fold) compared to the control gene. Furthermore, this decrease was blocked by co-transfection with a competitor RNA that was complementary to *let-7* miRNA, suggesting that this miRNA was involved in the decrease, as we would expect.

According to the paradigm presented in Chapter 16, we would predict that the amount of RL-Perf mRNA would decrease, because the perfect alignment between the mRNA and miRNA would lead to mRNA degradation. Indeed, Filipowicz and colleagues observed a five-fold reduction in the amount of this mRNA. Furthermore, we would predict that the amount of RL-3xBulge mRNA would not decrease significantly, because the imperfect alignment between the mRNA and miRNA would lead to interference with translation, rather than to mRNA destruction. And, in fact, the amount of this mRNA decreased only 20%.

These data are consistent with the hypothesis that the decline in RL-3xBulge expression is explained by blocking translation, rather than by degradation of mRNA. But it is also possible that the miRNA somehow targets the nascent

protein for degradation by proteolysis. If that were true, then hiding the nascent protein in the endoplasmic reticulum (ER) should shield it from destruction, and little or no drop in expression should be observed. To test this hypothesis, Filipowicz and colleagues coupled the RL-3xBulge gene to the hemaglutinin gene, which contained a signal sequence expressed at the N-terminus of the fusion protein. This signal sequence directed the nascent protein to the lumen of the ER. The protein product of this construct suffered the same decrease compared to the control as the RL-3xBulge product itself did. Thus, protein synthesis, rather than the protein product itself, appears to be the target of the *let-7* miRNA.

What part of the translation process is inhibited by *let-7* miRNA? To begin to answer this question, Filipowicz and colleagues collected polysomes (mRNAs being translated by multiple ribosomes, Chapter 18) from cells transfected with the RL-3xBulge gene. To detect the RL-3xBulge mRNA in the polysome profile, they performed Northern blots on polysome fractions (Figure 17.34). The more active the translation initiation on a given mRNA, the more ribosomes will be attached to the mRNA, and therefore the heavier the polysomes will be. The heaviest polysomes are found toward the right in Figure 17.34, and it is clear that the control RL mRNAs were in much larger polysomes (farther to the right, panel [a]) than the RL-3xBulge mRNAs (panel [b]). These results are depicted graphically in Figure 17.34c. The shift in polysome profile was mostly eliminated by co-transfection with an anti-*let-7* miRNA, which would block miRNA–mRNA interaction (results not shown). The shift was also eliminated when the RL-3xBulge mRNA was mutated to remove the 3′-UTR region that hybridizes to the miRNA. Taken together, these data indicate that translation initiation on RL-3xBulge mRNA is significantly inhibited compared to initiation on the control mRNA. Thus, initiation (binding of ribosomes to mRNA) seems to be the part of translation that is the target of the *let-7* miRNA.

Further study showed that the poly(A) tail on the mRNA played no role in *let-7* miRNA inhibition of translation: Translation of poly(A)$^+$ and poly(A)$^-$ mRNAs were equally inhibited by *let-7* miRNA. But the cap did play a big role. As we have seen, translation of uncapped mRNAs is very poor, so Filipowicz and colleagues endowed either the RL or FL mRNA with the internal ribosome entry site (IRES) from the encephalomyocarditis virus (EMCV), which allows cap-independent translation. Then they compared the effect of *let-7* miRNA on cap-dependent and -independent translation. As usual, *let-7* inhibited cap-dependent translation of FL-3xBulge mRNA, but it had no effect on the cap-independent translation of FL-3xBulge mRNA with an EMCV IRES. Thus, *let-7* miRNA appears to target cap-dependent initiation of translation.

To pin down the part of cap-dependent initiation that is affected by *let-7* miRNA, Filipowicz and colleagues built a

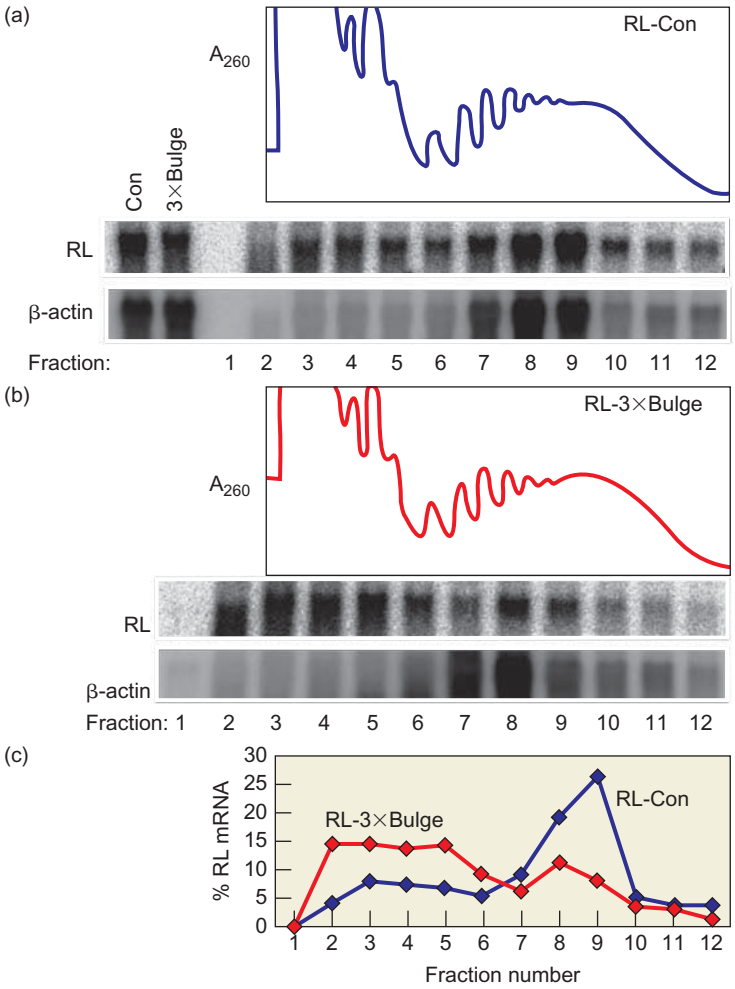


Figure 17.34 Polysomal profiles of RL mRNAs. Filipowicz and colleagues transfected human cells with genes that encoded either **(a)** the control RL mRNA (RL-Con) or **(b)** RL-3xBulge mRNA. Then they displayed the polysomes by sucrose gradient ultracentrifugation, subjected RNAs from fractions from the polysome profile to Northern blotting, and hybridized the blots to radioactive probes for RL or β-actin mRNA. The latter is an ordinary cellular mRNA, used as a positive control. The two lanes on the far left of the Northern blots in panel (a) contain RNAs from the inputs into the ultracentrifugation step. **(c)** The percentages of total radioactivity in each fraction from the control and RL-3xBulge polysome profiles are presented. (*Source:* (*a–c*) Reprinted with permission from *Science,* Vol. 309, Ramesh S. Pillai, Suvendra N. Bhattacharyya, Caroline G. Artus, Tabea Zoller, Nicolas Cougot, Eugenia Basyuk, Edouard Bertrand, and Witold Filipowicz, "Inhibition of Translational Initiation by Let-7 MicroRNA in Human Cells" Fig. 1 c&e, p. 1574, Copyright 2004, AAAS.)

DNA construct encoding a dicistronic mRNA with either eIF4E or eIF4G tethered in the intercistronic region just before the RL cistron. They performed the tethering as follows (Figure 17.35a): In the intercistronic region, they placed so-called BoxB stem-loops that have affinity for a peptide called the N peptide. Then they engineered genes for eIF4E and eIF4G, adding N peptide-hemagglutinin coding regions, so the initiation factors were each produced as fusion proteins tagged with the N peptide. These fusion proteins in turn bound to the BoxB stem-loops, so they

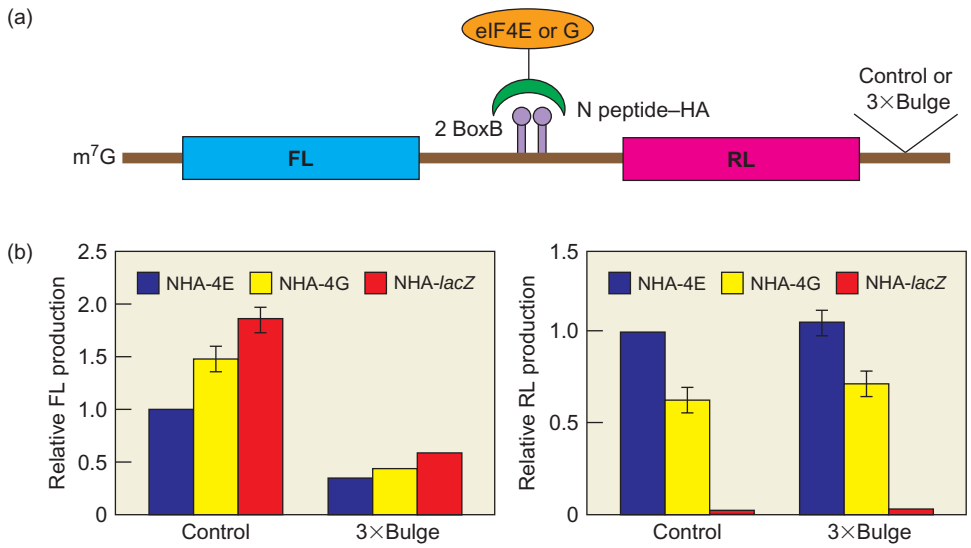


Figure 17.35 Effect of tethering translation initiation factors to the intercistronic region of a dicistronic mRNA. (a) Diagram of the construct with two BoxB stem loops (purple), between the two cistrons, bound to the N peptide part (green) of a fusion protein that also contained either eIF4E or eIF4G (orange). The 3′-UTR contained either the control RL sequence (Con) or the 3xBulge sequence. **(b)** Production of FL (left) and RL (right) from the control and 3xBulge mRNAs, as indicated at bottom, with various proteins tethered to the intercistronic region. The N peptide-hemaglutinin (NHA)-tagged protein tethered to the intercistronic region is indicated by color in the bar graphs: eIF4E, blue; eIF4G, yellow; *lacZ* product, red. (*Source:* Adapted from Ramesh, S., et al., 2004 Inhibition of translational initiation by let-7 microRNA in human cells. *Science* 309:1575, fig. 2.)

could stimulate translation of the RL cistron on the dicistronic mRNA. The translation of the FL cistron was cap-dependent, since this cistron came first in the capped mRNA. But translation of the RL cistron was cap-independent as long as one of the initiation factors was tethered to the intercistronic region. This protein apparently attracted all the other factors needed for initiation.

So Filipowicz and colleagues tested expression of the FL and RL parts of the fusion gene with either a control 3′-UTR or the 3xBulge 3′-UTR, and either of the initiation factors (or, as a negative control, the *lacZ* product, β-galactosidase) tethered to the intercistronic region. Figure 17.35b shows the results. As expected, translation of the FL cistron was cap-dependent, and the *let-7* miRNA inhibited translation of the FL cistron of the 3xBulge mRNA compared to the control mRNA. But, when either eIF4E or eIF4G was tethered to the intercistronic region, *let-7* miRNA did not inhibit translation of the RL cistron in the 3xBulge mRNA. (With the *lacZ* product, rather than an initiation factor, tethered in the intercistronic region, almost no translation occurred, even with the control mRNA.) Thus, having either eIF4E or eIF4G available (in this case by tethering) circumvents the *let-7*-mediated inhibition of translation initiation. This suggests that *let-7* blocks some step before eIF4E recruits eIF4G to the cap. One obvious candidate for this *let-7*-sensitive step is eIF4E binding to the cap.

These results in mammalian cells, showing that *let-7* miRNA interferes with translation initiation, differ from some of the results presented in Chapter 16, which indicated that *lin-4* miRNA does not alter the polysome profile of its target mRNA in *C. elegans* cells, and therefore does not appear to block translation initiation. As pointed out in Chapter 16, this discrepancy can be explained if different miRNAs have different modes of action, or if miRNAs work differently in different organisms, or both.

SUMMARY The *let-7* miRNA shifts the polysomal profile of target mRNAs in human cells toward smaller polysomes, indicating that this miRNA blocks translation initiation in human cells. Translation initiation that is cap-independent because of the presence of an IRES, or tethered initiation factors, is not affected by *let-7* miRNA, suggesting that this miRNA blocks binding of eIF4E to the cap of target mRNAs in human cells.

SUMMARY

Two events must occur as a prelude to protein synthesis: First, aminoacyl-tRNA synthetases join amino acids to their cognate tRNAs. They do this very specifically in a two-step reaction that begins with activation of the amino acid with AMP, derived from ATP. Second, ribosomes must dissociate into subunits at the end of each round of

translation. In bacteria, RRF and EF-G actively promote this dissociation, whereas IF3 binds to the free 30S subunit and prevents its reassociation with a 50S subunit to form a whole ribosome.

The initiation codon in prokaryotes is usually AUG, but it can also be GUG, or more rarely, UUG. The initiating aminoacyl-tRNA is *N*-formyl-methionyl-$tRNA_f^{Met}$. *N*-formyl-methionine (fMet) is therefore the first amino acid incorporated into a polypeptide, but it is frequently removed from the protein during maturation.

The 30S initiation complex is formed from a free 30S ribosomal subunit plus mRNA and fMet-$tRNA_f^{Met}$. Binding between the 30S prokaryotic ribosomal subunit and the initiation site of an mRNA depends on base pairing between a short RNA sequence called the Shine–Dalgarno sequence just upstream of the initiation codon, and a complementary sequence at the 3′-end of the 16S rRNA. This binding is mediated by IF3, with help from IF1 and IF2. All three initiation factors have bound to the 30S subunit by this time.

IF2 is the major factor promoting binding of fMet-$tRNA_f^{Met}$ to the 30S initiation complex. The other two initiation factors play important supporting roles. GTP is also required for IF2 binding at physiological IF2 concentrations, but it is not hydrolyzed in the process. The complete 30S initiation complex contains one 30S ribosomal subunit plus one molecule each of mRNA, fMet-$tRNA_f^{Met}$, GTP, IF1, IF2, and IF3. GTP is hydrolyzed after the 50S subunit joins the 30S complex to form the 70S initiation complex. This GTP hydrolysis is carried out by IF2 in conjunction with the 50S ribosomal subunit. The purpose of this hydrolysis is to release IF2 and GTP from the complex so polypeptide chain elongation can begin.

Eukaryotic 40S ribosomal subunits, together with the initiating Met-tRNA (Met-$tRNA_i^{Met}$), generally locate the appropriate start codon by binding to the 5′-cap of an mRNA and scanning downstream until they find the first AUG in a favorable context. The best context contains a purine at position −3 and a G at position +4. In 5–10% of the cases, most ribosomal subunits will bypass the first AUG and continue to scan for a more favorable one. Sometimes ribosomes apparently initiate at an upstream AUG, translate a short ORF, then continue scanning and reinitiate at a downstream AUG. This mechanism works only with short upstream ORFs. Some viral mRNAs that lack caps have IRESs that attract ribosomes directly to the mRNAs.

Secondary structure near the 5′-end of an mRNA can have positive or negative effects. A hairpin just past an AUG can force a ribosomal subunit to pause at the AUG and thus stimulate initiation. A very stable stem loop between the cap and an initiation site can block ribosomal subunit scanning and thus inhibit initiation.

The eukaryotic initiation factors have the following general functions: eIF1 and eIF1A aid in scanning to the initiation codon. eIF2 is involved in binding Met-$tRNA_i^{Met}$ to the ribosome. eIF2B activates eIF2 by replacing its GDP with GTP. eIF3 binds to the 40S ribosomal subunit and inhibits its reassociation with the 60S subunit. eIF4F is a cap-binding protein that allows the 40S ribosomal subunit to bind (through eIF3) to the 5′-end of an mRNA. eIF5 encourages association between the 43S complex (40S subunit plus mRNA and Met-$tRNA_i^{Met}$). eIF6 binds to the 60S subunit and blocks its reassociation with the 40S subunit.

eIF4F is a cap-binding protein composed of three parts: eIF4E has the actual cap-binding activity; it is accompanied by the two other subunits, eIF4A and eIF4G. eIF4A has RNA helicase activity that can unwind hairpins found in the 5′-leaders of eukaryotic mRNAs. It is aided in this task by another factor, eIF4B, and requires ATP for activity. eIF4G is an adapter protein that is capable of binding to a variety of other proteins, including eIF4E (the cap-binding protein), eIF3 (the 40S ribosomal subunit-binding protein), and Pab1p (a poly[A]-binding protein). By interacting with these proteins, eIF4G can recruit 40S ribosomal subunits to the mRNA and thereby stimulate translation initiation.

eIF1 and eIF1A act synergistically to promote formation of a stable 48S complex, involving initiation factors, Met-$tRNA_i^{Met}$, and a 40S ribosomal subunit that has scanned to the initiation codon of an mRNA. eIF1 and eIF1A appear to act by dissociating improper complexes between 40S subunits and mRNA and encouraging the formation of stable 48S complexes.

eIF5B is homologous to the prokaryotic factor IF2. It resembles IF2 in binding GTP and stimulating association of the two ribosomal subunits. eIF5B works with eIF5 in this reaction. eIF5B also resembles IF2 in using GTP hydrolysis to promote its own dissociation from the ribosome so protein synthesis can begin. But it differs from IF2 in that it cannot stimulate the binding of the initiating aminoacyl-tRNA to the small ribosomal subunit. That task is performed by eIF2 in eukaryotes.

Prokaryotic mRNAs are very short-lived, so control of translation is not common in these organisms. However, some translational control does occur. Messenger RNA secondary structure can govern translation initiation, as in the replicase gene of the MS2 class of phages, or in the mRNA for *E. coli* σ^{32}, whose translation is repressed by secondary structure that is relaxed by heating.

Small RNAs, in concert with proteins, can also affect mRNA secondary structure to control translation initiation, and riboswitches are one way this control can be exercised. The 5′-untranslated region of the *E. coli thiM* mRNA contains a riboswitch, including an aptamer that binds thiamine and its metabolites, including thiamine pyrophosphate (TPP). When TPP is abundant, it binds to this aptamer, causing a conformational shift in the mRNA that ties up the Shine–Dalgarno sequence in

secondary structure. This shift hides the SD sequence from ribosomes, and inhibits translation of the mRNA.

Eukaryotic mRNA lifetimes are relatively long, so there is more opportunity for translation control than in prokaryotes. The α-subunit of eIF2 is a favorite target for translation control. In heme-starved reticulocytes, HCR is activated, so it can phosphorylate eIF2α and inhibit initiation. In virus-infected cells, another kinase, DAI is activated; it also phosphorylates eIF2α and inhibits translation initiation.

Insulin and a number of growth factors stimulate a pathway involving a protein kinase called mTOR. One of the targets for mTOR is a protein called 4E-BP1. On phosphorylation by mTOR, this protein dissociates from eIF4E and releases it to participate in more active translation initiation. Another target of mTOR is S6K1. Once phosphorylated, activated S6K1, itself a protein kinase, phosphorylates targets that enhance translation. Splicing stimulates translation via SKAR, a component of the EJC. SKAR recruits activated S6K1 for the pioneering round of translation.

In *Xenopus* oocytes, Maskin binds to eIF4E and to CPEB bound to dormant cyclin B mRNAs. With Maskin bound to it, eIF4E cannot bind to eIF4G, so translation is inhibited. Upon activation of the oocytes, CPEB is phosphorylated, which stimulates polyadenylation and causes Maskin to dissociate from eIF4E. With Maskin no longer attached, eIF4E is free to associate with eIF4G, and translation can initiate.

Ferritin mRNA translation is subject to induction by iron. This induction seems to work as follows: A repressor protein (aconitase apoprotein), binds to a stem-loop iron response element (IRE) near the 5′-end of the 5′-UTR of the ferritin mRNA. Iron removes this repressor and allows translation of the mRNA to proceed.

The *let-7* miRNA shifts the polysomal profile of target mRNAs in human cells toward smaller polysomes, indicating that this miRNA blocks translation initiation in human cells. Translation initiation that is cap-independent because of the presence of an IRES, or tethered initiation factors, is not affected by *let-7* miRNA, suggesting that this miRNA blocks binding of eIF4E to the cap of target mRNAs in human cells.

REVIEW QUESTIONS

1. Describe and give the results of an experiment that shows that ribosomes dissociate and reassociate.
2. How does IF3 participate in ribosome dissociation?
3. What are the two bacterial methionyl-tRNAs called? What are their roles?
4. Why does translation of the MS2 phage replicase cistron depend on translation of the coat cistron?
5. Present data (exact base sequences are not necessary) to support the importance of base-pairing between the Shine–Dalgarno sequence and the 16S rRNA in translation initiation. Select the most convincing data.
6. Present data to show the effects of the three initiation factors in mRNA-ribosome binding.
7. Describe and give the results of an experiment that shows the role (if any) of GTP hydrolysis in forming the 30S initiation complex.
8. Describe and give the results of an experiment that shows the role of GTP hydrolysis in release of IF2 from the ribosome.
9. Present data to show the effects of the three initiation factors in fMet-tRNA$_f^{Met}$ binding to the ribosome.
10. Draw a diagram to summarize the initiation process in *E. coli.*
11. Explain what the Shine–Dalgarno sequence and the Kozak consensus sequence are and compare and contrast their roles.
12. Write the sequence of an ideal eukaryotic translation initiation site. Aside from the AUG, what are the most important positions?
13. Draw a diagram of the scanning model of translation initiation.
14. Present evidence that a scanning ribosome can bypass an AUG and initiate at a downstream AUG.
15. Under what circumstances is an upstream AUG in good context not a barrier to initiation at a downstream AUG? Present evidence.
16. Describe and give the results of an experiment that shows the effects of secondary structure in an mRNA leader on scanning.
17. Draw a diagram of the steps in translation initiation in eukaryotes, showing the effects of each class of initiation factor.
18. Describe and give the results of an experiment that identified the cap-binding protein.
19. Describe and give the results of an experiment that shows that cap-binding protein stimulates translation of capped, but not uncapped, mRNAs.
20. What is the subunit structure of eIF4F? Molecular masses are not required.
21. Describe and give the results of an experiment that shows the roles of eIF4A and eIF4B in translation.
22. How does the poliovirus genetic material resemble a typical cellular mRNA? How it is different? How does the virus take advantage of this difference? Compare and contrast this behavior with that of the hepatitis C virus.
23. How do we know that eIF1 and eIF1A do not cause conversion of complex I to complex II by stimulating scanning on the same mRNA?
24. Compare the initiation factors IF2 and eIF5B. What functions do they have in common? What function can IF2 perform that eIF5B cannot? What factor performs this function in eukaryotes?

25. Describe the mechanism by which the *rpoH* mRNA senses high temperature and turns on its own translation. What is the evidence for this model?
26. Describe the mechanism by which the riboswitch in the *E. coli thiM* gene controls translation.
27. Present a model for repression of translation by phosphorylation of eIF2α.
28. Present a model to explain the effect of 4E-BP1 phosphorylation on translation efficiency.
29. Describe and give the results of an experiment that shows the importance of the IRE in the ferritin mRNA to iron inducibility of ferritin production.
30. Present a hypothesis for iron inducibility of ferritin production in mammalian cells. Make sure your hypothesis explains why ferritin production is not inducible in cells in which the ferritin gene is driven by a strong promoter.
31. How is the human *let-7* miRNA thought to control expression of its target genes? Summarize the evidence for this model.

ANALYTICAL QUESTIONS

1. Describe a toeprint assay involving *E. coli* ribosomal subunits and a fictious mRNA in a cell-free extract that contains all the factors necessary for translation. What results would you expect to see with 30S ribosomal subunits alone? With 50S subunits alone? With both subunits and all amino acids except leucine, which is required in the 20th position of the polypeptide?
2. Predict the effects of the following mutations on phage R17 coat gene and replicase gene translation:
   a. An amber mutation (premature stop codon) six codons downstream of the coat gene initiation codon.
   b. Mutations in the stem loop around the coat gene initiation codon that weaken the base-pairing in the stem loop.
   c. Mutations in the interior of the replicase gene that cause it to base-pair with the coat gene initiation codon.
3. You are studying a eukaryotic gene in which translation normally begins with the second AUG in the mRNA. The sequence surrounding the two AUG codons is:

   CGGAUGCACAGGACAUCCUAUGGAGAUGA

   where the two AUG codons are underlined. Predict the effects of the following mutations on translation of this mRNA.
   a. Changing the first and second C's to G's.
   b. Changing the first and second C's to G's, and also changing the UAU codon before the second AUG codon to UAG.
   c. Changing the GAGAUGA sequence at the end to CAGAUGU
4. You are studying a eukaryotic mRNA that you believe exhibits control at the level of translation, particularly the initiation of translation. You think that the 5′-UTR plays a role in the control of translation. To definitively determine the role of the 5′-UTR, describe in detail experiments that you could perform to prove this. Be sure to include how you would experimentally determine if a protein binds to the 5′-UTR to prevent translation and the possible effects a mutation in the 5′-UTR might have on gene expression at the RNA level.

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