CHAPTER 16

Other RNA Processing Events and Post-Transcriptional Control of Gene Expression

A petunia flower showing the effects of silencing by adding extra copies of the purple color gene. © *Courtesy of Dr. Richard A. Jorgensen,* The Plant Cell.

In the previous two chapters, we examined splicing, capping, and polyadenylation, which covers most of what happens to pre-mRNAs in eukaryotic cells. However, in a few organisms, other specialized pre-mRNA processing events occur. For example, parasitic protozoa called trypanosomes, as well as some parasitic worms and the free-living protist *Euglena,* carry out *trans*-splicing of pre-mRNAs. This involves splicing together two independent transcripts. Trypanosomes also have mitochondria, called kinetoplasts, that edit their mRNAs by adding or deleting nucleotides after transcription. In contrast to these rather esoteric processing events, most organisms process their rRNAs and tRNAs by more conventional mechanisms. Also eukaryotes control some of their gene expression by regulating posttranscriptional processes, primarily mRNA degradation. Finally, eukaryotes can react to foreign genes

or double-stranded RNA by destroying the corresponding mRNA. All of these posttranscriptional events will be our subjects in this chapter.

16.1 Ribosomal RNA Processing

The rRNA genes of both eukaryotes and bacteria are transcribed as larger precursors that must be processed (cut into pieces) to yield rRNAs of mature size. However, this is not just a matter of removing unwanted material at either end of an overly long molecule. Instead, several different rRNA molecules are embedded in a long precursor, and each of these must be cut out. Let us consider rRNA processing, first in eukaryotes, then in bacteria.

Eukaryotic rRNA Processing

The rRNA genes in eukaryotes are repeated several hundred times and clustered together in the nucleolus of the cell. Their arrangement in amphibians has been especially well studied, and, as Figure 16.1a shows, they are separated by regions called **nontranscribed spacers (NTSs).** NTSs are distinguished from **transcribed spacers,** regions of the gene that are transcribed as part of the rRNA precursor and then removed in the processing of the precursor to mature rRNA species.

This clustering of the reiterated rRNA genes in the nucleolus made them easy to find and therefore provided Oscar Miller and his colleagues with an excellent opportunity to observe genes in action. These workers looked at amphibian nuclei with the electron microscope and uncovered a visually appealing phenomenon, shown in Figure 16.1b. The DNA containing the rRNA genes can be seen winding through the picture, but the most obvious feature of the micrograph is a series of "tree" structures. These include the rRNA genes (the trunk of the tree) and growing rRNA transcripts (the branches of the tree). We will see shortly that these transcripts are actually rRNA precursors, not mature rRNA molecules. The spaces between "trees" are the nontranscribed spacers. You can even tell the direction of transcription from the lengths of the transcripts within a given gene; the shorter RNAs are at the beginning of the gene and the longer ones are at the end.

We have seen that mRNA precursors frequently require splicing but no other trimming. On the other hand, rRNAs and tRNAs first appear as precursors that sometimes need splicing, but they also have excess nucleotides at their ends, or even between regions that will become separate mature

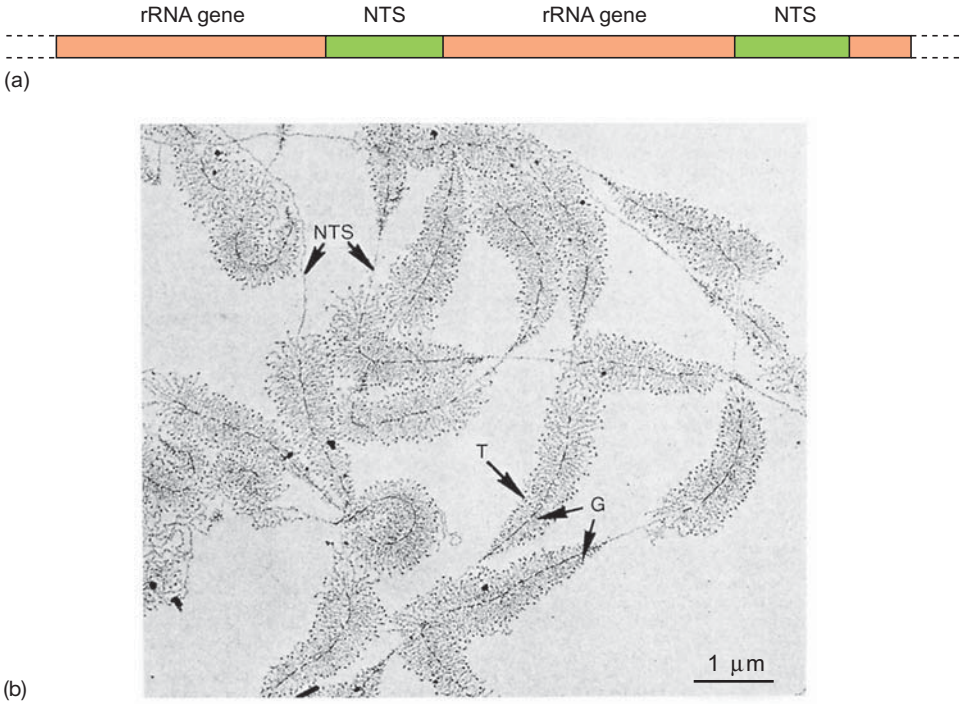


Figure 16.1 Transcription of rRNA precursor genes. (a) Map of a portion of the newt (amphibian) rRNA precursor gene cluster, showing the alternating rRNA genes (orange) and nontranscribed spacers (NTS, green). **(b)** Electron micrograph of part of a newt nucleolus, showing rRNA precursor transcripts (T) being synthesized in a "tree" pattern on the tandemly duplicated rRNA precursor genes (G). At the base of each transcript is an RNA polymerase I, not visible in this picture. The genes are separated by nontranscribed spacer DNA (NTS). (*Source:* (b) O.L. Miller, Jr., B.R. Beatty, B.A. Hamkalo, and C.A. Thomas, Electron microscopic visualization of transcription. *Cold Spring Harbor. Symposia on Quantitative Biology* 35 (1970) p. 506.)

RNA sequences. These excess regions must also be removed. This trimming of excess regions from an RNA precursor is another kind of **processing.** It is similar to splicing in that unnecessary RNA is removed, but it differs from splicing in that no RNAs are stitched together.

For example, mammalian RNA polymerase I makes a **45S rRNA precursor,** which contains the **28S, 18S,** and **5.8S rRNAs,** embedded between transcribed spacer RNA regions. The processing of the precursor (Figure 16.2) takes place in the **nucleolus,** the nuclear compartment where rRNAs are made and ribosomes are assembled. The first step is to cut off the spacer at the 5′-end, leaving a 41S intermediate. The next step involves cleaving the 41S RNA into two pieces, 32S and 20S, that contain the 28S and 18S sequences, respectively. The 32S precursor also retains the 5.8S sequence. Finally, the 32S intermediate is split to yield the mature 28S and 5.8S RNAs, which base-pair with each other, and the 20S intermediate is trimmed to mature 18S size.

What is the evidence for this sequence of events? As long ago as 1964, Robert Perry used a **pulse-chase** procedure to establish a precursor–product relationship between the 45S precursor and the 18S and 28S mature rRNAs. He labeled mouse L cells for a short time (a short pulse) with [^{3}H]uridine and found that the labeled RNA sedimented as a broad peak centered at about 45S. Then he "chased" the label in this RNA into 18S and 28S rRNAs. That is, he added excess unlabeled uridine to dilute the labeled nucleoside and observed that the amount of label in the 45S precursor decreased as the amount of label in the mature 18S and 28S rRNAs increased. This suggested that one or more RNA species in the 45S peak was a precursor to 18S and 28S rRNAs. In 1970, Robert Weinberg and Sheldon Penman found the key intermediates by labeling poliovirus-infected HeLa cells with [^{3}H]methionine and [^{32}P]phosphate and separating the labeled RNAs by gel electrophoresis. Ordinarily, processing intermediates are too short-lived to accumulate to detectable levels, but poliovirus infection slowed processing down enough that the intermediates could be seen. The major species observed were 45S, 41S, 32S, 28S, 20S, and 18S (Figure 16.3). Dual labeling was possible because rRNA precursors in eukaryotes are methylated.

In 1973, Peter Wellauer and Igor Dawid visualized the precursor, intermediates, and products of human rRNA processing by electron microscopy. Each RNA species had its own capacity for intramolecular base pairing, so each had its own secondary structure. Once David and Wellauer

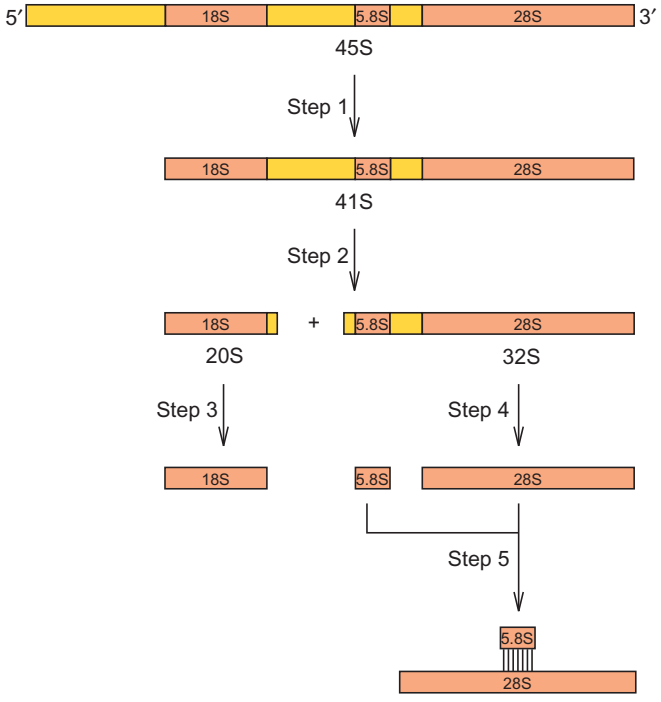


Figure 16.2 Processing scheme of 45S human (HeLa) rRNA precursor. Step 1: The 5′-end of the 45S precursor RNA is removed, yielding the 41S precursor. Step 2: The 41S precursor is cut into two parts, the 20S precursor of the 18S rRNA, and the 32S precursor of the 5.8S and 28S rRNAs. Step 3: The 3′-end of the 20S precursor is removed, yielding the mature 18S rRNA. Step 4: The 32S precursor is cut to liberate the 5.8S and 28S rRNAs. Step 5: The 5.8S and 28S rRNAs associate by base-pairing.

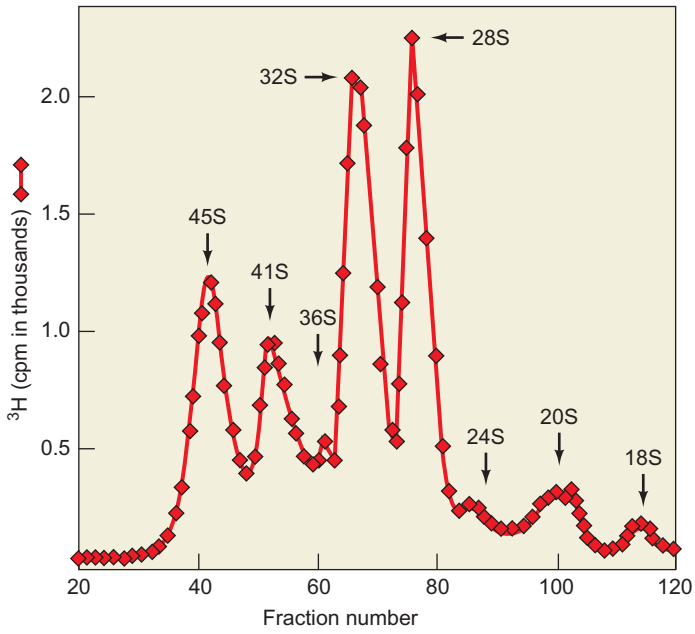


Figure 16.3 Isolation of 45S rRNA-processing intermediates from poliovirus-infected HeLa cells. Penman and colleagues labeled RNA in virus-infected cells with [^{3}H]methionine, which labeled the many methyl groups in rRNAs and their precursors. They isolated nucleolar RNA (mostly rRNA) from these cells, subjected it to gel electrophoresis, sliced the gel, determined the radioactivity in each slice, then plotted these radioactivity values in cpm versus slice, or fraction number. The mobilities of the RNA species were compared with those of markers of known sedimentation coefficients. (*Source:* Adapted from Weinberg, R.A. and S. Penman, Processing of 45S nucleolar RNA. *Journal of Molecular Biology* 47:169 (1970).)

had identified these "signatures" of all the RNA species, they could recognize them in the 45S precursor and thereby locate the 28S and 18S species in the precursor. Although they originally got the order backwards, we now know that the arrangement is: 5′-18S-5.8S-28S-3′. The details of this processing scheme are not universal; even the mouse does things a little differently, and the frog precursor is only 40S, which is quite a bit smaller than 45S. Still, the basic mechanism of rRNA processing, including the order of mature sequences in the precursor, is preserved throughout the eukaryotic kingdom.

The rRNA-processing steps are orchestrated in the nucleolus by a class of **small nucleolar RNAs (snoRNAs),** associated with proteins in **small nucleolar ribonucleoproteins, (snoRNPs).** There are many hundreds of snoRNPs, and quite a few of them participate in rRNA processing by modifying nucleotides within the rRNA precursor. The rRNA precursor contains about 110 2′-*O*-methyl groups and about 100 pseudouridines. (In pseudouridine, the ribose joins to the 5-carbon of the uracil, rather than the 1-nitrogen; Chapter 19). Because these modified nucleotides persist in the mature rRNAs, it appears that they help define what regions of the precursor to remove and what regions to preserve. The RNA parts (**guide snoRNAs**) of the snoRNPs base-pair to specific sites within the rRNA precursor and dictate either methylation or pseudouridylation at those sites.

SUMMARY Ribosomal RNAs are made in eukaryotic nucleoli as precursors that must be processed to release the mature rRNAs. The order of RNAs in the precursor is 18S, 5.8S, 28S in all eukaryotes, although the exact sizes of the mature rRNAs vary from one species to another. In human cells, the precursor is 45S, and the processing scheme creates 41S, 32S, and 20S intermediates. snoRNPs play vital roles in these processing steps by methylating and pseudouridylating specific sites within the rRNA precursor.

Bacterial rRNA Processing

The bacterium *E. coli* has seven *rrn* operons that contain rRNA genes. Figure 16.4a presents an example, *rrnD,* which has three tRNA genes in addition to the three rRNA genes. Transcription of the operon yields a 30S precursor, which must be cut up to release the three rRNAs and three tRNAs.

RNase III is the enzyme that performs at least the initial cleavages that separate the individual large rRNAs. One type of evidence leading to this conclusion is genetic: A mutant with a defective RNase III gene accumulates 30S rRNA precursors. In 1980, Joan Steitz and her colleagues compared the sequences of the spacers between the rRNAs

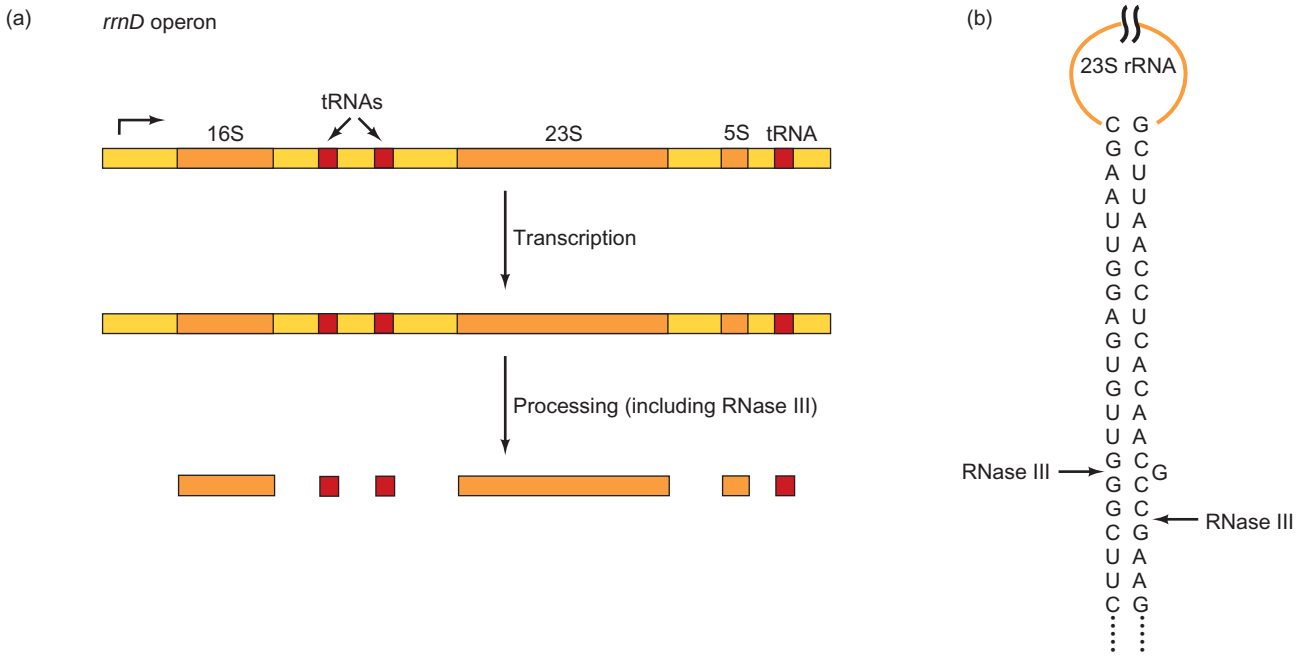


Figure 16.4 Processing bacterial rRNA precursors. (a) Structure of the *E. coli rrnD* operon. This operon is typical of the rRNA-encoding operons of *E. coli* in that it includes regions that code for tRNAs (red), as well as rRNA-coding regions (orange), embedded in transcribed spacers (yellow). As usual with bacterial operons, this one is transcribed to produce a long composite RNA. This RNA is then processed by enzymes, including RNase III, to yield mature products. **(b)** Sequence analysis has shown that the spacers surrounding the 23S rRNA gene are complementary, so they can form an extended hairpin with the 23S rRNA region at the top. The observed cleavage sites for RNase III are in the stem, offset by 2 bp. The regions surrounding the 16S rRNA gene can also form a hairpin stem, with a somewhat more complex structure.

in two different precursors (from the *rrnX* and *rrnD* operons) and found considerable similarity. These sequences revealed complementary sequences flanking both 16S and 23S rRNA regions of the precursors. This complementarity predicts two extended hairpins (Figure 16.4b) involving stems created by base pairing between two spacers, with the rRNA regions looping out in between. The RNase III cleavage sites in this model are in the stems. Another ribonuclease, **RNase E,** is responsible for removing the 5S rRNA from the precursor.

SUMMARY Bacterial rRNA precursors contain tRNAs as well as all three rRNAs. The rRNAs are released from their precursors by RNase III and RNase E.

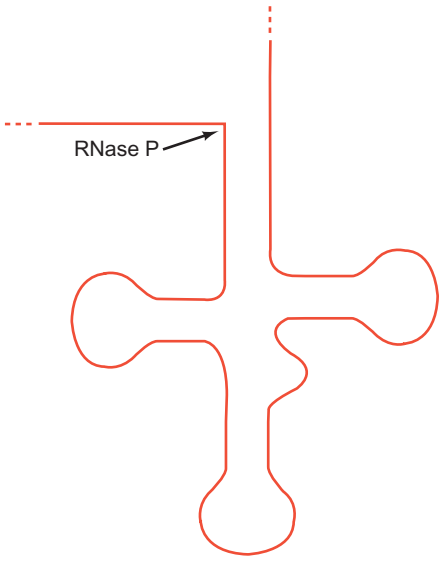


Figure 16.5 RNase P action. RNase P makes a cut at the site that will become the mature 5′-end of a tRNA. Thus, this enzyme is all that is needed to form mature 5′-ends.

16.2 Transfer RNA Processing

Transfer RNAs are made in all cells as overly long precursors that must be processed by removing RNA at both ends. In the nuclei of eukaryotes, these precursors contain a single tRNA; in bacteria, a precursor may contain one or more tRNAs, and sometimes a mixture of rRNAs and tRNAs, as we saw in Figure 16.4. Because the tRNA processing schemes in eukaryotes and bacteria are so similar, we will consider them together.

Cutting Apart Polycistronic Precursors

The first step in processing bacterial RNAs that contain more than one tRNA is to cut the precursor up into fragments with just one tRNA each. This means cutting between tRNAs in precursors that have two or more tRNAs, or cutting between tRNAs and rRNAs in precursors, such as the one in Figure 16.4, that have both tRNAs and rRNAs. The enzyme that performs both these chores seems to be RNase III.

Forming Mature 5′-Ends

After RNase III has cut the tRNA precursor into pieces, the tRNA still contains extra nucleotides at both 5′- and 3′-ends. As such, it resembles the primary transcripts of eukaryotic tRNA genes, which are monocistronic (single-gene) precursors with extended 5′- and 3′-ends. Maturation of the 5′-end of a bacterial or eukaryotic tRNA involves a single cut just at the point that will be the 5′-end of the mature tRNA, as shown in Figure 16.5. The enzyme that catalyzes this cleavage is **RNase P.**

RNase P from both bacteria and eukaryotic nuclei is a fascinating enzyme. It contains two subunits, but unlike other dimeric enzymes we have studied, one of these subunits is made of RNA, not protein. In fact, the majority of the enzyme is RNA because the RNA (the **M1 RNA**) has a molecular mass of about 125 kD, and the protein has a mass of only about 14 kD. When Sidney Altman and his colleagues first isolated this enzyme and discovered that it is a ribonucleoprotein, they faced a critical question: Which part has the catalytic activity, the RNA or the protein? The heavy betting at that time was on the protein because all enzymes that had ever been studied were made of protein, not RNA. In fact, early studies on RNase P showed that the enzyme lost all activity when the RNA and protein parts were separated.

Then, in 1982, Thomas Cech and colleagues found autocatalytic activity in a self-splicing intron (Chapter 14). Shortly thereafter, Altman and Norman Pace and their colleagues demonstrated the catalytic activity of the M1 part of RNase P in 1983. As Figure 16.6 illustrates, the trick was magnesium concentration. The early studies had been performed with 5–10 mM Mg^{2+}, under these conditions, both the protein and RNA parts of RNase P are required for activity. Figure 16.6 shows the effect of Mg^{2+} concentration over the range 5 mM to 50 mM using M1 RNA alone. Altman, Pace, and colleagues used two different substrates: pre-$tRNA^{Tyr}$ and pre-4.5S RNA from *E. coli*. Figure 16.6, lanes 1–3 show the differences among 5, 10 and 20 mM Mg^{2+}, respectively. At 5 mM Mg^{2+}, neither substrate showed any maturation by cleavage of the extra nucleotides from the 5′-end. Even at 10 mM Mg^{2+}, the cleavage of pre-tRNA was barely detectable. By contrast, at 20 mM Mg^{2+}, approximately half the pre-tRNA was cleaved to mature form, releasing the extra nucleotides as a single fragment, labeled "5′-Tyr" in the figure. Increasing the Mg^{2+} concentration to 30, 40, and 50 mM

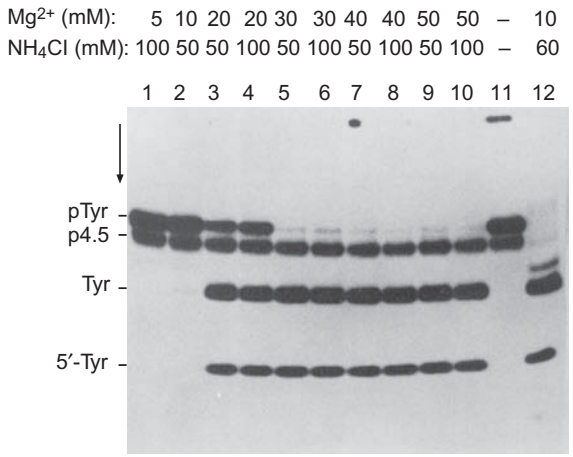


Figure 16.6 The M1 RNA of *E. coli* RNase P has enzymatic activity. Altman and Pace and colleagues purified the M1 RNA from RNase P and incubated it with ^{32}P-labeled pre-tRNA^{Tyr} (pTyr) and p4.5S RNA from *E. coli* (p4.5) for 15 min at the Mg^{2+} and NH_4Cl concentrations indicated at top. Then they electrophoresed the RNAs and visualized them by autoradiography. Lane 11, no additions; lane 12, crude *E. coli* RNase P. At the higher Mg^{2+} concentrations, the M1 RNA by itself cleaved the pTyr to form mature 5′-ends, but had no effect on the p4.5 substrate under any of the conditions used. (*Source:* Guerrier-Takada, C., K. Gardiner, T. Marsh, N. Pace, and S. Altman, The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 35 (Dec 1983) p. 851, f. 4A. Reprinted by permission of Elsevier Science.)

Mg^{2+} (lanes 5, 7, and 9, respectively) further enhanced 5′-processing of the pre-tRNA, but did not cause any pre-4.5S processing. Lane 12 demonstrates that crude RNase P (the dimeric form of the enzyme that contains both the RNA and protein subunits) can cleave both substrates at 10 mM Mg^{2+}.

Eukaryotic nuclear RNase P is very much like the bacterial enzyme. For example, the yeast nuclear RNase P contains a protein and an RNA part, and the RNA has the catalytic activity. However, Peter Gegenheimer and his colleagues, in papers beginning in 1988, showed that spinach chloroplast RNase P appears not to have an RNA at all. This enzyme is not inhibited by micrococcal nuclease, as it should be if it contains a catalytic RNA, and it has the density expected of pure protein, not a ribonucleoprotein that is mostly RNA. In 2008, Walter Rossmanith demonstrated that human mitochondrial RNase P also lacks an RNA component.

The archaeon *Nanoarchaeum equitans* gets along without RNase P. It synthesizes its tRNAs without 5′-leaders, so no RNase P is required to remove them.

SUMMARY Extra nucleotides are removed from the 5′-ends of pre-tRNAs in one step by an endonucleolytic cleavage catalyzed by RNase P. RNase P's from bacteria and eukaryotic nuclei have a catalytic RNA subunit called M1 RNA. Spinach chloroplast RNase P appears to lack an RNA subunit.

Forming Mature 3′-Ends

Transfer RNA 3′-end maturation is considerably more complex than 5′-maturation because not one, but six RNases take part. Murray Deutscher and other investigators have shown that the following RNases can remove nucleotides from the 3′-ends of tRNAs in vitro: *RNase D, RNase BN, RNase T, RNase PH, RNase II,* and *polynucleotide phosphorylase (PNPase)*. Genetic experiments by Deutscher and colleagues have also demonstrated that each of these enzymes is necessary for the most efficient 3′-end processing. If the genes encoding any of these enzymes were inactivated, the efficiency of tRNA processing suffered. Inactivation of all of the genes at once was lethal to bacterial cells. On the other hand, the presence of any one of the enzymes was sufficient to ensure viability and tRNA maturation, although the efficiency varied depending on the active RNase.

A combination of genetic and biochemical experiments has shown that RNase II and PNPase cooperate to remove the bulk of the 3′-trailer from pre-tRNA. This opens the way for RNases PH and T to complete the job by removing the last two nucleotides. RNase T is the most active in removing the last nucleotide.

The situation in eukaryotes seems a bit simpler. A single enzyme, **tRNA 3′-processing endoribonuclease (3′-tRNase)** cleaves the excess nucleotides from the 3′-end of a tRNA precursor. In 2003, Masayuki Nashimoto and colleagues purified a 3′-tRNase from pig liver. Comparison of a partial sequence of the purified protein to the human genomic database revealed a close similarity to a poorly characterized human protein (**ELAC2**), mutations in which are risk factors for prostate cancer. Nashimoto and colleagues cloned and expressed the human ELAC2 gene in bacteria and tested the protein product for 3′-tRNase activity in vitro. It was able to efficiently remove the excess nucleotides from the end of human tRNA^{Arg}, showing that ELAC2 is at least one of the 3′-tRNase enzymes in humans.

SUMMARY RNase II and polynucleotide phosphorylase cooperate to remove most of the extra nucleotides at the end of an *E. coli* tRNA precursor, but stop at the +2 stage, with two extra nucleotides remaining. RNases PH and T are most active in removing the last two nucleotides from the RNA, with RNase T being the major participant in removing the very last nucleotide. In eukaryotes, a single enzyme, tRNA 3′-processing endoribonuclease (3′-tRNase), processes the 3′-end of a pre-tRNA.

16.3 *Trans*-Splicing

In Chapter 14 we considered the sort of splicing that occurs in almost all eukaryotic species. This splicing can be called ***cis*-splicing,** because it involves two or more exons that exist together in the same gene. As unlikely as it may seem, in another alternative, ***trans*-splicing,** the exons are not part of the same gene at all and may not even be found on the same chromosome.

The Mechanism of *Trans*-Splicing

Trans-splicing occurs in several oganisms, including parasitic and free-living worms (e.g., *Caenorhabditis elegans*), but it was first discovered in **trypanosomes,** a group of parasitic flagellated protozoa, one species of which causes African sleeping sickness. The genes of trypanosomes are expressed in a manner we would never have predicted based on what we have discussed in this book so far. Piet Borst and his colleagues laid the groundwork for these surprising discoveries in 1982 when they sequenced the 5′-end of an mRNA encoding a trypanosome surface coat protein and the 5′-end of the gene that encodes this same protein and discovered that they did not match. The mRNA had 35 extra nucleotides that were missing from the gene. As molecular biologists sequenced more and more trypanosome mRNAs, they discovered that they all had the same 35-nt leader, called the **spliced leader (SL),** but none of the genes encoded the SL. Instead, the SL is encoded by a separate gene that is repeated about 200 times in the trypanosome genome. This gene encodes only the SL, plus a 100-nt sequence that is joined to the leader through a consensus 5′-splice sequence. Thus, this minigene is composed of a short SL exon, followed by what looks like the 5′-part of an intron.

How can we explain the production of an mRNA derived from two widely separated DNA regions that are sometimes even found on separate chromosomes? Two classes of explanations are plausible. First (Figure 16.7a), the SL (with or without its intron) could be transcribed, and this transcript could then serve as a primer for transcription of any one of the coding regions elsewhere in the genome. Alternatively (Figure 16.7b), RNA polymerases could transcribe an SL and a coding region separately, and these two independent transcripts could then be spliced together.

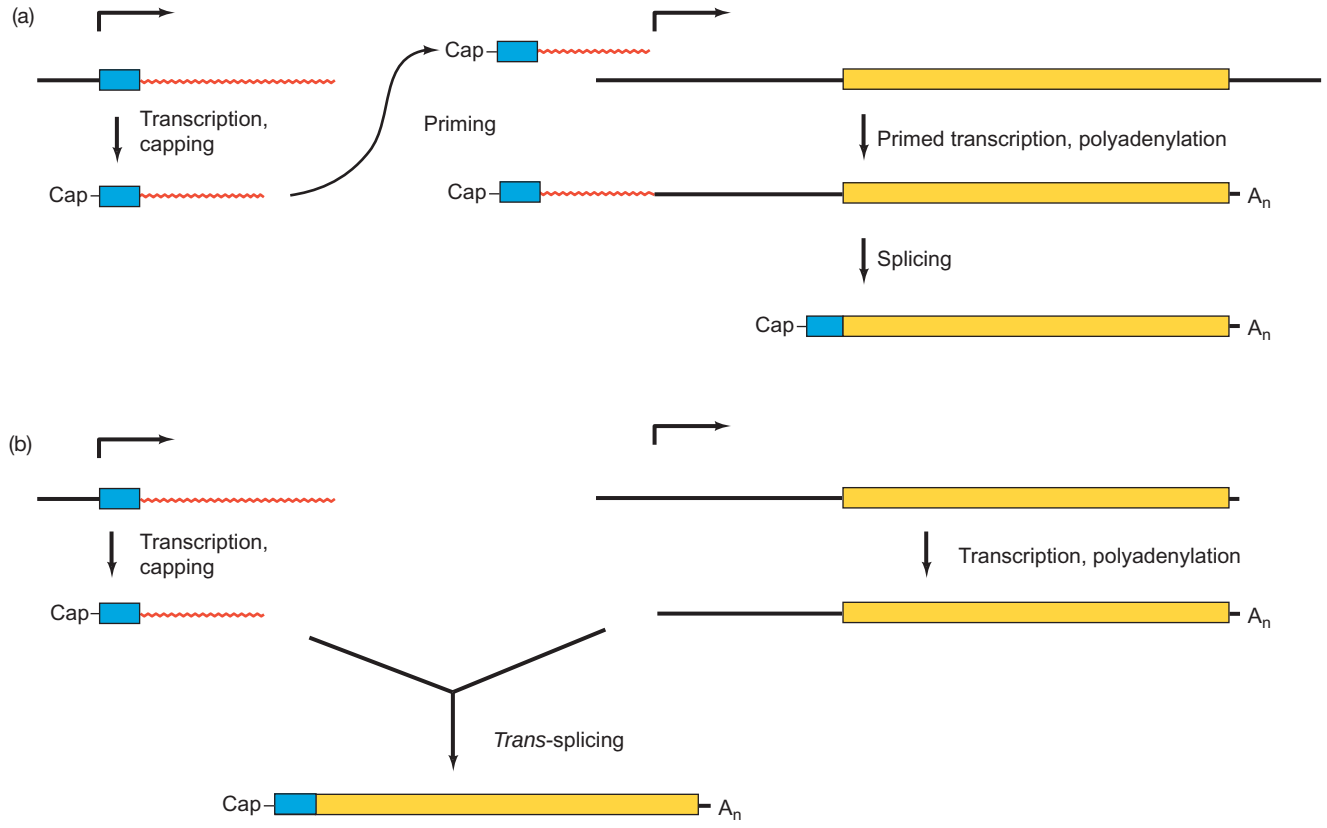


Figure 16.7 Two hypotheses for joining the SL to the coding region of an mRNA. (a) Priming by the SL intron. The SL (blue), with its attached half-intron (red), is transcribed to yield a 135-nt RNA. This RNA then serves as a primer for transcription of a coding region (yellow), including its attached half-intron (black). This produces a transcript including the SL plus the coding region, with a whole intron in between. The intron can then be spliced out to yield the mature mRNA. **(b)** *Trans*-splicing. The SL with its attached half-intron is transcribed; independently, the coding region with its half-intron is transcribed. Then these two separate RNAs undergo *trans*-splicing to produce the mature mRNA.

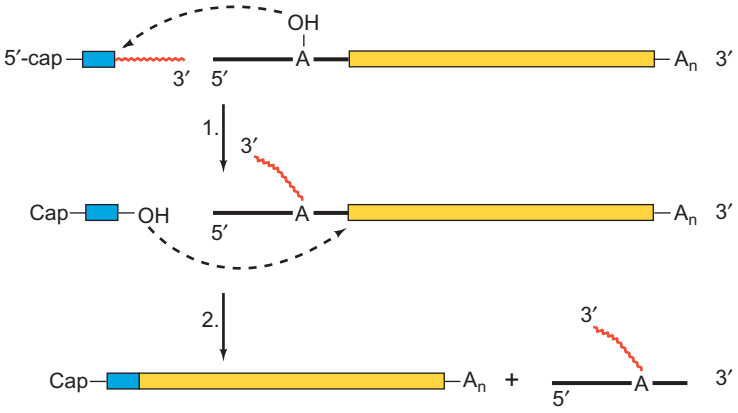


Figure 16.8 Detailed *trans*-splicing scheme for a trypanosome mRNA. Step 1: The branchpoint adenosine within the half-intron (black) attached to the coding exon (yellow) attacks the junction between the leader exon (blue) and its half-intron (red). This creates a Y-shaped intron–exon intermediate analogous to the lariat intermediate created by *cis*-splicing. Step 2: The leader exon attacks the splice site between the branched intron and the coding exon. This produces the spliced, mature mRNA plus the Y-shaped intron.

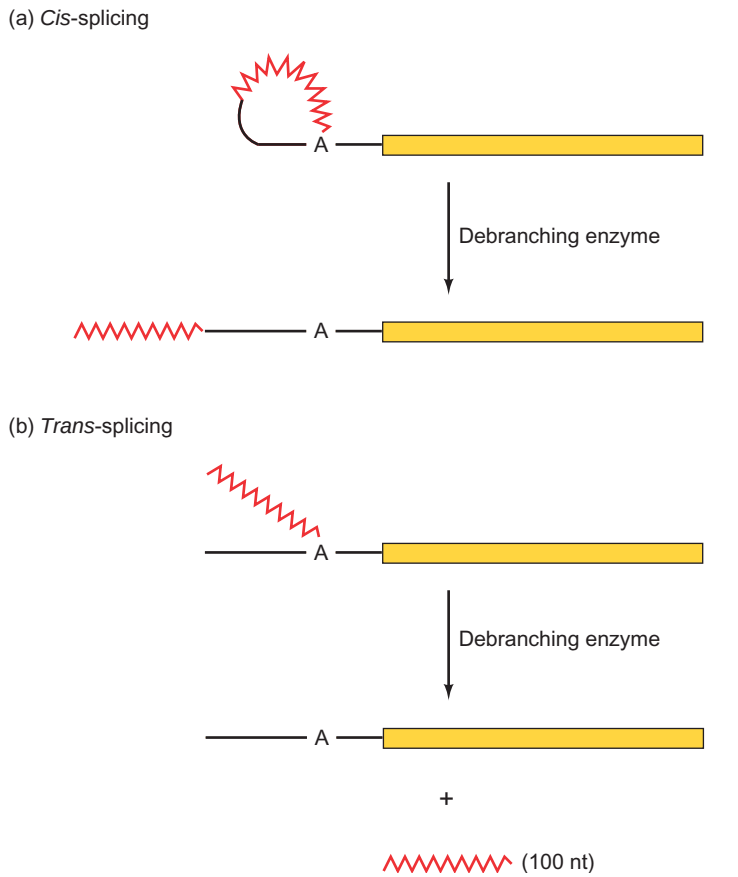


Figure 16.9 Treating hypothetical splicing intermediates with debranching enzyme. (a) *Cis*-splicing. The debranching enzyme simply opens the lariat up to a linear form. **(b)** *Trans*-splicing. Because the 100-nt half-intron (red) is open at its 3′-end instead of being involved in a lariat, debranching enzyme releases it as an independent RNA.

If such *trans*-splicing really occurs, then we would not expect to see lariat-shaped intermediates. Instead, we should find Y-shaped intermediates that form when the branchpoint in the intron attacks the 5′-end of the intron attached to the short leader exon, as illustrated in Figure 16.8. Finding the Y-shaped intermediate would go a long way toward proving that *trans*-splicing really takes place. Nina Agabian and colleagues reported evidence for the intermediate in 1986.

The unique feature of the Y-shaped structure, which distinguishes it from a normal, lariat intermediate, is that the 3′-end of the SL intron in the Y-shaped structure is free (see Figure 16.8). This means that treatment of the Y-shaped splicing intermediate with debranching enzyme, which breaks the 2′–5′-phosphodiester bond at the branchpoint, should yield a 100-nt fragment as a by-product (Figure 16.9). This contrasts with the results we expect from a lariat-shaped intermediate, which would simply be linearized. Figure 16.10 shows the results of a Northern blot of total RNA and poly(A)$^+$ RNA after treatment with debranching enzyme probed with an oligonucleotide specific for the 100-nt fragment. In both cases, the expected 100-nt fragment appeared, thus corroborating the *trans*-splicing hypothesis.

Trans-splicing is very widespread in some organisms. In *C. elegans*, for example, all or nearly all mRNAs are *trans*-spliced to a small group of spliced leaders. And more than 15% of these *trans*-spliced mRNAs are encoded in groups of two to eight genes that can be considered a kind of operon. Such a group of genes resembles a prokaryotic operon in that they belong to a transcription unit controlled by a single

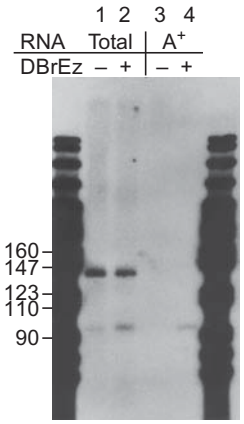


Figure 16.10 Release of the SL half-intron from a larger RNA by debranching enzyme. Agabian and colleagues labeled trypanosome RNA with ^{32}P and treated total RNA, or poly(A)$^+$ RNA, with debranching enzyme (DBrEz) as indicated at top. Then they electrophoresed the products, blotted them, and probed the blot with an oligonucleotide specific for the 100-nt SL half-intron, which is clearly detectable in both enzyme-treated RNA samples. (*Source:* Murphy W.J., K.P. Watkins, and N. Agabian, Identification of a novel Y branch structure as an intermediate in trypanosome mRNA processing. Evidence of *trans*-splicing. *Cell* 47 (21 Nov 1986) p. 521, f. 5. Reprinted by permission of Elsevier Science.)

promoter. But it differs from a true operon in that the primary transcript is ultimately broken into pieces by *trans*-splicing, with each coding region being supplied with its own spliced leader. Indeed, *trans*-splicing makes such eukaryotic "operons" possible by providing each of the internal coding regions with its own cap. Otherwise, only the first coding region would receive a cap upon transcription, and therefore would be the only one to be efficiently translated. This is not a problem in bacteria, which have unique translation start sites for each gene within a polycistronic mRNA (Chapter 7), but it would be in eukaryotes, whose mRNAs generally do not have internal translation start sites and instead depend on caps to recruit ribosomes (Chapter 17).

SUMMARY Trypanosome mRNAs are formed by *trans*-splicing between a short leader exon and any one of many independent coding exons. *Trans*-splicing is common in organisms such as *C. elegans,* in which polycistronic pre-mRNAs are broken up into their individual gene transcripts by *trans*-splicing each of those parts of the pre-mRNA to a common spliced leader.

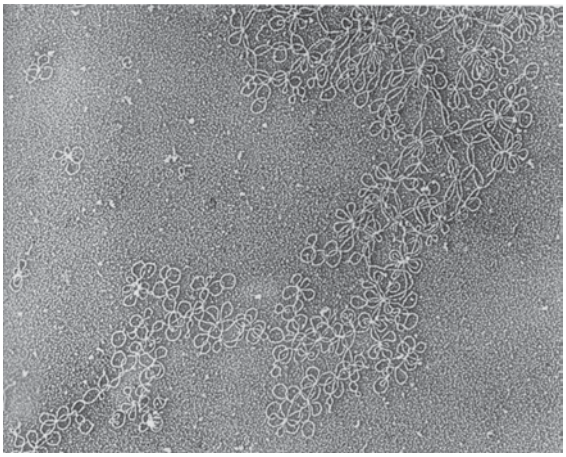


Figure 16.11 Part of the network of kinetoplast minicircles and maxicircles from *Leishmania tarentolae*. (Source: Cell 61 (1 June 1990) cover (acc. Sturm & Simpson, pp. 871–84). Reprinted by permission of Elsevier Science.)

COX II DNA: ···GTATAAAAGTAGA G A ACCTGG···

COX II RNA: ···GUAUAAAAGUAGAUUGUAUACCUGG···

Figure 16.12 Comparison of the sequence of part of the *COX II* gene of a trypanosome with its mRNA product. Four U's in the mRNA are not represented by T's in the gene. These four U's are presumably added to the RNA by editing.

16.4 RNA Editing

Trans-splicing is not the only bizarre occurrence in trypanosomatids. These organisms also have unusual mitochondria called **kinetoplasts,** which contain two types of circular DNA linked together into large networks (Figure 16.11). There are 25–50 identical **maxicircles,** 20–40 kb in size, which contain the mitochondrial genes, and about 10,000 1–3-kb **minicircles,** which have a role in mitochondrial gene expression. In 1986, Rob Benne and his colleagues discovered that the sequence of the cytochrome oxidase (*COX II*) mRNA from trypanosomes does not match the sequence of the *COX II* gene; the mRNA contains four nucleotides that are missing from the gene (Figure 16.12). Furthermore, these missing nucleotides cause a frameshift (a shift in the frame in which a ribosome reads the mRNA; see Chapter 18) that should seemingly inactivate the gene. But somehow the mRNA has been supplied with these four nucleotides, averting the frameshift.

Of course, one possibility is that the gene Benne and colleagues sequenced did not actually code for the mRNA, but was a **pseudogene,** a duplicate copy of a gene that has been mutated so it does not function and is no longer used. The active gene could reside elsewhere, and these workers could have missed it. The problem with this explanation is that, try as they might, Benne and his coworkers could find no other *COX II* gene in either the kinetoplast or the nucleus. Furthermore, they found the same missing nucleotides in the *COX II* genes of two other trypanosomatids. For these and other reasons, Benne and coworkers concluded that the mRNAs of trypanosomatids are copied from incomplete genes called **cryptogenes** and then edited by adding the missing nucleotides, which are all UMPs.

By 1988, a number of trypanosomatid kinetoplast genes and corresponding mRNAs had been sequenced, revealing editing as a common phenomenon in these organisms. In fact, some RNAs are very extensively edited (**panedited**). For example, a 731-nt stretch of the *COIII* mRNA of *Trypanosoma brucei* contains 407 UMPs added by editing; editing also deletes 19 encoded UMPs from this stretch of the *COIII* mRNA. Part of this sequence is presented in Figure 16.13.

Mechanism of Editing

We have been assuming that editing is a posttranscriptional event. This seems like a good bet because unedited transcripts can be found along with edited versions of the same mRNAs. Moreover, editing occurs in the poly(A) tails of mRNAs, which are added posttranscriptionally.

One important clue about the mechanism of editing is that partially edited transcripts have been isolated, and these are always edited at their 3′-ends but not at their 5′-ends. This suggests strongly that editing proceeds in a 3′→5′ direction. Kenneth Stuart and colleagues first

Figure 16.13 Part of the edited sequence of the *COIII* mRNA of *T. brucei*. The U's added by editing are shown in gray; the T's present in the gene, but absent (as U's) in the mRNA are shown in blue above the sequence. (*Source:* Adapted from *Cell* 53:cover, 1988.)

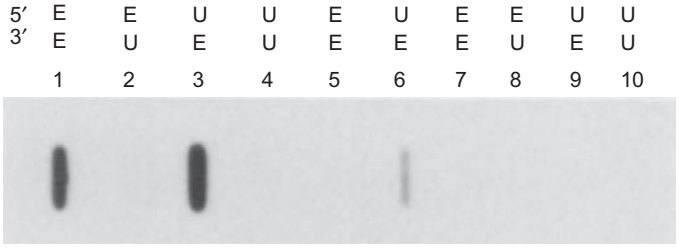


Figure 16.14 PCR analysis of direction of editing. Stuart and colleagues performed RT-PCR with kinetoplast RNA and edited (E) or unedited (U) 5′- and 3′-primers for the cytochrome c oxidase III transcript, as indicated at top. Then they slot-blotted the PCR products and hybridized them to a labeled probe and detected hybridization by autoradiography. PCR templates: lanes 1–4, RNA from wild-type cells; lanes 5–6, a 3′-edited cDNA (positive control); lanes 7–10, RNA from a mutant that lacks mitochondrial DNA (negative control). (*Source:* Abraham, J.M., J.E. Feagin, and K. Stuart, Characterization of cytochrome c oxidase III transcripts that are edited only in the 39 region. Cell 55 (21 Oct 1988) p. 269, f. 2a. Reprinted by permission of Elsevier Science.)

reported this phenomenon in 1988. Their experimental tool was RT-PCR, starting with reverse transcriptase to make the first DNA strand from an RNA template, followed by standard PCR (see Chapter 4).

In one experiment, Stuart and coworkers used pairs of PCR primers in which both were edited primers, both unedited primers, or one of each. A completely edited RNA will hybridize only to edited primers and give a PCR signal, whereas it will not hybridize to unedited primers, so any PCR protocol including at least one unedited primer will not give a signal from this RNA. By contrast, a completely unedited RNA will react only with unedited primers. But the real test is to use an unedited 5′-primer and an edited 3′-primer to detect 3′-edited transcripts, or an edited 5′-primer and an unedited 3′-primer to detect 5′-edited transcripts. If editing goes from 3′ to 5′ in the transcript, then 3′-edited transcripts, but not 5′-edited transcripts, should be detected. The advantage of the PCR method is that it amplifies very small amounts of RNA, such as partially edited RNAs, to easily detectable bands of DNA.

Figure 16.14 depicts the results of this analysis. Lanes 1–4 show the PCR products of *Trypanosoma brucei* kinetoplast RNA with different combinations of primers. We see signals only when both primers were edited, or the 3′-primer was edited. We see no signal when only the 5′-primer was edited. Thus, 3′-editing occurred in the absence of 5′-editing, but 5′-editing did not occur without 3′-editing. This is consistent with editing in the 3′→5′ direction. Lanes 5–6 and 7–10 are positive and negative controls, respectively.

This experiment is valuable, but it has a flaw: None of the lanes involving the unedited 3′-primer shows a signal. We might have expected to see a signal in lane 4, which used unedited 5′- and 3′-primers, but none was observed. This could (and probably does) mean that the concentration of totally unedited RNA is so small that it is undetectable using this method. But it could also mean that there is something wrong with the 3′-unedited primer. Thus, this experiment could have been improved by including a positive control for the 3′-unedited primer—some RNA, such as an in vitro transcript of the gene, which would be totally unedited and should therefore give a signal. If it did, it would remove any doubt about the quality of the 3′-unedited primer. Such controls are especially important in PCR experiments, which have enormous power to amplify tiny quantities of nucleic acids, including contaminants.

What determines where the editing system should add or delete UMPs? Larry Simpson and colleagues found the answer in 1990 when they discovered **guide RNAs** (**gRNAs**) encoded in *Leishmania* maxicircles. They began with a computer search of the 21-kb part of the maxicircle DNA sequence that was known at that time. This search revealed seven short sequences that could produce short RNAs (gRNAs) complementary to parts of five different edited mitochondrial mRNAs. In principle, such gRNAs could direct the insertion and deletion of UMPs over a stretch of several dozen nucleotides in the mRNA, as illustrated in Figure 16.15a and b. Once that editing is done, another gRNA could hybridize near the 5′-end of the newly edited region and direct editing of a new segment, as Figure 16.15c and d demonstrate. Working in this way from the 3′-end of the mRNA toward the 5′-end, successive gRNAs bind to regions edited by their predecessor gRNAs and direct further editing until they have finished the whole editing job. The sequences of the gRNAs reinforce the conclusion that editing proceeds in the 3′→5′direction: Only the gRNAs at

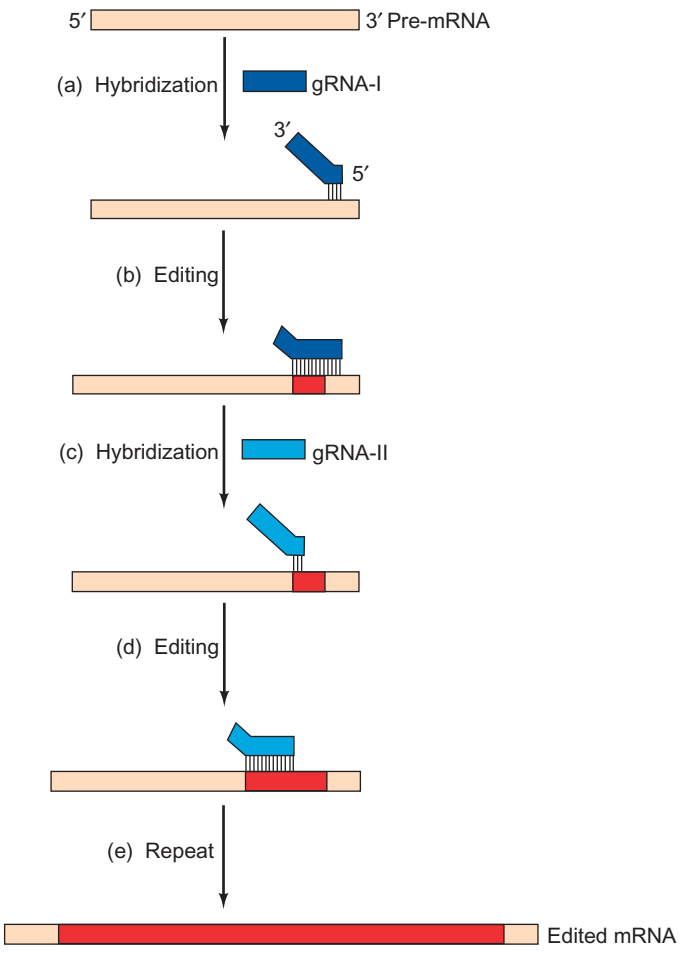


Figure 16.15 Model for the role of gRNAs in editing. (a) In the first step, gRNA-I (dark blue) hybridizes through its 5′-end to a region of the pre-mRNA that requires no editing. Its 3′-end also hybridizes through an oligo(U) region, but that is not illustrated here. **(b)** Most of the rest of the gRNA-I directs editing of part of the pre-mRNA. The edited portion is shown in red, and the pre-mRNA has grown in length, due to the inserted UMPs. **(c)** A new gRNA, gRNA-II (light blue), displaces gRNA-I by hybridizing to the 5′-end of the newly edited region of the pre-mRNA. **(d)** gRNA-II directs editing of a new part of the pre-mRNA. **(e)** The previous steps are repeated with additional gRNAs until the RNA is completely edited.

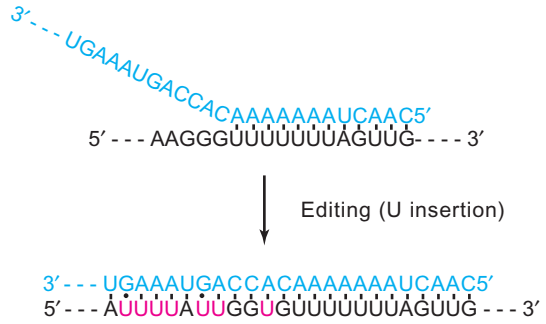


Figure 16.16 Editing of part of a hypothetical RNA. The gRNA (blue) binds via Watson–Crick base pairs to an edited portion of a pre-mRNA. The 3′-end of the gRNA then serves as the template for insertion of U residues (pink). Most of the base pairs between newly inserted U's and the gRNA are Watson–Crick A–U pairs, but two are wobble G–U pairs, denoted by dots.

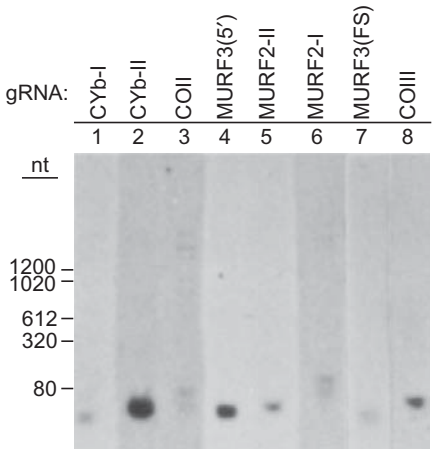


Figure 16.17 Evidence for gRNAs. Simpson and colleagues Northern blotted RNA from the mitochondria of *Leishmania tarentolae* and probed the blots with labeled oligonucleotides that would hybridize to gRNAs. The gRNAs are identified at top. (*Source:* Blum, B., N. Bakalara, and L. Simpson, A model for RNA editing in kinetoplastid mitochondria: "Guide" RNA molecules transcribed from maxicircle DNA provide the edited information. *Cell* 60 (26 Jan 1990) p. 191, f. 3a. Reprinted by permission of Elsevier Science.)

the 3′-border of editing can hybridize to unedited sequences. All the other gRNAs hybridize to edited sequences. This makes sense only if editing goes 3′→5′.

One notable feature of the base-pairing between gRNAs and mRNA is the existence of G–U base pairs, as well as standard Watson–Crick base pairs. In Chapter 18 we will learn that G–U base pairs are also common during codon–anticodon pairing in translation, and one of the two bases can accommodate these nonstandard base pairs by wobbling slightly from the position it would occupy in Watson–Crick base pairs. The importance of these G–U base pairs in editing probably derives from the fact that they are weaker than Watson–Crick base pairs. This means that the 5′-end of a new gRNA, by forming Watson–Crick base pairs with the newly edited region of an mRNA, can displace the 3′-end of the base-paired region of an old gRNA, whose base-pairing with the mRNA includes weak G–U pairs (Figure 16.16).

Later in 1990, Nancy Sturm and Larry Simpson found that minicircles also encode gRNAs. But besides the coding potential, Simpson and colleagues found direct evidence for the existence of gRNAs. They electrophoresed kinetoplastid RNA, Northern blotted it, and hybridized it to labeled oligonucleotide probes designed to detect gRNAs, according to the sequences of putative gRNA genes in maxicircles. Figure 16.17 shows that this procedure detected small RNAs, most of which appeared to be shorter than 80 nt.

The precise mechanism of editing, the cutting and pasting required to insert and delete UMPs, remained unclear

for several years, but the enzyme activities found in kinetoplasts provided some hints. For example, kinetoplasts have a **terminal uridylyl transferase** (**TUTase**) that could add extra UMPs (uridylates) to the mRNA during editing. Because the mRNA has to be cut to accept these new UMPs, it must also be ligated together again, and kinetoplasts also contain an **RNA ligase.** The major remaining question concerned the source of uridylates for editing. UTP could provide them. On the other hand, uridylates at the ends of gRNAs could be transferred to the pre-mRNA by transesterification. That is, the uridylates could be plucked off of the ends of gRNAs and transferred directly to the pre-mRNA.

Then, in 1994, Scott Seiwert and Stuart used a mitochondrial extract and a gRNA to edit a synthetic pre-mRNA. They found that deletion of UMPs required three enzymatic activities (Figure 16.18a): (1) an endonuclease that follows directions from the gRNA and cuts the pre-mRNA at the site where a UMP needs to be removed; (2) a 3′-exonuclease that is specific for terminal uridines; and (3) an RNA ligase. In 1996, using a similar in vitro system, Stuart and colleagues demonstrated that UMP insertion follows a similar three-step pathway (Figure 16.18b): (1) a gRNA-directed endonuclease cuts at the site where UMP insertion is required; (2) an enzyme (probably TUTase) transfers UMPs from UTP (not from gRNA), as directed by the gRNA; and (3) an RNA ligase puts the two pieces of RNA back together.

It is interesting that the gRNAs are encoded in the mitochondrial DNAs, while the proteins required for editing are encoded in the nucleus and imported into the mitochondria.

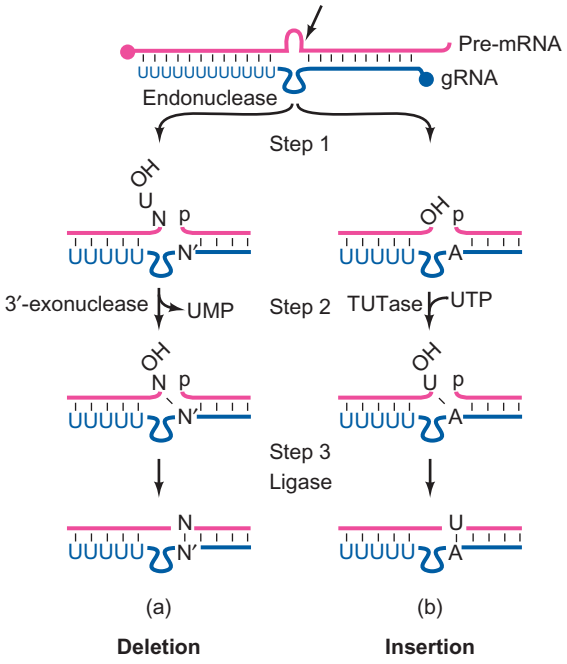


Figure 16.18 Mechanism of RNA editing. The mechanisms of **(a)** U deletion, and **(b)** U insertion are shown, starting with a hybrid between a pre-mRNA (pink) and a gRNA (dark blue) at top. The bulge in the gRNA denotes a stretch of bases that do not match those found in the pre-mRNA, and will be used as a template for editing. The arrow indicates the position at which the nuclease cuts the pre-mRNA for editing. **(a)** U deletion. Step 1: An endonuclease clips the pre-mRNA just to the 3′-side of the U to be deleted. Step 2: An exonuclease removes the UMP at the end of the left-hand RNA fragment. Base pairing occurs between base N in the pre-mRNA and base N′ in the gRNA. Step 3: RNA ligase puts the two halves of the pre-mRNA back together. **(b)** U insertion. Step 1: An endonuclease clips the pre-mRNA at the site where the gRNA dictates that a U should be inserted. Step 2: TUTase transfers a UMP from UTP to the 3′-end of the left-hand RNA fragment. This U base-pairs with an A in the gRNA. Step 3: RNA ligase puts the pre-mRNA back together. (*Source:* Adapted from Seiwert, S.D., Pharmacia Biotech in Science Prize. 1996 grand prize winner. RNA editing hints of a remarkable diversity in gene expression pathways. Science 274:1637, 1996.)

SUMMARY Trypanosomatid mitochondria encode incomplete mRNAs that must be edited before they can be translated. Editing occurs in the 3′→5′ direction by successive action of one or more guide RNAs. The 5′-end of the first gRNA hybridizes to an unedited region at the 3′-border of editing in the pre-mRNA; the 5′-ends of the rest of the gRNAs hybridize to edited regions progressively closer to the 5′-end of the region to be edited in the pre-mRNA. All of these gRNAs provide A's and G's as templates for the incorporation of U's missing from the mRNA. Sometimes the gRNA is missing an A or G to pair with a U in the mRNA, in which case the U is removed. The mechanism of removing U's involves: (1) cutting the pre-mRNA just beyond the U to be removed; (2) removal of the U by an exonuclease; and (3) ligating the two pieces of pre-mRNA together. The mechanism of adding U's uses the same first and last step, but the middle step (step 2) involves addition of one or more U's from UTP by TUTase instead of removing U's.

Editing by Nucleotide Deamination

RNA editing is not just something strange that happens in weird organisms, it also plays a vital role in higher organisms—even mammals. As yet, there has been no indication that mammals carry out the type of uridine addition and deletion that occurs in trypanosomes, but abundant evidence has been found for another kind of editing: deamination of adenosine, which converts adenosine to inosine, which has an oxygen in place of adenine's amino group. Because inosine forms base pairs with cytidine in the same way as guanosine, the deamination of adenosine changes the meaning of a codon. For example, an ACG (threonine) codon becomes an ICG codon, which would be read by the ribosome as GCG (alanine).

This kind of RNA editing is directed by an enzyme called **adenosine deaminase acting on RNA** (**ADAR**). Humans and mice contain three ADAR genes: *ADAR1*, *ADAR2,* and *ADAR3*. The products of the first two are ubiquitous in the body, but the third gene product is found only in the brain. These enzymes are very specific. It would be disastrous if they deaminated every adenosine in an mRNA, so they select only certain adenosines in certain mRNAs. For example, ADAR2 deaminates one adenosine in the glutamate-sensitive ion-channel receptor subunit B (GluR-B) mRNA, with greater than 99% efficiency. This alteration in the mRNA changes a glutamine codon to an arginine codon. Is this an important change? We know it is because an ion channel containing the GluR-B protein with a glutamine instead of an arginine is too permeable to calcium ions. We would therefore predict that mice with a defective *ADAR2* gene would have serious problems. Indeed, mice homozygous for a defective *ADAR2* gene do not carry out the appropriate *GluR-B* mRNA editing. They seem to develop normally, but die shortly after weaning.

Peter Seeburg and colleagues wondered what would happen if the mouse *GluR-B* gene were simply changed so that it encoded arginine at the edited position; then, no editing of this gene's transcript would be necessary. When they performed this experiment, they found that their mice were viable, even if they had a homozygous-defective *ADAR2* gene. Thus, this experiment also demonstrated that the only critical target of ADAR2 is the *GluR-B* transcript.

The *Drosophila* genome contains only one *ADAR* gene. When this gene is mutated so the flies lack all ADAR activity, they do not carry out any mRNA editing at known editing sites. These mutant flies are viable, but they have difficulty walking, cannot fly, and suffer progressive neural degeneration, particularly in the brain. Thus, the phenotype of this mutation is similar to the phenotype of mutations in the gene for ADAR2 in mammals. The *Drosophila* work bolsters the hypothesis that mRNA editing by ADAR is essential for normal central nervous system development.

ADAR1 also appears to be essential for mammalian life. Kazuko Nishikura and coworkers mutated mouse stem cells to heterozygous mutant ($ADAR1^{+/-}$), then injected these cells into normal mouse blastocysts in an attempt to create chimeric mice (see Chapter 5). But they found it impossible even to generate chimeric mice with a sizeable proportion of mutant cells. No embryo with more than a limited complement of mutant cells survived to birth. Thus, even heterozygous mutations in *ADAR1* appear to be embryonic lethal.

Why do embryos with a low ADAR1 activity die? Most tissues in the affected embryos appeared normal, but red blood cells (erythrocytes) did not. They remained nucleated, like erythrocytes derived from the yolk sac, long after erythropoiesis (creation of erythrocytes) would normally have shifted from the yolk sac to the liver, which generates erythrocytes that lose their nuclei. Thus, some aspect of erythropoiesis depends on a full complement of ADAR1 in the embryo.

Interestingly, certain tumors lose ADAR activity. In particular, a very malignant human brain tumor called glioblastoma multiforme (GBM) has very low ADAR2 activity, and a corresponding underediting in the *GluR-B* mRNA. Some epileptics also have this underedited mRNA, and GBM patients often are afflicted with epileptic seizures.

Another kind of editing is carried out by **cytidine deaminase acting on RNA** (**CDAR**), which converts cytidine to uridine. This C→U editing is defective in about 25% of the benign peripheral nerve sheath tumors found in neurofibromatosis type I patients. C→U editing also appears to occur in HIV transcripts in human cells. Still another kind of editing that occurs in HIV-infected human cells is G→A editing. But this kind of editing cannot be explained by a single-step deamination, and it is unclear how it is accomplished.

SUMMARY Some adenosines in mRNAs of higher eukaryotes, including fruit flies and mammals, must be deaminated to inosine posttranscriptionally for the mRNAs to code for the proper proteins. Enzymes known as adenosine deaminases active on RNAs (ADARs) carry out this kind of RNA editing. In addition, some cytidines must be deaminated to uridine for an mRNA to code properly.

16.5 Post-Transcriptional Control of Gene Expression: mRNA Stability

In our discussions of the mechanisms of prokaryotic and eukaryotic transcription, we saw many examples of transcriptional control. It makes sense to control gene expression by blocking the first step—transcription. That is the least wasteful method because the cell expends no energy making an mRNA for a protein that is not needed.

Although transcriptional control is the most prevalent form of control of gene expression, it is by no means the only way. We have already seen in Chapter 15 that poly (A) stabilizes and confers translatability on an mRNA, and special sequences in the 3′-untranslated region of an mRNA, called cytoplasmic polyadenylation elements (CPEs), govern the efficiency of polyadenylation of maternal messages during oocyte maturation. In this way, these CPEs serve as controllers of gene expression.

But an even more important posttranscriptional control of gene expression is control of mRNA stability. In fact, Joe Harford has pointed out that "cellular mRNA levels often correlate more closely with transcript stability than with transcription rate."

Casein mRNA Stability

The response of mammary gland tissue to the hormone prolactin provides a good example of control of mRNA stability. When cultured mammary gland tissue is stimulated with prolactin, it responds by producing the milk protein casein. One would expect an increase in casein mRNA concentration to accompany this casein buildup, and it does. The number of casein mRNA molecules increases about 20-fold in 24 h following the hormone treatment. But this does not mean the rate of casein mRNA synthesis has increased 20-fold. In fact it only increases about two- to threefold. The rest of the increase in casein mRNA level depends on an approximately 20-fold increase in stability of the casein mRNA.

Jeffrey Rosen and his colleagues performed a **pulse-chase** experiment to measure the **half-life** of casein mRNA. The half-life is the time it takes for half the RNA molecules to be degraded. Rosen and colleagues radioactively labeled casein mRNA for a short time in vivo in the presence or absence of prolactin. In other words, they gave the cells a pulse of radioactive nucleotides, which the cells incorporated into their RNAs. Then they transferred the cells to medium lacking radioactivity. This chased the radioactivity out of the RNA, as labeled RNAs broke down and were replaced by unlabeled ones. After various chase times, the experimenters measured the level of labeled casein mRNA by hybridizing it to a cloned casein gene. The faster the labeled casein mRNA disappeared, the shorter its half-life. The conclusion, shown in Table 16.1, was that the half-life of casein mRNA increased dramatically, from 1.1 h to 28.5 h, in the presence of prolactin. At the same time, the half-life of total polyadenylated mRNA increased only 1.3- to 4-fold in response to the hormone. It appears prolactin causes a selective stabilization of casein mRNA that is largely responsible for the enhanced expression of the casein gene. Note that pulse-chase experiments can do more than measure the half-life of a molecule. They can also show precursor-product relationships, as a labeled precursor is chased into labeled products. We saw a good example—rRNA precursor and products—earlier in this chapter.

Table 16.1 Effect of Prolactin on Half-Life of Casein mRNA

	RNA Half-life (h)	
Species of RNA	**− Prolactin**	**+ Prolactin**
rRNA	>790	>790
Poly(A)$^+$ RNA (short-lived)	3.3	12.8
Poly(A)$^+$ RNA (long-lived)	29	39
Casein mRNA	1.1	28.5

Source: Reprinted from Guyette, W.A., R.J. Matusik, and J.M. Rosen, Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression. *Cell* 17:1013, 1979. Copyright © 1979, with permission from Elsevier Science.

SUMMARY A common form of posttranscriptional control of gene expression is control of mRNA stability. For example, when mammary gland tissue is stimulated by prolactin, the synthesis of casein protein increases dramatically. However, most of this increase in casein is not due to an increase in the rate of transcription of the casein gene. Instead, it is caused by an increase in the half-life of casein mRNA.

Transferrin Receptor mRNA Stability

One of the best studied examples of posttranscriptional control concerns iron homeostasis (control of iron concentration) in mammalian cells. Iron is an essential mineral for all eukaryotic cells, yet it is toxic in high concentrations. Consequently, cells have to regulate the intracellular iron concentration carefully. Mammalian cells do this by regulating the amounts of two proteins: an iron import protein called the **transferrin receptor** (**TfR**), and an iron storage protein called **ferritin.** Transferrin is an iron-bearing protein that can get into a cell via the transferrin receptor on the cell surface. Once the cell imports transferrin, it passes the iron to cellular proteins, such as cytochromes, that need iron. Alternatively, if the cell receives too much iron, it stores the iron in the form of ferritin.

Thus, when a cell needs more iron, it increases the concentration of transferrin receptors to get more iron into the cell and decreases the concentration of ferritin, so not as much iron will be stored and more will be available. On the other hand, if a cell has too much iron, it decreases the concentration of transferrin receptors and increases the concentration of ferritin. It employs posttranscriptional strategies to do both these things: It regulates the rate of translation of ferritin mRNA, and it regulates the stability of the transferrin receptor mRNA. We will deal with the regulation of ferritin mRNA translation in Chapter 17. Here we are concerned with the latter process: controlling the stability of the mRNA encoding the transferrin receptor.

Joe Harford and his colleagues reported in 1986 that depleting intracellular iron by chelation resulted in an increase in transferrin receptor (TfR) mRNA concentration. On the other hand, increasing the intracellular iron concentration by adding hemin or iron salts decreased the TfR mRNA concentration. The changes in TfR mRNA concentrations with fluctuating intracellular iron concentration are not caused primarily by changes in the rate of synthesis of TfR mRNA. Instead, these alterations in TfR mRNA concentration largely depend on changes in the TfR mRNA half-life. In particular, the TfR mRNA half-life increases from about 45 min when iron is plentiful to many hours when iron is in short supply. We will examine the data on mRNA half-life but first we need to inspect the structure of the mRNA, which makes possible the modulation in its lifetime.

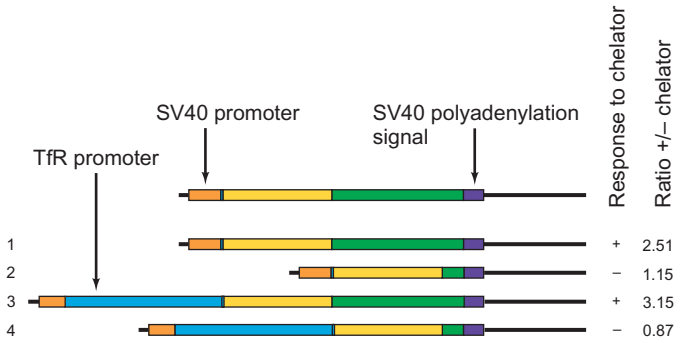


Figure 16.19 Effect of the 3′-UTR on the iron-responsiveness of cell surface concentration of TfR. Owen and Kühn made the TfR gene constructs diagrammed here. The DNA regions within the boxes are color-coded as follows: SV40 promoter, orange; TfR promoter, blue; TfR 5′-UTR, black; TfR-coding region, yellow; TfR 3′-UTR, green; SV40 polyadenylation signal, purple. These workers then transfected cells with each construct and assayed for concentration of TfR on the cell surface, using fluorescent antibodies. The ratio of cell surface TfR in the presence and absence of the iron chelator (desferrioxamine) is given at right, along with a qualitative index of response to chelator (+ or −). (*Source:* Adapted from Owen, D. and L.C. Kühn, Noncode 39 sequences of the transferrin receptor gene are required for mRNA regulation by iron. *The EMBO Journal* 6:1288, 1987.)

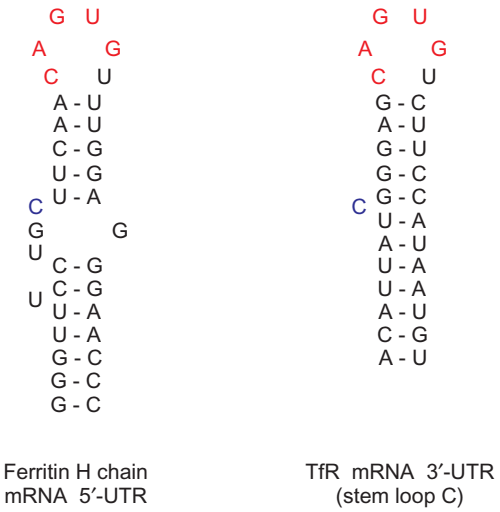


Figure 16.20 Comparison of stem loop structures in the 3′-UTR of the human TfR mRNA with the IRE in the 5′-UTR of the human ferritin mRNA. Only one (stem-loop C) of the five TfR mRNA stem-loops is shown. The conserved, looped-out C and the conserved bases in the loop are in blue and red, respectively. (*Source*: Adapted from Casey, J.L., M.W. Hentze, D.M. Koeller, S.W. Caughman. T.A. Rovault, R.D. Klausner, and J.B. Harford, Iron-responsive elements: Regulatory RNA sequences that control mRNA levels and translation. Science 240:926, 1988.)

Iron Response Elements Lukas Kühn and his colleagues cloned a human TfR cDNA in 1985 and found that it encoded an mRNA with a 96-nt 5′-untranslated region (**5′-UTR**), a 2280-nt coding region, and a 2.6-kb 3′-untranslated region (**3′-UTR**). To test the effect of this long 3′-UTR, Dianne Owen and Kühn deleted 2.3 kb of the 3′-UTR and transfected mouse L cells with this shortened construct. They also made similar constructs with the normal TfR promoter replaced by an SV40 viral promoter. Then they used a monoclonal antibody specific for the human TfR and a fluorescent secondary antibody to detect TfR on the cell surfaces. Figure 16.19 summarizes the results. With the wild-type gene, the cells responded to an iron chelator by increasing the surface concentration of TfR about threefold. Owen and Kühn observed the same behavior when the TfR gene was controlled by the SV40 promoter, demonstrating that the TfR promoter was not responsible for iron responsiveness. On the other hand, the gene with the deleted 3′-UTR did not respond to iron; the same concentration of TfR appeared on the cell surface in the presence or in the absence of the iron chelator. Thus, the part of the 3′-UTR deleted in this experiment apparently included the iron response element.

Of course, the appearance of TfR receptor on the cell surface does not necessarily reflect the concentration of TfR mRNA. To check directly for an effect of iron on TfR mRNA concentration, Owen and Kühn performed S1 analysis (Chapter 5) of TfR mRNA in cells treated and untreated with iron chelator. As expected, the iron chelator increased the concentration of TfR mRNA considerably. But this response to iron disappeared when the gene had a deleted 3′-UTR.

What part of the 3′-UTR confers responsiveness to iron? Harford and colleagues narrowed the search when they discovered that deletion of just 678 nt from the middle of the 3′-UTR eliminated most of the iron responsiveness.

Computer analysis of the critical 678-nt region of the 3′-UTR revealed that its most probable structure includes five hairpins, or stem-loops, as illustrated in Figure 16.20. Even more interesting is the fact that the overall structures of these stem-loops, including the base sequences in the loops, bear a strong resemblance to a stem loop found in the 5′-UTR of the ferritin mRNA. This stem-loop, called an **iron response element (IRE)**, is responsible for the ability of iron to stimulate translation of the ferritin mRNA. The implication is that these TfR IREs are the mediators of the responsiveness of TfR expression to iron.

Harford and colleagues went on to show by gel mobility shift assays (Chapter 5) that human cells contain a protein or proteins that bind specifically to the human TfR IREs (Figure 16.21). This binding could be competed with excess TfR mRNA or ferritin mRNA, which also has an IRE, but it could not be competed by β-globin mRNA, which has no IRE. Thus, the binding is IRE-specific. This finding underscores the similarity between the ferritin and TfR IREs and suggests that they may even bind the same protein(s). However, binding of the protein(s) to the two mRNAs has different effects, as we have seen.

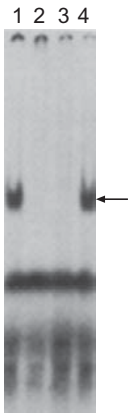


Figure 16.21 Gel mobility shift assay for IRE-binding proteins. Harford and colleagues prepared a labeled 1059-nt transcript corresponding to the region of the human TfR mRNA 3′-UTR that contains the five IREs. They mixed this labeled RNA with a cytoplasmic extract from human cells (with or without competitor RNA), electrophoresed the complexes, and visualized them by autoradiography. Lane 1, no competitor; lane 2, TfR mRNA competitor; lane 3, ferritin mRNA competitor; lane 4, β-globin mRNA competitor. The arrow points to a specific protein–RNA complex, presumably involving one or more IRE-binding proteins. (*Source*: Koeller, D.M., J.L. Casey, M.W. Hentze, E.M. Gerhardt, L.-N.L. Chan, R.D. Klausner, and J.B. Harford, A cytosolic protein binds to structural elements within the nonregulatory region of the transferrin receptor mRNA. *Proceedings of the National Academy of Sciences USA* 86 (1989) p. 3576, f. 3.)

SUMMARY The transferrin receptor-TfR concentration is low when iron concentration is high, and this loss of TfR is largely due to decreased stability of the TfR mRNA. This response to iron depends on the 3′-UTR of the mRNA, which contains five stem loops called iron response elements (IREs).

The Rapid Turnover Determinant Knowing that iron regulates the TfR gene by controlling mRNA stability, and knowing that a protein binds to one or more IREs in the 3′-UTR of TfR mRNA, we assume that the IRE-binding protein protects the mRNA from degradation. This kind of regulation demands that the TfR mRNA be inherently unstable. If it were a stable mRNA, relatively little would be gained by stabilizing it further. In fact, the mRNA *is* unstable, and Harford and coworkers have demonstrated that this instability is caused by a **rapid turnover determinant** that also lies in the 3′-UTR.

What is this rapid turnover determinant? Because the human and chicken TfR genes are controlled in the same manner, they probably have the same kind of rapid turnover determinant. Therefore, a comparison of the 3′-UTRs of these two mRNAs might reveal common features that would suggest where to start the search. Harford and colleagues compared the 678-nt region of the TfR mRNA from human with the corresponding region of the chicken TfR

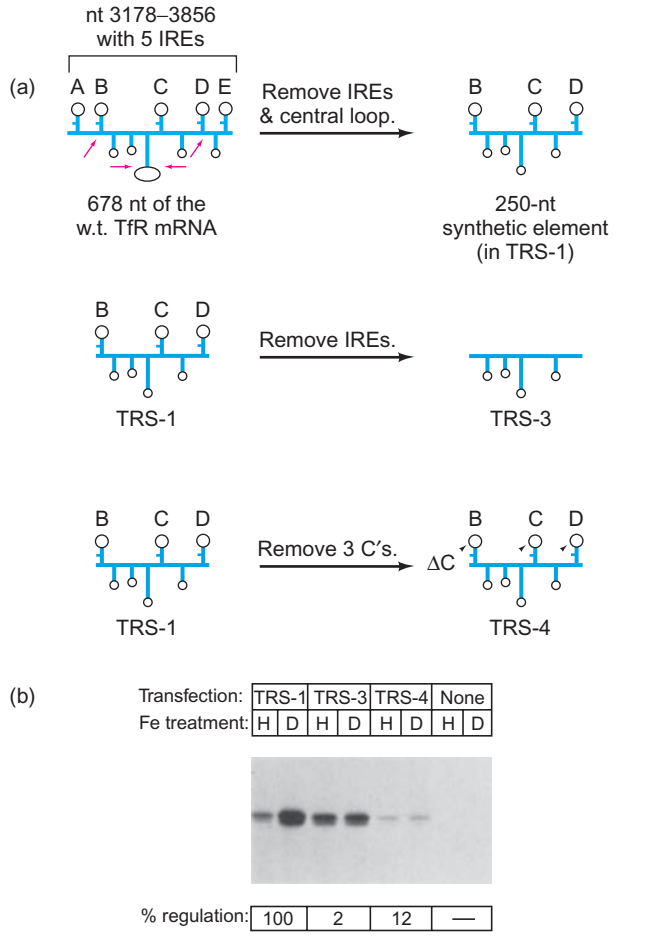


Figure 16.22 Effects of deletions in the IRE region of the TfR 3′-UTR on iron responsiveness. (a) Creation of deletion mutants. Harford and colleagues generated the TRS-1 mutant by removing IREs A and E, and the large central loop, as shown by the arrows. From TRS-1, they generated TRS-3 by removing the remaining three IREs, and TRS-4 by deleting a single C at the 5′-end of each IRE loop. **(b)** Testing mutants for iron response. These workers transfected cells with each construct, treated half the cells with hemin (H) and the other half with desferrioxamine (D), and assayed for TfR biosynthesis by immunoprecipitation. The autoradiograph is shown, with transfected construct and iron treatment shown at top. A summary of the percentage regulation by iron is given at bottom. This is the fold induction by iron chelator vs. hemin (D/H) compared with wild-type, which is defined as 100% regulation. TRS-3 shows essentially no regulation and a constitutively high level of TfR synthesis, suggesting a stable mRNA. TRS-4 shows little regulation and a low level of TfR synthesis, suggesting an unstable mRNA. (*Source:* Casey, J.L., D.M. Koeller, V.C. Ramin, R.D. Klausner, and J.B. Harford, Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 39 untranslated region of the mRNA. *EMBO Journal* 8 (8 Jul 1989) p. 3695, f. 3B.)

mRNA and found a great deal of similarity in the region containing the IREs. Figure 16.22a (left) depicts the human structure. Both have two IREs in the 5′-part of the region, then a stem with a large loop (250 nt in human and 332 nt in chicken), then the other three IREs. The 5′-and 3′-IRE-containing regions in the human mRNA are very similar to

the corresponding regions in the chicken mRNA, but the loop region in between and the regions farther upstream and downstream have no detectable similarity. This suggested that the rapid turnover determinant should be somewhere among the IREs. Harford and coworkers identified some of its elements by mutagenizing the TfR mRNA 3′-UTR and observing which mutations stabilized the mRNA.

The first mutants they looked at were simple 5′- or 3′-deletions. They transfected cells with these constructs and assayed for iron regulation by comparing the TfR mRNA and protein levels after treatment with either hemin or the iron chelator desferrioxamine. They measured mRNA levels by Northern blotting and protein levels by immunoprecipitation. They found that deletion of the 250-nt central loop or deletion of IRE A had no effect on iron regulation. However, deletion of both IREs A and B eliminated iron regulation: The levels of TfR mRNA and protein were the same (and high) with both treatments. Thus, the TfR mRNA is stable when IRE B is removed, so this IRE seems to be part of the rapid turnover determinant. The 3′-deletions gave a similar result. Deletion of IRE E had little effect on iron regulation, but deletion of both IREs D and E stabilized the TfR mRNA, even in the presence of hemin. Thus, IRE D appears to be part of the rapid turnover determinant.

Based on these findings, we would predict that IRE A, IRE E, and the central loop could be deleted without altering iron regulation. Accordingly, Harford and colleagues made a synthetic element they called TRS-1 that was missing these three parts as illustrated in Figure 16.22a. As expected, mRNAs containing this element retained full iron responsiveness. Next, these workers made two alterations to TRS-1 (Figure 16.22a). The first, TRS-3, had lost all three of its IREs. All that remained were the other stem-loops, pictured pointing downward in Figure 16.22. The other, TRS-4, had lost only three bases, the C's at the 5′-end of the loop in each IRE. Figure 16.22b shows the effects of these two alterations. TRS-3, with no IREs, had lost virtually all iron responsiveness, and the TfR RNA appeared to be much more stable than the wild-type mRNA. That is, there was abundant TfR even in the presence of hemin. TRS-4, with a C missing from each IRE, had lost most of its iron responsiveness, but the mRNA remained unstable. That is, there was not much TfR even in the presence of the iron chelator. Thus, this mRNA retained its rapid turnover determinant, but had lost the ability to be stabilized by the IRE-binding protein. In fact, as we would expect, gel mobility shift assays showed that TRS-4 could not bind the IRE-binding protein.

To pin down the rapid turnover determinant still further, Harford and colleagues made two new constructs in which they deleted one or the other of the two (downward-pointing) non-IRE stem-loops on either side of the large central stem-loop. Then they tested these constructs by transfection and immunoprecipitation as before. Both constructs showed almost total loss of iron responsiveness and a constitutively high level of TfR expression (the same pattern shown by TRS-3 in Figure 16.22). Thus, both of the deleted stem-loops appear to be essential to confer rapid turnover of the mRNA. To demonstrate that this effect was not due to an inability of the mRNAs to interact with the IRE-binding protein, these workers assayed protein–RNA binding as before by gel mobility shift. Both constructs were just as capable of binding to the IRE-binding protein as was the wild-type mRNA, and excess unlabeled IRE successfully competed with the labeled constructs for binding.

SUMMARY IREs A and E, and the large central loop of the TfR 3′-UTR can be deleted without altering the response to iron. However, removing IREs A and B, or IREs D and E, or all five IREs renders the TfR mRNA constitutively stable. Thus, IREs B and D, at least, are part of the rapid turnover determinant. Removing a C from IREs B–D renders the TfR mRNA constitutively unstable and unable to bind the IRE-binding protein.

TfR mRNA Stability and Degradation Pathway The data presented so far strongly suggest that iron regulates the TfR mRNA half-life, rather than the rate of mRNA synthesis. To provide direct evidence for this hypothesis, Ernst Müllner and Lukas Kühn measured the rate of TfR mRNA decay in the presence and absence of the iron chelator desferrioxamine. They found that the TfR mRNA was very stable when the iron concentration was low. On the other hand, at high iron concentration the TfR mRNA decayed much faster. These two half-lives were 30 and 1.5 h, respectively, so iron appears to destabilize the TfR mRNA by approximately 30/1.5, or 20-fold.

Harford and colleagues investigated the mechanism by which TfR mRNA is degraded and found that the first event appears to be an endonucleolytic cut within the IRE region. Unlike the degradation of many other mRNAs, there seems to be no requirement for deadenylation (removal of poly[A]) before TfR degradation can begin.

These workers began their study by treating human plasmacytoma cells (ARH-77 cells) with hemin and showing by Northern blotting that the level of TfR mRNA dropped precipitously in 8 h. When they exposed the blot for a longer time, they found that a new RNA species, about 1000–1500 nt shorter than full-length TfR mRNA, appeared during the period in which the TfR mRNA was breaking down. This RNA was also found in the poly(A)$^-$ fraction, suggesting that it had lost its poly(A). But the size of this shortened RNA suggested that it had lost much more than just its poly(A). The simplest explanation was that it had been cut by an endonuclease within its 3′-UTR, which removed over 1000 3′-terminal nucleotides, including the poly(A).

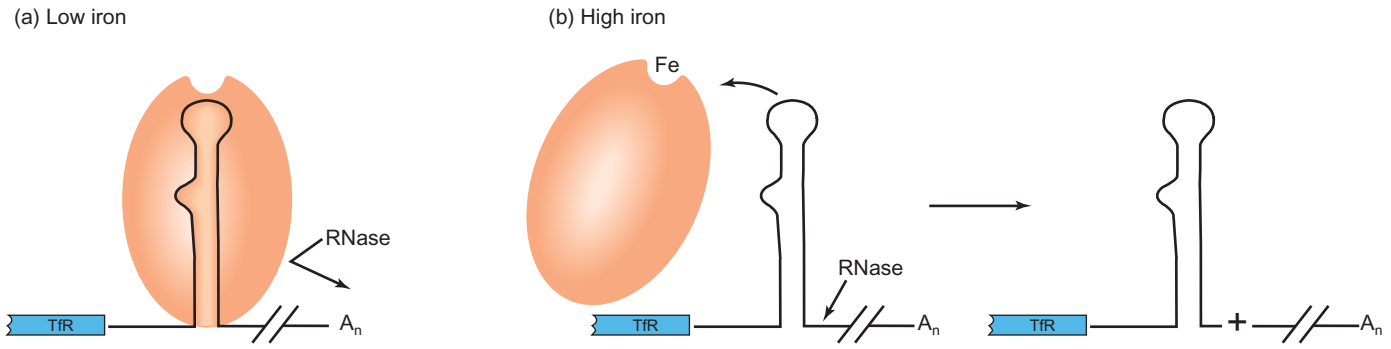


Figure 16.23 Model for destabilization of TfR mRNA by iron. **(a)** Under low-iron conditions, the aconitase apoprotein (orange) binds to the IREs in the 3′-UTR of the TfR mRNA. This protects the RNA from degradation by RNases. **(b)** Under high-iron conditions, iron binds to the aconitase apoprotein, removing it from the IREs, and opening the IREs up to attack by RNase. The RNase clips the mRNA at least once, exposing its 3′-end to further degradation.

All the data we have considered are consistent with the following hypothesis (Figure 16.23): When iron concentrations are low, an IRE-binding protein, or **iron regulatory protein (IRP),** binds to the rapid turnover determinant in the 3′-UTR of the TfR mRNA. This protein protects the mRNA from degradation. When iron concentrations are high, iron binds to the IRE-binding protein, causing it to dissociate from the rapid turnover determinant, opening it up to attack by a specific endonuclease that clips off a 1-kb fragment from the 3′-end of the TfR mRNA. This destabilizes the mRNA and leads to its rapid degradation.

One of the proteins (IRP1) that bind to the IREs in both the transferrin receptor mRNA and the ferritin mRNA (Chapter 17) has now been identified as a form of **aconitase,** an enzyme that converts citrate to isocitrate in the citric acid cycle. The enzymatically active form of aconitase is an iron-containing protein that does not bind to the IREs. However, the apoprotein form of aconitase, which lacks iron, binds to the IREs in mRNAs.

SUMMARY When the iron concentration is high, the TfR mRNA decays rapidly. When the iron concentration is low, the TfR mRNA decays much more slowly. This difference in mRNA stability is about 20-fold and plays a major role in control of the gene's expression. The initiating event in TfR mRNA degradation seems to be an endonucleolytic cleavage of the mRNA more than 1000 nt from its 3′-end, within the IRE region. This cleavage does not require prior deadenylation of the mRNA. Iron controls TfR mRNA stability as follows: When iron concentration is low, aconitase exists at least partly in an apoprotein form that lacks iron. This protein binds to the IREs in the TfR mRNA and protects the RNA against attack by RNases. But when iron concentration is high, the aconitase apoprotein binds to iron and therefore cannot bind to the TfR mRNA IREs. This leaves the RNA vulnerable to degradation.

16.6 Post-Transcriptional Control of Gene Expression: RNA Interference

For years, molecular biologists have been using antisense RNA to inhibit expression of selected genes in living cells. At first, the rationale was that the antisense RNA, which is complementary to mRNA, would base-pair to the mRNA and inhibit its translation. The strategy usually worked, but the rationale was incomplete. As Su Guo and Kenneth Kemphues established in 1995, injecting sense RNA into cells worked just as well as antisense RNA in blocking expression of a particular gene. Then, in 1998, Andrew Fire and Craig Mello and their colleagues showed that double-stranded RNA (**dsRNA**) worked much better than either sense or antisense RNA. In fact, the main reason sense and antisense RNAs worked appears to be that they were contaminated with (or produced) small amounts of dsRNA, and the dsRNA actually did the most to block gene expression.

Also, beginning in 1990, molecular biologists began noticing that placing transgenes into various organisms sometimes had the opposite of the desired effect. Instead of turning on the transgene, organisms sometimes turned off, not only the transgene, but the normal cellular copy of the gene as well. One of the first examples was an attempt to intensify the purple color of a petunia by supplying extra copies of the pigment-producing genes. But in up to 25% of the transformed plants, blossoms were white or patchy purple and white—the opposite of the intended

Figure 16.24 Silencing of a purple color gene in petunia by adding extra copies of the color gene. The central white stripe in each petal shows where silencing occurred. (*Source*: Courtesy of Dr. Richard A. Jorgensen, The Plant Cell.)

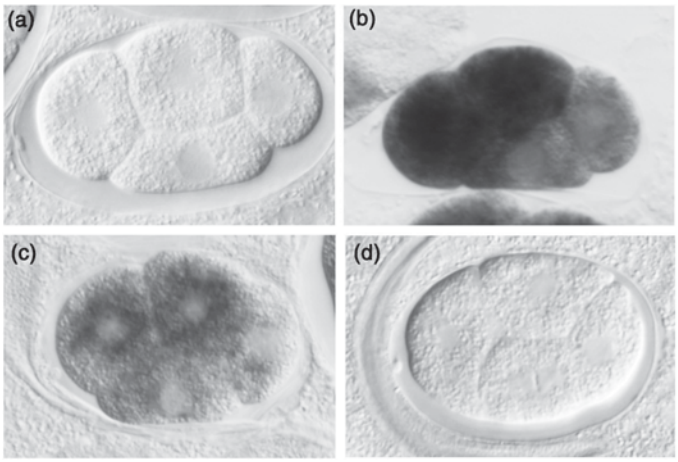


Figure 16.25 Double-stranded RNA-induced RNA interference causes destruction of a specific mRNA. Fire and colleagues injected antisense or dsRNA corresponding to the *C. elegans mex-3* mRNA into *C. elegans* ovaries. After 24 h, they fixed the embryos in the treated ovaries and subjected them to in situ hybridization (Chapter 5) with a probe for *mex-3* mRNA. **(a)** Embryo from a negative control parent with no hybridization probe. **(b)** Embryo from a positive control parent that was not injected with RNA. **(c)** Embryo from a parent that was injected with *mex-3* antisense RNA. A considerable amount of *mex-3* mRNA remained. **(d)** Embryo from a parent that was injected with dsRNA corresponding to part of the *mex-3* mRNA. No detectable *mex-3* mRNA remained. (*Source*: Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature* 391 (1998) f. 3, p. 809. Copyright © Macmillan Magazines Ltd.)

effect (Figure 16.24). This phenomenon was called by several names: cosuppression and **post-transcriptional gene silencing** (**PTGS**) in plants, **RNA interference** (**RNAi**) in animals such as nematodes *(Caenorhabditis elegans)* and fruit flies, and **quelling** in fungi. To avoid confusion, we will refer to this phenomenon as RNAi from now on, regardless of the species under study.

Mechanism of RNAi

Fire and colleagues showed that injecting *C. elegans* gonads with dsRNA (the **trigger dsRNA**) caused RNAi in the resulting embryos. Furthermore, they detected a loss of the corresponding mRNA (the **target mRNA**) in embryos undergoing RNAi (Figure 16.25). However, the dsRNA had to include exon regions; dsRNA corresponding to introns and promoter sequences did not cause RNAi. Finally, these workers demonstrated that the effect of the dsRNA crossed cell boundaries, at least in *C. elegans*. That is, the effect spread throughout the whole organism.

Is this loss of a particular mRNA in response to the corresponding dsRNA caused by repression of transcription of the gene or destruction of the mRNA? In 1998, Fire and colleagues, as well as others, demonstrated that RNAi is a post-transcriptional process that involves mRNA degradation. Several investigators reported the presence of short pieces of dsRNA called **short interfering RNA** (**siRNA**) in cells undergoing RNAi. In 2000, Scott Hammond and collaborators purified a nuclease from *Drosophila* embryos undergoing RNAi that digests the targeted mRNA. The partially purified preparation that contained this nuclease activity also contained a 25-nt RNA fraction that could be detected on Northern blots with probes for either the sense or antisense strand of the targeted mRNA. Degradation of the 25-nt RNA with micrococcal nuclease destroyed the ability of the preparation to digest the mRNA. These data suggested that a nuclease digests the trigger dsRNA into fragments about 25 nt long, and these fragments then associate with a nuclease and provide guide sequences that allow the nuclease to target the corresponding mRNA.

Phillip Zamore and collaborators developed a system based on *Drosophila* embryo lysates that carried out RNAi in vitro. This system allowed these workers to look at individual steps in the RNAi process. The embryos had been injected with trigger dsRNA corresponding to luciferase mRNA, so they targeted that mRNA for destruction. First, Zamore and collaborators showed that RNAi requires ATP. They depleted their extract of ATP by incubating it with hexokinase and glucose, which converts ATP to ADP and transfers the lost phosphate group to glucose. The ATP-depleted extract no longer carried out the degradation of the target, luciferase mRNA.

Next, these workers performed experiments in which they labeled one strand of the dsRNA at a time (or both) and showed that labeled short siRNAs of 21–23 nt appeared, no matter which strand was labeled (Figure 16.26). The appearance of the siRNAs did not require the presence of mRNA (e.g., compare lanes 2 and 3), so these short RNAs apparently derived from dsRNA, not mRNA. When capped antisense luciferase RNA was labeled (lanes 11 and 12),

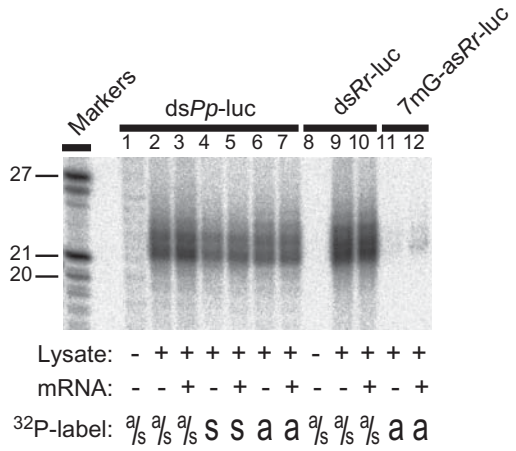


Figure 16.26 Generation of 21–23-nt RNA fragments in an RNAi-competent *Drosophila* embryo extract. Zamore and collaborators added ds luciferase RNA from *Photinus pyralis* (*Pp*-luc RNA) or from *Renilla reniformis* (*Rr*-luc RNA), as indicated at top, to lysates in the presence or absence of the corresponding mRNA, as indicated at bottom. The dsRNAs were labeled in the sense strand (s), in the antisense strand (a), or in both strands (a/s), as indicated at bottom. RNA markers from 17–27 nt long were included in the lane at left. Lanes 11 and 12 contained labeled, capped antisense *Rr*-luc RNA in the absence and presence of mRNA, respectively. (*Source:* Zamore, P.D., T. Tuschl, P.A. Sharp, and D.P. Bartel, RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101 (2000) f. 3, p. 28. Reprinted by permission of Elsevier Science.)

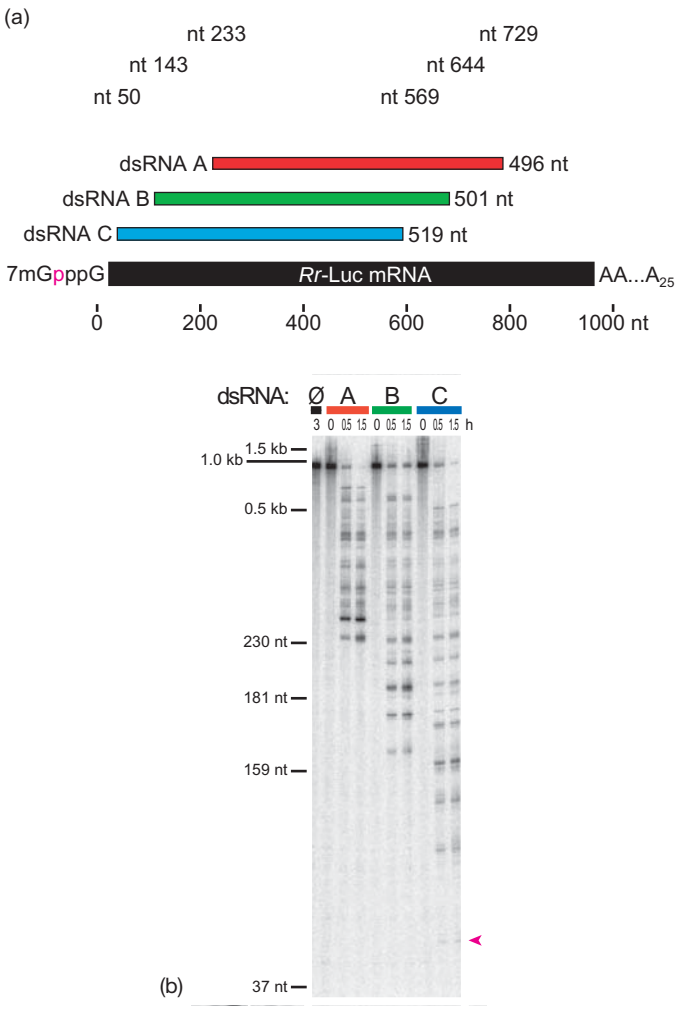


Figure 16.27 The trigger dsRNA dictates the boundaries of cleavage of mRNA in RNAi. Zamore and collaborators added the three dsRNAs pictured in panel **(a)** to an embryo extract along with an *Rr*-luc mRNA, 5′-labeled in one of the phosphates of the cap. **(b)** Experimental results. The 5′-end-labeled mRNA degradation products were electrophoresed. The dsRNAs included in the reactions are indicated and color-coded at top. The first lane, marked 0, contained no dsRNA. Reactions were incubated for the times (in h) indicated at top. The arrowhead indicates a faint cleavage site that lies outside the position of RNA C. Otherwise, the sites cleaved lie within the positions of the three dsRNAs on the mRNA. (*Source:* Zamore, P.D., T. Tuschl, P.A. Sharp, and D.P. Bartel, 2000. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101 (2000) f. 5, p. 30. Reprinted by permission of Elsevier Science.)

a small amount of siRNAs appeared, and that amount increased in the presence of mRNA (lane 12). This result suggested that the labeled antisense RNA was hybridizing to the added mRNA to generate a dsRNA that could be degraded to the short RNA pieces. In summary, all these results suggest that a nuclease degrades the trigger dsRNA into short pieces. Further work has shown that these siRNAs are about 21–23 nt long.

Next, Zamore and collaborators showed that the trigger dsRNA dictated where the corresponding mRNA would be cleaved. They added three different trigger dsRNAs, whose ends differed by about 100 nt, to their RNAi extracts, then added 5′-labeled mRNA, allowed RNA cleavage to occur, and electrophoresed the products. Figure 16.27 shows the results: The dsRNA (C) whose 5′-end was closest to the 5′-end of the mRNA yielded the shortest fragments; the next dsRNA(B), whose 5′-end was about 100 nt farther downstream, yielded mRNA fragments about 100 nt longer; and the third dsRNA, whose 5′-end was about another 100 nt farther downstream, yielded mRNA fragments about another 100 nt longer. This close relationship between the position of the trigger dsRNA relative to the mRNA, and the position at which cleavage began, strongly suggests that the dsRNA determined the sites of cleavage of the mRNA.

Next, Zamore and collaborators performed high-resolution gel electrophoresis of the mRNA degradation products from Figure 16.27. The results, presented in Figure 16.28, are striking. The major cleavage sites in the mRNA are mostly at 21–23-nt intervals, producing a set of RNA fragments whose lengths differ by multiples of 21–23 nt. The one obvious exception is the site marked by an arrowhead, which lies only 9 nt from the previous cleavage site. This exceptional site lies within a run of seven uracil residues, which is interesting in light of the fact that 14 of 16 cleavage sites mapped were at uracils. After this exceptional site, the 21–23-nt interval resumed

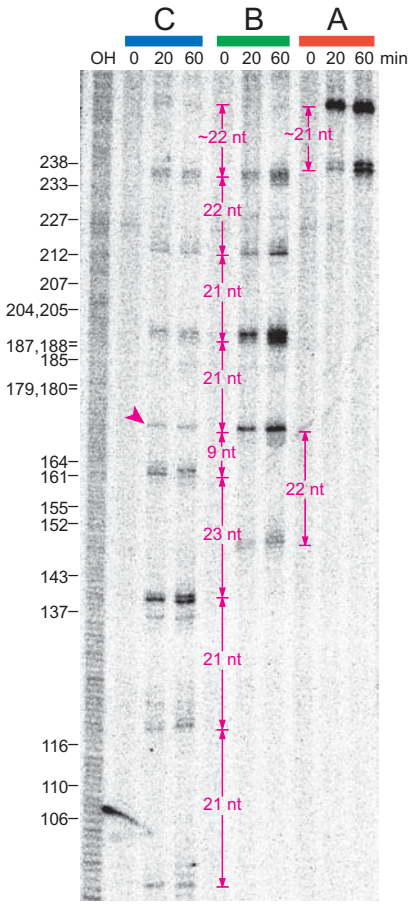


Figure 16.28 Cleavages of target mRNA in RNAi occur at 21–23-nt intervals. Zamore and collaborators performed high-resolution denaturing polyacrylamide gel electrophoresis on the products of RNAi in the presence of all three of the trigger dsRNAs from Figure 16.27. The cleavages, with one notable exception (arrowhead), occurred at 21–23-nt intervals. The exceptional band indicates a cleavage at only a 9-nt interval, but cleavages thereafter were at 21–23-nt intervals. (*Source:* Zamore, P.D., T. Tuschl, P.A. Sharp, and D.P. Bartel, RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101 (2000) f. 6, p. 31. Reprinted by permission of Elsevier Science.)

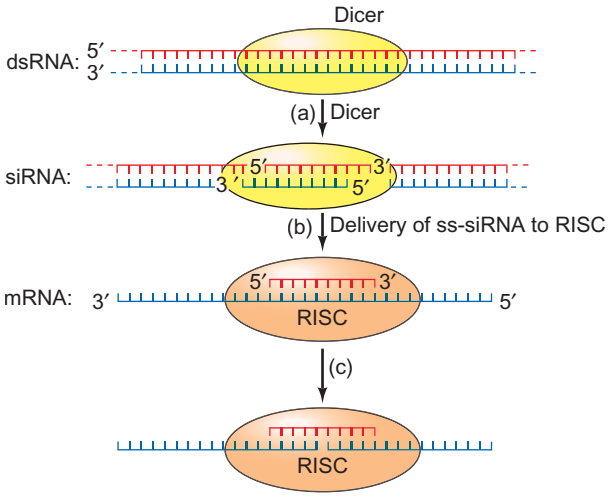


Figure 16.29 A simplified model for RNAi. (a) Dicer (yellow) recognizes and binds to a double-stranded RNA (red and blue), then cleaves the RNA into siRNAs about 21–23 nt long (depicted here as 10 nt long, for simplicity), with 2-nt 3′-overhangs. The ends of the central siRNA are labeled to illustrate the 3′-overhangs. **(b)** One of the siRNA strands (red) associates with RISC (orange) and base-pairs to a target mRNA (blue). **(c)** The siRNA strand in the RISC complex serves as a guide RNA to direct the cleavage of the target mRNA in the middle of the sequence opposite the siRNA.

for the rest of the mapped cleavage sites. These results support the hypothesis that the 21–23-nt siRNAs determine where the mRNA will be cut and suggest that cleavage takes place preferentially at uracils.

In 2001, Hammond and colleagues reported that they had purified from *Drosophila* the enzyme that cleaves the trigger double-stranded RNA into short pieces. They named it **Dicer,** because it dices double-stranded RNA up into uniform-sized pieces. Dicer is a member of the RNase III family discussed earlier in this chapter. In fact, Hammond and colleagues narrowed their search for Dicer by looking for enzymes in this family because RNase III was the only known nuclease specific for dsRNA. Like RNase III, Dicer leaves 2-nt 3′-overhangs (protruding 3′-ends) at the ends of the double-stranded siRNAs, and phosphorylated 5′-ends.

Three early lines of evidence implicated Dicer in RNA cleavage in RNAi. First, *dicer,* the gene that encodes Dicer, produces a protein that can cut dsRNA into 22-nt pieces. Second, antibodies against this protein bind to an enzyme in *Drosophila* extracts that cuts dsRNA into short pieces. Finally, when *dicer* dsRNA is introduced into *Drosophila* cells, it partially blocks RNAi. It is ironic that Hammond and colleagues could use RNAi to block RNAi! But, of course, if you think about it, the blockage could never be complete.

Dicer also has RNA helicase activity, so it can separate the two strands of the siRNAs it creates, at least in principle. However, Dicer does not carry out the second step in RNAi, cleavage of the target mRNA. That appears to be the job of another enzyme, called **slicer,** which resides in a complex called the **RNA-induced silencing complex** (**RISC**). Figure 16.29 summarizes what we have learned so far about the mechanism of RNAi.

Hammond and others have implicated another *Drosophila* protein, **Argonaute,** known from genetic experiments to be required for RNAi, in the second (slicer) step. Argonaute does not have an RNase III motif, so molecular biologists discounted it at first as a slicer candidate. However, structural, biochemical, and genetic studies of Argonaute carried out by Leemor Joshua-Tor, Gregory Hannon, and their colleagues in 2004 showed that Argonaute almost certainly has slicer activity.

These workers had shown in structural studies in 2003 that Argonaute2 of *Drosophila* contains two characteristic

domains, **PAZ,** and **PIWI.** (PAZ, from PIWI, Argonaute, and Zwili, was found only in Argonaute and Dicer; PIWI was discovered in *Drosophila*. The acronym stands for P-element-induced wimpy testis.) They had also determined the structure of PAZ, and had shown that it contained a module resembling a so-called OB fold, which can bind single-stranded RNAs. They also demonstrated by cross-linking studies with labeled siRNAs and cloned GST–PAZ fusion proteins that the PAZ domain was capable of binding to single-stranded siRNAs, or to the 2-nt single-stranded overhangs at the 3′-ends of double-stranded siRNAs. This implicated Argonaute in the slicer reaction, at least as a docking site for the siRNA, but not necessarily as the slicer enzyme itself.

Next, Joshua-Tor, Hannon, and colleagues performed x-ray crystallography on the Argonaute-like protein of the archaeon *Pyrococcus furiosus*. (No full-length eukaryotic Argonaute structure could be obtained.) They found that three domains of the protein (the middle domain, PIWI, and the N-terminal domain) form a crescent shape at the bottom of the structure, with the PIWI domain in the middle. The PAZ domain lies above the crescent and is connected to it by a stalk domain. Figure 16.30 depicts this structure, and illustrates that the crescent forms a groove, capped by the PAZ domain. This groove is big enough to accommodate a double-stranded RNA, and it is lined with basic residues, which could form electrostatic bridges to an RNA substrate.

However, the most telling part of the structure is that the PIWI domain resembles a similar domain in RNase H, which cleaves the RNA strand in an RNA–DNA hybrid. Thus, RNase H can recognize a double-stranded polynucleotide and cleave one of its strands (the RNA). In addition to their overall architectural similarities, both proteins have a cluster of three acidic residues (two aspartates and one glutamate). In RNase H, this carboxylate cluster binds a Mg^{2+} ion that plays a key role in catalyzing the cleavage of the RNA strand. These similarities are very interesting because slicer has an analogous activity: It must also recognize a double-stranded polynucleotide (an siRNA–mRNA hybrid) and cleave one of its strands (the mRNA). Thus, Argonaute has all the attributes we expect of slicer: a domain (PIWI) with a site that appears to be capable of cleaving one strand of an siRNA–mRNA hybrid, and another domain (PAZ) that can bind to the end of the siRNA.

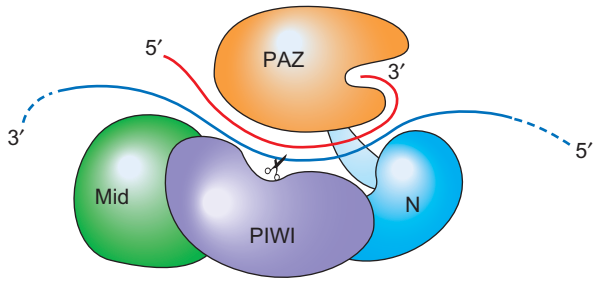


Figure 16.30 Model for slicer activity of Argonaute. The hybrid involving an siRNA and a target mRNA is held in the active site, at least partly due to the interaction between the 3′-end of the siRNA and the PAZ domain of Argonaute. This places the target mRNA in position to be cut by the slicer active site, represented by the scissors. Cleavage occurs opposite the middle of the siRNA, which serves as a guide RNA. The PAZ, middle, PIWI, and N-terminal domains of Argonaute are labeled. (*Source:* Adapted from Science, Vol. 305, Ji-Joon Song, Stephanie K. Smith, Gregory J. Hannon, and Leemor Joshua-Tor, "Crystal Structure of Argonaute and Its Implications for RISC Slicer Activity," Fig. 4, p. 1436, AAAS.)

To investigate further the role of Argonaute in mammals, Hannon, Joshua-Tor, and colleagues performed genetic and biochemical studies on the Argonaute genes and proteins in the mouse. Mammals have four Argonaute proteins, designated Argonaute 1–4. The investigators transfected cells with genes encoding Argonautes 1–3, along with an siRNA that targets firefly luciferase mRNA. Then they immunoprecipitated the RISC complexes and tested them for ability to cleave luciferase mRNA in vitro. Only **Argonaute2** (**Ago2**) had this capability.

Next, these workers knocked out the Ago2 gene in mice and observed that all such animals died in the embryonic stage of development, with severe developmental defects and delay. The reason for this profound phenotype is that Ago2 participates, not only in RNAi, but in a normal (and critical) developmental process involving microRNAs, which we will discuss later in this chapter. Furthermore, mouse embryo fibroblasts (MEFs) from wild-type cells showed normal RNAi, but MEFs from Ago2 knockout mice were defective in RNAi, as expected if Ago2 is important in RNAi.

All of the studies cited so far are consistent with the hypothesis that Ago2 has slicer activity, but none addressed this question directly. However, if Argonaute really has slicer activity, then mutating any of the three acidic amino acids at the putative active site should block cleavage of mRNA by RISC. Hannon, Joshua-Tor, and colleagues mutated each of the two key aspartate residues and found that either mutation abolished the RNAi-mRNA cleavage step both in vitro and in vivo. Taken together, all this evidence strongly implicates Ago2 as the slicer enzyme.

In 2005, Joshua-Tor and colleagues demonstrated definitively that human Ago2 really does have slicer activity. They reconstituted a minimal RISC with human recombinant Ago2 and an siRNA, which could accurately cleave a substrate RNA complementary to the siRNA. Figure 16.31 shows the results. The first siRNA (siRNA1) caused cleavage of the substrate RNA (S500) about 180 nt from its 3′-end, yielding a 3′-product about 180 nt long and a 5′-product about 320 nt long. The second siRNA (siRNA2) caused cleavage of the S500 about 140 nt from its 5′-end, yielding a 5′-product about 140 nt long and a 3′-product about 360 nt long. As expected, no products were produced in the absence of siRNA. Nor did products appear in the absence of Mg^{2+}, showing that a divalent metal ion is required for slicer activity.

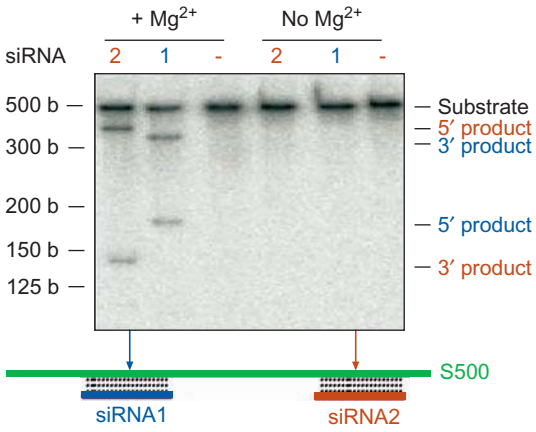


Figure 16.31 Ago2 plus an siRNA form a minimal RISC with slicer activity in vitro. Joshua-Tor and colleagues mixed recombinant human Ago2 (produced in bacteria) with either of two siRNAs that were specific for two different sites on a target 500-nt RNA, as shown at bottom. Then they added the labeled target RNA in the presence or absence of Mg^{2+} ions, as indicated at top. The siRNA used (either #1, or #2, or neither) is also indicated at top. Finally, they displayed the labeled RNA products by gel electrophoresis. Cleavage depended on Mg^{2+} and on an siRNA. The two siRNAs yielded different products, whose sizes were predicted from the known sites on the target RNA to which they hybridized. (*Source:* Reprinted from *Nature Structural & Molecular Biology*, vol 12, Fabiola V Rivas, Niraj H Tolia, Ji-Joon Song, Juan P Aragon, Jidong Liu, Gregory J. Hannon, Leemor Joshua-Tor, "Purified Argonaute2 and an siRNA form recombinant human RISC," fig. 1d, p. 341, Copyright 2005, reprinted by permission from Macmillan Publishers Ltd)

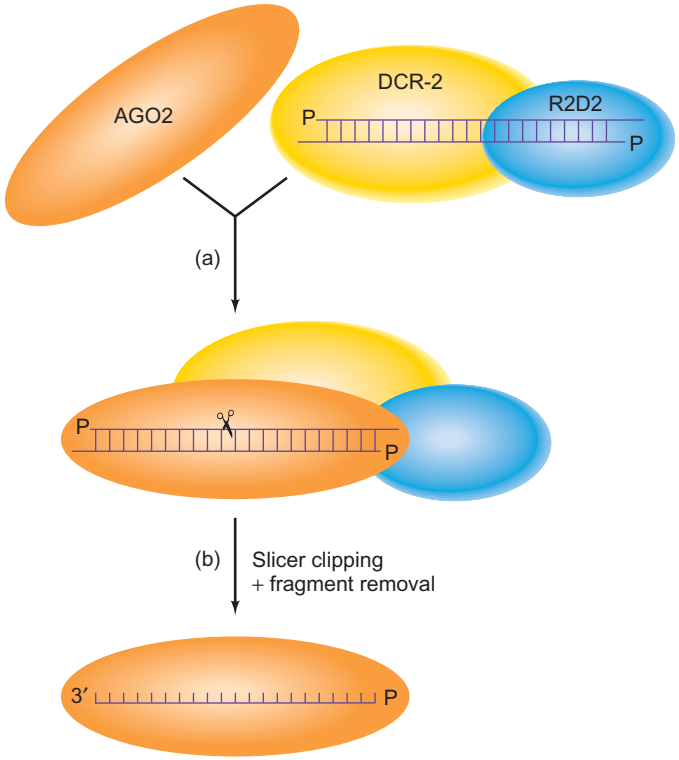


Figure 16.32 Delivery of single-stranded siRNA to RISC. The names of the proteins are from *Drosophila*, in which this process has been well studied. **(a)** Ago2 is attracted to a Dicer (DCR-2)-R2D2-dsRNA, forming a pre-RISC complex. The ds siRNA has already been created by DCR-2, leaving phosphorylated 5′-ends and 2-nt 3′-overhangs. **(b)** The slicer activity of Ago2 cuts the passenger strand (top) in half, weakening its base-pairing to the guide strand. The passenger strand fragments are lost, leaving the guide strand bound to Ago2, which is the catalytic center of the mature RISC. Other proteins besides Ago2 are part of mature RISC, though they are not shown here.

For mRNA cleavage to occur, a catalytically active RISC must form (Figure 16.32). We have seen that an Argonaute protein contains the slicer active site in a RISC, and we also know that a single-stranded siRNA must be present to serve as a guide to select mRNAs to degrade. So Ago2 plus siRNA constitutes a minimal RISC, at least in mammalian cells. But this complex does not form directly. Instead, siRNA must be delivered to Ago2 by a **RISC loading complex (RLC).** The composition of the RLC is presumed to include at least Dicer and a Dicer-associated protein, cutely-named **R2D2,** in addition to siRNA, and it could also include **Armitage,** which is essential for converting an RLC to a RISC in *Drosophila*.

What is the role of R2D2? It is not required for double-stranded siRNA formation, as Dicer can carry out this process efficiently without R2D2 in vitro. However, gel mobility shift and protein–RNA cross-linking experiments have shown that Dicer alone cannot retain contact with siRNAs once it has made them, but Dicer plus R2D2 can. Furthermore, R2D2 contains two double-stranded RNA-binding domains, and mutations in these domains render the Dicer–R2D2 complex incapable of binding double-stranded siRNAs. Thus, it appears that R2D2 is an essential part of the RLC because it can shepherd the siRNA between the time it is formed by Dicer and the time it is delivered to the RISC.

How are the two strands of the ds-siRNA separated to yield the ss-siRNA that ultimately associates with the RISC? An early hypothesis was that Armitage, which has RNA helicase activity, separated the two strands. However, that would require ATP, and the two RNA strands can be separated without ATP, at least in *Drosophila*. Figure 16.32 presents a model that incorporates that fact and other data. A complex composed of double-stranded siRNA plus Dicer (DCR-2 in *Drosophila*) and R2D2 attracts an Argonaute protein (Ago2 in *Drosophila*). Then Ago2 cleaves the **passenger strand** (the discarded strand) of the siRNA in the middle, weakening its grip on the **guide strand** (the strand that will associate with the RISC), so the passenger strand fragments are lost. This leaves a RISC active center composed of Ago2 and the siRNA guide strand.

What determines which strand is the guide strand, and which is the discarded passenger strand of the siRNA? This distinction is made in a complex that forms before the RLC, and contains Dicer and R2D2, each of which binds to an end of the double-stranded siRNA. The two proteins appear to bind asymmetrically, with Dicer associated with

the less stable end (the one in which the base pairs are easiest to dissociate). And the strand with its 5′-end bound to Dicer is the one that becomes the guide strand.

X-ray crystallography studies on complexes between siRNAs and Argonaute-like proteins have shown that the siRNA guide strand binds with 3′-end in the PAZ domain. This places the active site of Argonaute between residues 10 and 11 of the siRNA, so the mRNA would be cleaved right in the middle of the siRNA–mRNA hybrid.

What is the physiological significance of RNAi? True double-stranded RNA does not normally occur in eukaryotic cells, but it does occur during infection by certain RNA viruses that replicate through dsRNA intermediates. So one important function of RNAi may be to inhibit the replication of viruses by degrading their mRNAs. But Fire and other investigators have also found that some of the genes required for RNAi are also required to prevent certain transposons from transposing within the genome. Indeed, Titia Sijen and Ronald Plasterk showed in 2003 that transposition of the Tc1 transposon in *C. elegans* germ cells is silenced by RNAi. What double-stranded RNA triggers this RNAi? It appears that transcription of the terminal inverted repeats of the transposon yields an RNA that can form a stem-loop structure, which is double-stranded in the stem portion. Thus, RNAi can protect cells not only against viruses, but also against transposition that can threaten the genomic integrity of germ cells.

RNAi can also silence transgenes and their genomic homologs. How is double-stranded RNA made from transgenes? It seems that some transcription of both strands of transgenes occurs, in contrast to the behavior of normal genes. This symmetric transcription yields enough double-stranded RNA to trigger RNAi.

Aside from its natural functions, RNAi has been a terrific boon to molecular biologists because it enables them to inactivate genes at will, simply by introducing double-stranded RNAs corresponding to the target genes. This process, known as **knockdown**, is usually much more convenient than the laborious process of producing knockout organisms, as described in Chapter 5. Also, it has not escaped the notice of the biotechnology industry that RNAi represents a potential bonanza. We know of many genes which, when overactive, can have devastating effects. For example, many **oncogenes** become hyperactive in various cancer cells, and that hyperactivity is what drives the cancer cells to lose control over their growth. RNAi directed against these oncogenes could control their activities, and thereby restore growth control to the cancer cells.

In spite of all this optimism, some caution is warranted because data began accumulating in 2004 that RNAi is not as exquisitely specific as had been thought. Genes that do not match the trigger double-stranded RNA perfectly are still targeted for repression to some extent. We do not know yet whether this nonspecificity will seriously compromise the effectiveness of RNAi in research and medicine.

Furthermore, if scientists want to use RNAi to investigate human gene function, or even to combat human disease, they will have to take account of another fact: Unlike in roundworms and fruit flies, the RNAi induced by adding dsRNA to mammalian cells is transient. But there is a way around this problem: Lasting RNAi can be induced by transforming mammalian cells with genes encoding RNAs with inverted repeats that form hairpins. These genes provide a continuous supply of double-stranded RNA in the form of hairpins, and that is enough to keep the RNAi process going. By 2004, researchers had already built libraries of genes encoding **short hairpin RNAs** (**shRNAs**) that targeted almost 10,000 human genes. These represent a valuable resource for research, and perhaps even intervention in human disease.

SUMMARY RNA interference (RNAi) occurs when a cell encounters dsRNA from a virus, a transposon, or a transgene (or experimentally added dsRNA), and results in destruction of the mRNA corresponding to the trigger dsRNA. The mechanism of RNAi in *Drosophila* is as follows: The trigger dsRNA is degraded into 21–23-nt fragments (siRNAs) by an RNase III-like enzyme called Dicer. The double-stranded siRNA, with Dicer and the Dicer-associated protein R2D2 recruit Ago2 to form a pre-RISC complex that can separate the siRNA into its two component strands: the guide strand, which will base-pair with the target mRNA in the RNA-induced silencing complex (RISC) and guide cleavage of the mRNA, and the passenger strand, which will be discarded. Ago2 cleaves the passenger strand, which then falls off the pre-RISC complex. The guide strand of the siRNA then base-pairs with the target mRNA in the active site in the PIWI domain of Ago2, which is an RNase H-like enzyme, also known as slicer. Slicer cleaves the target mRNA in the middle of the region of its base-pairing with the siRNA. In an ATP-dependent step, the cleaved mRNA is ejected from the RISC, which can then accept a new molecule of mRNA to be degraded.

Amplification of siRNA

One aspect of RNAi in some organisms, including plants and nematodes, has been difficult to explain: its great sensitivity. Just a few molecules of dsRNA can set in motion a process that totally silences a gene, not only in one cell, but in a whole organism—and even the descendants of that organism. This phenomenon led to the proposal that the process is catalytic. Indeed, Dicer does create many molecules of siRNA out of the trigger dsRNA and the target mRNA, but that seems insufficient to explain the power of

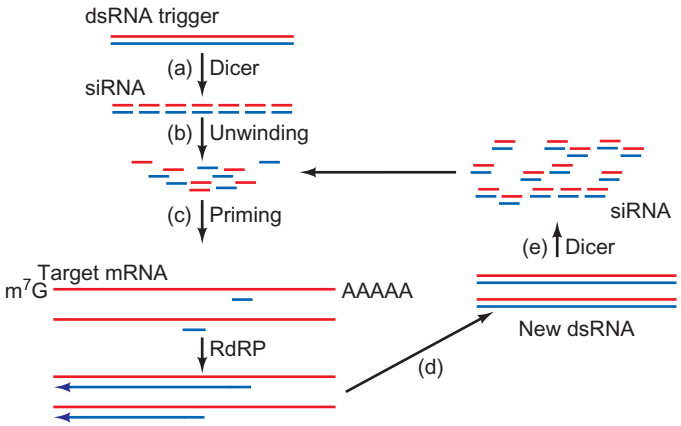


Figure 16.33 Amplification of siRNA. **(a)** Dicer chops up trigger dsRNA to make siRNA. **(b)** The antisense strands of siRNA hybridize to target mRNA. **(c)** RdRP uses the siRNA antisense strands as primers and target mRNA as template to make long antisense strands. **(d)** The product of step (c) is new trigger dsRNA. **(e)** Dicer chops up the new trigger dsRNA to make more siRNA, which can start a new round of priming and siRNA amplification. (*Source:* Adapted from Nishikura. *Cell* 107 (2001) f. 1, p. 416.)

RNAi in organisms like *C. elegans*. Fire and colleagues solved this riddle by showing that *C. elegans* cells employ an enzyme: **RNA-directed RNA polymerase** (**RdRP**) that uses antisense siRNAs as primers to make many copies of siRNA, as shown in Figure 16.33.

To test this hypothesis, Fire and colleagues used an RNase protection assay with a labeled sense strand probe to detect antisense siRNA in *C. elegans* fed on bacteria expressing trigger dsRNA at high levels. They used two different triggers and found large amounts of new siRNA produced in both cases. In addition, they discovered some **secondary siRNAs** outside the bounds of the trigger RNA. It is significant that these secondary siRNAs always corresponded only to the mRNA region upstream of the trigger sequence. This finding makes sense in the context of RdRP activity, because the trigger siRNA should prime synthesis toward the 5′ (upstream)-end of the mRNA. Thus, the discovery of secondary siRNAs also supports the hypothesis that an RdRP amplifies the siRNA, using the target mRNA as the template.

Thus, a mechanism does exist for amplifying the input dsRNA, and this could explain the great power of RNAi. The first round of this mechanism depends on priming by antisense siRNA on an mRNA template. This model can explain the earlier finding of Fire and colleagues that modification of the antisense, but not the sense, strand of the trigger dsRNA blocks RNAi. The model is also compatible with the earlier discovery of an RdRP in tomato cells, and the presence of homologous genes in fungi, and other plants, that are required for efficiency of RNAi.

SUMMARY In certain organisms, including *C. elegans*, siRNA is amplified during RNAi. This happens when antisense siRNAs hybridize to target mRNA and prime synthesis of full-length antisense RNA by an RNA-dependent RNA polymerase. This new dsRNA is then digested by Dicer into new pieces of siRNA.

Role of the RNAi Machinery in Heterochromatin Formation and Gene Silencing

In 2002, evidence began accumulating that implicated the RNAi machinery in heterochromatin formation and gene silencing, known as **transcriptional gene silencing** (**TGS**), as well as in RNAi itself. Then investigators found that siRNA-induced gene silencing can target a gene's control region through DNA and histone methylation.

RNAi and Heterochromatization Shiv Grewal, Robert Martienssen, and their colleagues deleted the RNAi genes encoding Dicer, Argonaute, and RdRP (*dcr1, ago1,* and *rdp1*, respectively) in the fission yeast *Schizosaccharomyces pombe* and found that all of these mutants were defective in the silencing that normally affects transgenes inserted near the centromere. That is, these transgenes became active in the RNAi mutants. Note that no trigger dsRNAs for the transgenes had been added, so RNAi was not directly involved in silencing the transgenes.

The investigators also looked to see whether the repeated DNA sequences (*cen3* sequences) at the centromere were transcribed in wild-type cells and in the mutants. Using Northern blots, they found no trace of such transcripts in wild-type cells, but they found three abundant transcripts in the RNAi mutants. A more detailed investigation using RNA dot blots showed that the reverse transcript of the *cen3* sequences appeared in wild-type and mutant cells, but the forward transcript appeared only in the mutants. Furthermore, nuclear run-on analysis demonstrated the same pattern: forward transcripts only in the mutants. Thus, the concentration of *cen3* transcripts is controlled at the transcriptional, rather than the post-transcriptional, level.

Next, the investigators examined specific core histone methylation in centromeric repeats using ChIP with antibodies against methylated histone H3 lysine 4 and lysine 9. As we learned in Chapter 13, methylated lysine 4 of histone H3 is associated with active genes, whereas methylated lysine 9 correlates with heterochromatin and gene inactivity. As expected from the activities we have already discussed, wild-type cells had lysines 4 and 9 that were both methylated in the centromeric region, but all three RNAi mutants showed an aberrant pattern of centromeric histone H3 methylation:

a high level of lysine 4 methylation, but a very low level of lysine 9 methylation. The same pattern was found in a *ura4+* transgene placed in the outermost centromere region (*otr*): a high level of lysine 9 methylation in wild-type cells, but a greatly depressed level in all three RNAi mutants.

Is RNAi responsible for histone methylation, and the resulting heterochromatization at the centromere? If so, we would expect at least some RNAi proteins to interact with centromeric chromatin, and we would also expect to find siRNAs corresponding to centromeric RNA. Martienssen and colleagues did indeed find that the Rdp1 part of the RNAi machinery binds to centromeric chromatin. And B.J. Reinhard and David Bartel had already found evidence to support the second prediction of the hypothesis when they cloned apparent Dicer products from wild-type cells and showed that all 12 clones came from transcripts of the centromeric region.

Thus, at least one component of the RNAi machinery is found at the centromere, and siRNAs are made from centromeric transcripts. All these data, and more, led Martienssen and colleagues to propose that RNAi is involved in heterochromatic silencing at the centromere (Figure 16.34). In particular, they proposed that the abundant reverse transcripts of the *otr* region base-pair with forward transcripts produced occasionally by RNA polymerase II, or perhaps by RdRP, to form trigger dsRNA. Dicer then digests this dsRNA to produce siRNA, and the siRNA associates with an Argonaute1 protein (**Ago1**) in a complex called **RITS** (for RNA-induced transcriptional silencing complex). This complex can then attract RdRP in a complex known as RDRC (for RNA-directed RNA polymerase complex) which amplifies the double-stranded siRNA. By base-pairing either to the DNA directly or to transcripts of the DNA, the siRNA then escorts RITS to corresponding sites on the genome. RITS then causes recruitment of a histone H3 lysine 9 methyltransferase. Once a lysine 9 is methylated, it can recruit Swi6, which is required for forming heterochromatin. Other proteins may be required, but the end result is spreading of heterochromatin to the *otr* region of the centromere. Whatever the mechanism, it is likely to be highly conserved, because mammalian pericentromeric heterochromatin structure also involves histone H3 lysine 9 modification and some RNase-sensitive substance, which could be one or more of the RNAi intermediates.

Does the RITS complex associate directly with DNA, or is it attracted by transcripts of chromatin regions that are targeted for silencing? In 2006, Danesh Moazed and colleagues provided evidence for the importance of transcripts in this process by showing that artificially tethering RITS to a nascent transcript of the *ura4*$^+$ gene resulted in silencing of this normally active gene.

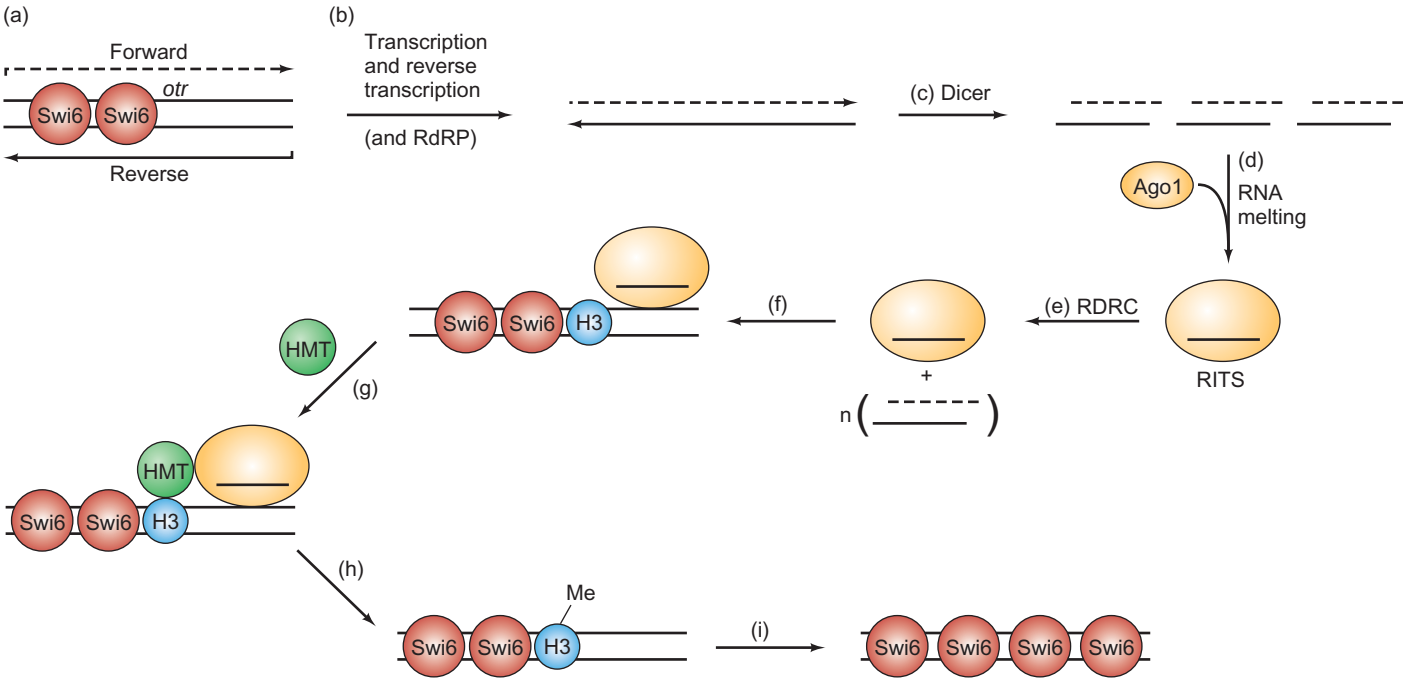


Figure 16.34 A model for the involvement of the RNAi machinery in the heterochromatization at the *S. pombe* centromere. **(a)** The outermost region *(otr)* of the centromere is constantly being transcribed to produce reverse transcripts, and production of forward transcripts probably also occurs at a low (undetectable) level. **(b)** After transcription and reverse transcription (or after reverse transcription and RdRP action), we have double-stranded RNA (dsRNA). **(c)** Dicer cuts the dsRNA into siRNAs. **(d)** Ago1 (yellow, perhaps along with other proteins) associates with single-stranded siRNAs to produce the RITS. **(e)** The RdRP in the RDRC amplifies the siRNA, producing double-stranded siRNAs. **(f)** The RITS, through its siRNA, associates with the *otr,* either through direct interaction with the DNA, or through interaction with transcripts in this region. **(g)** The RITS attracts a histone methyltransferase (HMT, green) to the *otr.* **(h)** The HMT methylates the lysine 9 of a histone H3 (blue). Of course, this histone is part of a nucleosome, which is not shown here, for simplicity. **(i)** This methylation in turn attracts more Swi6 (red), which helps to spread heterochromatization.

It seems paradoxical that, in order for a region like a centromere to be silenced, it has to be expressed. How, then, does expression occur after mitosis to preserve heterochromatization in the genomes of both progeny cells? A solution to this paradox was proposed by Rob Martienssen and colleagues and Grewal and colleagues in 2008. Together, the work of these two groups showed that serine 10 of histone H3 in centromeric heterochromatin in *S. pombe* becomes phosphorylated during mitosis, and that this results in the loss of methylation of lysine 9 of histone H3, and therefore in the loss of the Swi6 protein that is necessary for heterochromatization. As a result, the chromatin opens up enough that it is transcribed during the S phase. This produces centromere transcripts, presumably in both directions, that attract the RNAi machinery, so the centromere can be heterochromatized again during the ensuing long G_2 phase.

This hypothesis views heterochromatin as more dynamic than the traditional view of a static, condensed, inactive structure. Does it also open up the possibility of real expression of centromeric DNA? Apparently not. For one thing, centromeric transcription is confined to the S phase, in which gene expression is very restricted. For another, the centromeric transcripts are rapidly degraded, either by the RNAi machinery, or by other RNA-degrading systems that recognize aberrant transcripts.

Grewal and colleagues noted that centromere-like sequences are also found at sites such as the silent mating-type region, which lies far from the centromere but is also silenced by heterochromatization. In separate experiments, these workers showed that the RNAi machinery is required for initiating heterochromatization at the silent mating-type region, but is expendable for maintaining and inheriting the silencing. Swi6 is apparently sufficient for such heterochromatin maintenance.

The role of the RNAi machinery in centromeric events is not confined to lower organisms. In 2004, Tatsuo Fukagawa and colleagues reported tests on a chicken–human hybrid cell line whose only human chromosome was chromosome 21. These workers then made the Dicer gene tetracycline-repressible in these hybrid cells and observed what happened, particularly to human chromosome 21, when Dicer expression was blocked by tetracycline. The most obvious effect of the loss of Dicer was that the cells died after about five days.

Moreover, the specific pathologies of these cells point to problems with the centromere: The cells showed abnormal mitoses with evidence of premature sister chromatid separation. As in yeast cells with defective RNAi, these vertebrate cells exhibited abnormal buildup of transcripts of the centromeric repeat region of human chromosome 21. They also showed abnormal localization of some, but not all, centromeric proteins. The problems at the centromere were presumably caused by the loss of Dicer, and this in turn led to the failure of cell division and to cell death.

We assume that the events that occur in the centromeric region in fission yeast, illustrated in Figure 16.34, help to explain these results in cells from higher organisms. However, one caveat to bear in mind is that mammals appear to lack an RdRP. So any dsRNA that appears at the centromere in mammals must be made by bidirectional transcription of this region, or of a homologous region elsewhere in the genome.

Another major difference between heterochromatization in fission yeast and in plants and mammals is that the latter organisms experience DNA methylation in addition to histone methylation. The methyl groups are added to the C's of **CpG sequences** in both strands, and these help to attract the proteins that induce heterochromatization. Again, the presence of double-stranded RNA appears to play a key role by recruiting the RNAi machinery, which stimulates DNA methylation.

One significant advantage of this mechanism is that it is permanent. Once the DNA is methylated on the C's of both strands of a CpG sequence, this methylation is inherited from one cell generation to the next, as the methylated C on one strand ensures that the new C on the opposite strand will also be methylated after DNA replication. Although this methylation is permanent, it is not a true genetic change, which would be a change of one base to another (e.g., a C changed to a T). Instead, we call it an **epigenetic** modification of the DNA. It is every bit as important as a genetic change because it can cause the silencing of a gene or even heterochromatization of a whole region of a chromosome.

RNAi may also play a role in X chromosome inactivation in mammals. In each cell of a female mammal, one of the X chromosomes is inactivated by heterochromatization. This prevents the very deleterious consequences of elevated levels of X chromosome products. One of the first steps in X chromosome inactivation is histone H3 lysine 9 methylation. And this methylation occurs immediately after the appearance of a noncoding transcript of the *Xist* locus. We also know that *Xist* is controlled by the antisense RNA, *Tsix,* and by *Xist* promoter methylation. The presence of *Tsix* and *Xist* transcripts in the same cell would of course invoke the RNAi system, and that could recruit the histone methylase that kicks off the formation of heterochromatin.

SUMMARY The RNAi machinery is involved in heterochromatization at yeast centromeres and silent mating-type regions and is also involved in heterochromatization in other organisms. At the outermost regions of centromeres of fission yeast, active transcription of the reverse strand occurs. Occasional forward transcripts, or forward transcripts made by RdRP, base-pair with the reverse transcripts to kick off RNAi, which in turn recruits a histone methyltransferase, which methylates lysine 9 of histone H3, which recruits Swi6, which causes heterochromatization. In plants and mammals, this process is abetted by DNA methylation, which can also attract the heterochromatization machinery.

Transcriptional Gene Silencing Induced by siRNA Directed at a Gene's Control Region Kevin Morris and colleagues found in 2004 that mammalian genes can also be silenced by the RNAi machinery and, as we have seen with heterochromatization in plants and mammals, this silencing involves DNA methylation. Furthermore, in contrast to normal RNAi, this silencing involves an siRNA directed at the control region, rather than the coding region, of a gene.

Morris and colleagues targeted a green fluorescent protein reporter gene driven by the human elongation factor 1α gene (EF1A) promoter-enhancer region. They transduced human cells with feline immunodeficiency virus (FIV) containing this reporter construct, which caused integration of the reporter gene and its control region into the human genome. The FIV vector also made the nuclear membrane permeable to the siRNA, which otherwise would not have been taken up by the mammalian nuclei.

Because the siRNA in this case was directed against the gene's control region, and not its coding region, we would predict that it could not cause mRNA destruction or block translation. Indeed, we would predict that it would block transcription, and indeed that is what Morris and colleagues showed. Using real-time RT-PCR (Chapter 4), they demonstrated almost total disappearance of the GFP transcript upon transducing cells with the EF52 siRNA, which targets the control region of the fusion gene. By contrast, an siRNA that targets the coding region of the GFP mRNA caused a relatively modest 78% reduction in the concentration of the GFP transcript (Figure 16.35a).

Because a common feature of transcriptional silencing in mammals is histone and DNA (cytosine) methylation, Morris and colleagues tested the effect of trichostatin (TSA) and 5-azacytidine (5-azaC), which inhibit histone and DNA methylation, respectively. These drugs completely reversed the silencing caused by the EF52 siRNA, but had no effect on silencing caused by the GFP coding region siRNA. These results supported the hypothesis that DNA and/or histone methylation are involved in silencing caused by the EF52 siRNA.

To check whether the silencing by the EF52 siRNA was at the transcription level, Morris and colleagues performed nuclear run-on assays (Chapter 5). Figure 16.35b shows that EF52 did indeed dramatically reduce the number of initiated GFP transcripts, while it had no effect on irrelevant glyceraldehyde-phosphate dehydrogenase (GAPDH) transcripts.

To see whether DNA in the gene's control region was really methylated during transcriptional silencing, Morris and colleagues used *Hin*P1I, a restriction enzyme that cuts at a site that includes a CpG. If the C in this sequence is unmethylated, *Hin*P1I will cut, but if it is methylated it will not. There is a *Hin*P1I site in the control region of the EF1A gene. Thus, if this site is methylated, it will be protected from *Hin*P1I cleavage, and PCR using primers on opposite sides of the site will produce a product. On the other hand, if the site is unmethylated, *Hin*P1I will cut it, and no PCR product will appear.

Figure 16.36 shows the results of this experiment. The control in lane 1 shows that a plasmid with a *Hin*P1I site methylated in vitro really does yield a PCR product, even after attempted cleavage with *Hin*P1I. Lanes 2 and 3 are controls with DNA from cells that had been transduced with an irrelevant siRNA or a GFP coding region siRNA,

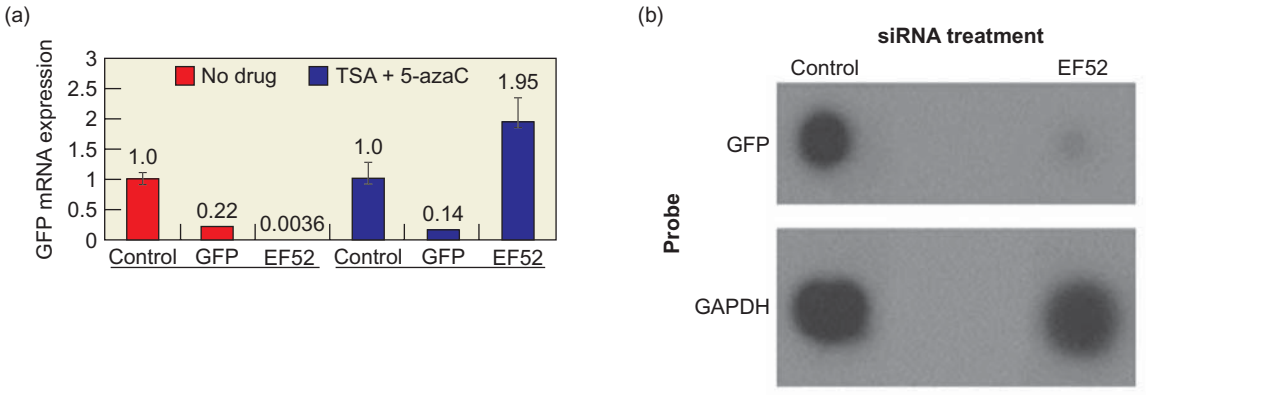


Figure 16.35 Silencing by an siRNA targeting the EF1A gene control region. (a) Real-time PCR assay for GFP mRNA in human cells bearing a GFP gene driven by the EF1A gene promoter-enhancer region. Cells were transduced with FIV bearing the GFP gene construct, and then siRNAs were added in the absence (no drug), or presence of TSA and 5-azaC. Then real-time PCR was performed to measure the concentration of GFP mRNA. The bars (and corresponding quantifications) show the results with no siRNA (control), an siRNA that targets the coding region of the mRNA (GFP), and an siRNA that targets the EF1A gene control region (EF52). **(b)** Nuclear run-on assay for transcription. Nuclei were isolated from cells transduced with the EF1A-GFP construct, plus either the EF52 siRNA or no siRNA (control). Labeled nuclear run-on mRNA was synthesized and hybridized to blots of GFP DNA, or GAPDH DNA, as indicated at left. The EF52 siRNA silenced the GFP gene, but not the GAPDH gene, at the transcriptional level. (*Source:* Reprinted with permission from *Science,* Vol. 305, Kevin V. Morris, Simon W.-L. Chan, Steven E. Jacobsen, and David J. Looney, "Small Interfering RNA-Induced Transcriptional Gene Silencing in Human Cells," Fig. 1, p. 1290, Copyright 2004, AAAS.)

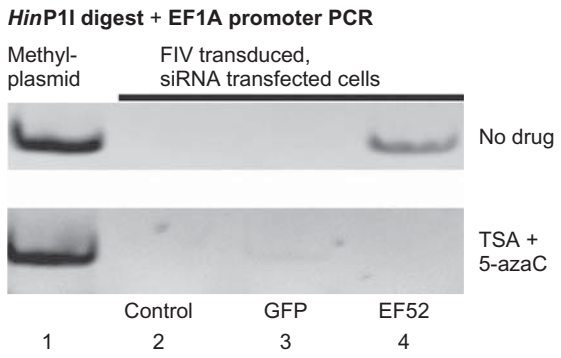


Figure 16.36 Demonstration of methylation of the EF1A gene control region in response to siRNA. Morris and colleagues tested for methylation of a CpG sequence in the EF1A control region by cleavage with *Hin*P1I, which cleaves unmethylated, but not methylated sites including CpG sequences. They performed the cleavage on DNA from cells either untreated (top row, "No drug") or treated (bottom row) with TSA plus 5-azaC to block methylation of CpG sequences. After treatment with *Hin*P1I, they performed PCR with primers flanking the CpG site. Only uncut (methylated) DNA should yield a signal. Lane 1, positive control with synthetically methylated site. Lane 2, negative control with irrelevant siRNA. Lane 3, negative control with an siRNA directed against the GFP coding region, rather than the control region. Lane 4, experimental result with an siRNA that targets the control region. With this siRNA, the CpG is methylated (uncut, and therefore yields a PCR signal) in the absence of drug, but is not methylated when the methylation blocker was included. (*Source:* Reprinted with permission from *Science,* Vol. 305, Kevin V. Morris, Simon W.-L. Chan, Steven E. Jacobsen, and David J. Looney, "Small Interfering RNA-Induced Transcriptional Gene Silencing in Human Cells," Fig. 1, p. 1290, Copyright 2004, AAAS.)

respectively. Lane 4 shows the results with cells transduced with the EF52 siRNA. The top row shows that the DNA must have been methylated, because it was protected from *Hin*P1I cleavage, and a PCR product appeared. However, the bottom row shows that the methylation-blocking drugs TSA and 5-azaC, blocked methylation, rendering the *Hin*P1I site cleavable, so no PCR product appeared.

All of the experiments described so far used cells that were transduced with FIV, which inserted the EF1A gene into the human genome, but not in its natural location. To check for siRNA silencing of the endogenous human gene, Morris and colleagues performed the same kinds of experiments as in Figures 16.35 and 16.36, but with cells rendered permeable to siRNAs with MPG, a fusion peptide that contains an HIV-1 transmembrane peptide linked to the nuclear localization signal from SV40 virus. In these experiments, no EF1A gene was introduced into the cells, so only the endogenous gene was present, and it was silenced (though not as dramatically as in the previous experiments) by the EF52 siRNA. As before, this silencing was accompanied by DNA methylation, and could be blocked by methylation inhibitors.

Where does the siRNA in these experiments come from? After all, it is directed at the control region, not the coding region, of the gene, so it cannot come from a normal gene transcript. Morris and colleagues showed that the sense strand part of the siRNA probably came from a 5′-extended transcript of the EF1a gene—that is, a transcript that started in the promoter, upstream of the normal transcription start site. They detected this extended transcript with an **RNA pull-down** procedure that used a 5′-biotin-labeled promoter antisense RNA and avidin bound to magnetic beads. The biotin-labeled promoter antisense RNA hybridized in vivo to the RNA transcribed through the promoter region, and the avidin-tagged beads bound to the biotin, allowing the whole RNA-RNA-bead complex to be isolated ("pulled down") magnetically.

Quantification of the promoter-associated RNA and the normal EF1a transcripts by real-time RT-PCR yielded a ratio of about 1:570. Thus, about one in 570 transcripts of the EF1a gene begins within the promoter. A 5′-RACE procedure (Chapter 5) showed that these promoter-associated transcripts begin about 230 bp upstream of the normal transcription start site, and a 3′-RACE procedure showed that these transcripts extend as far in the 3′-direction as the normal transcripts and are spliced and polyadenylated.

Does the promoter-associated RNA play a role in transcriptional gene silencing (TGS)? To answer this question, Morris and colleagues targeted the promoter-associated RNA for destruction by RNase H (Chapter 14), by transfecting cells with a promoter-associated RNA-specific phosphorothioate oligonucleotide, which acts like a deoxyribo-oligonucleotide in this procedure. The destruction of the EF1a promoter-associated RNA abolished transcriptional silencing by added promoter-associated siRNA. By contrast, RNase H-mediated destruction of a promoter-associated RNA from another gene (CCR5) had no effect on TGS of the EF1a gene. Thus, a promoter-associated RNA appears to be essential for TGS.

One of the epigenetic changes that occurs in the EF1a control region during gene silencing is a trimethylation of lysine 27 of histone H3 (H3K27me3) in a nucleosome at that site. Does the promoter-associated RNA play a role in this epigenetic change? A pull-down assay showed that it does. When the EF1a promoter-associated RNA was destroyed by oligonucleotide and RNase treatment, the chromatin could no longer be precipitated with an anti-H3K27me3 antibody. On the other hand, treatment with the irrelevant oligonucleotide directed at the CCR5 control region did not block precipitation of the EF1a promoter-associated nucleosome with an anti-H3K27me3 antibody.

Thus, the presence of the promoter-associated RNA is required for the silencing methylation of H3K27. The exact nature of that requirement is still unclear, but one can imagine that the promoter-associated RNA would hybridize to an antisense RNA (perhaps the antisense strand of an siRNA). This hybrid would in turn recruit a chromatin remodeling complex, including the H3K27 methyltransferase, which would trimethylate H3K27, helping to silence the gene.

All of the silencing we have discussed so far is due to epigenetic modification (usually methylation) of chromatin.

Another silencing mechanism targets nuclear RNA: Endogenous double-stranded siRNAs can enter the nucleus and cause degradation of nuclear RNAs by the familiar RNAi mechanism. Scott Kennedy and colleagues showed in 2008 that siRNAs bind to an Argonaute protein (NRDE-3 in *C. elegans*) in the cytoplasm. NRDE-3 has a **nuclear localization signal** that targets it to the nucleus, so the siRNA-NRDE-3 complex can enter the nucleus and collaborate in the destruction of cognate nuclear pre-mRNAs. Note that the nuclear location distinguishes this mechanism from ordinary RNAi, which occurs in the cytoplasm.

SUMMARY Individual genes in mammals can also be silenced by an RNAi mechanism that targets the control region, rather than the coding region, of the gene. This silencing process involves DNA and histone methylation, rather than mRNA destruction. One requirement for such histone methylation in siRNA-induced gene silencing, at least in some genes, is production of a 5′-extended transcript that begins within the gene's control region (a promoter-associated transcript). This transcript presumably associates with an antisense RNA, and then recruits a chromatin remodeling complex, including a histone methyltransferase, which methylates H3K27 on a nearby nucleosome, helping to silence the gene. Genes can also be silenced by a nuclear RNAi process that involves Argonaute proteins that are targeted to the nucleus by a nuclear localization signal.

Transcriptional Gene Silencing in Plants The short RNAs required for TGS in fission yeast and animals are made by RNA polymerase II. But in TGS in flowering plants, two other polymerases, **RNA polymerase IV** and **RNA polymerase V,** which are evolutionarily derived from polymerase II, play the key roles. Polymerase IV produces the 24-nt heterochromatic siRNAs whose yeast and animal counterparts are made by polymerase II. The role of polymerase V is more subtle, and was therefore more difficult to unravel.

Polymerase V produces transcripts of non-coding regions that are more than 200 nt long, have either caps or triphosphates at their 5′-ends, and are not polyadenylated. Transcripts in a given region have multiple 5′-ends, which suggests they are made in a promoter-independent manner. In 2008, Craig Pikaard and colleagues demonstrated the involvement of polymerase V in transcriptional gene silencing by mutating the largest subunit of the enzyme. They observed, in addition to loss of polymerase V activity, loss of transcripts of certain non-coding regions, and defective silencing in overlapping and adjacent chromatin regions. Furthermore, they found that some of the hallmarks of heterochromatin, including histone and DNA methylation, were lost in cells lacking polymerase V activity.

How do the polymerase V transcripts attract the silencing machinery? Pikaard and colleagues proposed a model very similar to that in Figure 16.34, except that polymerases IV and V play roles performed by polymerase II in fungi and animals. The polymerase V transcripts attract a complex composed of Argonaute 4 (Ago4) and siRNA (made by polymerase IV). This complex in turn attracts the silencing machinery. In 2009, Pikaard and colleagues provided more support for this hypothesis, as follows. First, they performed ChIP analysis with chromatin from *Arabidopsis* plants that produce mutant Ago4 and polymerase V. They found that both wild-type Ago4 and polymerase V bound to transposon genes that are normally silenced, but mutations in either the *Ago4* gene or the *nrpe1* gene, which encodes the largest polymerase V subunit, abolished this association. Thus, Ago4 and polymerase V are necessary for Ago4 to associate with chromatin that is to be silenced.

To test whether polymerase V transcripts are required to recruit Ago4 to chromatin, Pikaard and colleagues performed ChiP analysis in wild-type plants, and in plants bearing a mutation at the active site of the largest subunit of polymerase V. The mutant polypeptide is stable and can still bind normally to the second-largest subunit, but it is utterly incapable of making transcripts. ChIP analysis showed no binding of Ago4 to target chromatin sites in the mutant plants. This binding could be restored by transforming plants with the wild-type *nrpe1* gene, but not with the mutant gene. Thus, transcription by polymerase V is required to recruit Ago4, in accord with the hypothesis.

It is important to note that polymerase V transcripts are found throughout the genome of *Arabidopsis thaliana,* a member of the mustard family, in heterochromatic and euchromatic regions alike. How then do the euchromatic regions avoid silencing? Pikaard and colleagues proposed that polymerase V transcripts are necessary, but not sufficient, for silencing. The silencing process also requires siRNAs. Therefore, because euchromatic regions do not give rise to siRNAs, they are not silenced.

Earlier in this chapter, we discussed the paradox that silenced chromatin must be transcribed in order to be silenced. The existence of polymerases IV and V gives flowering plants a way to deal with this problem: These polymerases appear not to initiate at promoters, and they are not subject to the same rules as polymerase II. Thus, they can presumably initiate transcription even in chromatin regions that are silenced with respect to polymerase II.

SUMMARY Flowering plants have two nuclear RNA polymerases, polymerase IV and polymerase V, that are not found in animals and fungi. Polymerase IV makes siRNAs corresponding to chromatin regions

to be silenced. Polymerase V makes longer RNAs from regions throughout the plant genome. These longer RNAs attract siRNA-Ago4 complexes, but only to regions that are targets for silencing, from which these siRNAs were made. These complexes in turn attract the enzymes required to methylate both DNA and histones, which in turn leads to heterochromatization.

16.7 Piwi-Interacting RNAs and Transposon Control

In Chapter 23 we will learn that DNA elements known as transposons can transpose, or jump from place to place in a genome. In doing so, they can interrupt and inactivate genes, or even break chromosomes. Thus, transposition is a dangerous process that can lead to cell death or disease, such as cancer. Accordingly, it is important that cells be able to control transposition. This is particularly true in germ cells, which give rise to gametes that will pass genes on to the next generation. The serious mutations or cell death caused by transposition in germ cells reduce reproductive success and therefore threaten a species's survival.

It is not surprising, therefore, that organisms have evolved mechanisms for dealing with transposons, and that these can be targeted to germ cells. In fact, germ cells produce another class of small RNAs, 24 to 30 nt long, called **Piwi-interacting RNAs** (**piRNAs**). Like siRNAs and miRNAs, piRNAs associate with Argonaute proteins, but these proteins belong to a different branch, or clade, of the Argonaute superfamily than the Ago proteins we have been discussing. The piRNAs bind to members of the Piwi clade, while siRNAs and miRNAs bind to members of the Ago clade.

The piRNAs of fruit flies and mammals tend to be complementary to either the sense or antisense strand of transposons from the same organism. These piRNAs derive from clusters of piRNA genes, apparently via transcription of a long cluster and subsequent processing of the precursor RNA into mature piRNAs. Some, if not most, of this processing may actually occur simultaneously with inactivation of transposons, by a so-called ping-pong amplification loop, as follows (Figure 16.37):

In *Drosophila*, Piwi proteins such as **Piwi** and **Aubergine** tend to associate with piRNAs that are complementary to transposon mRNAs; these piRNAs usually have a U in the first position. This piRNA-Piwi or -Aubergine complex can associate through base-pairing with a transposon mRNA, which triggers slicer cutting 10 nt upstream of an A that is base-paired to the U at the 5′-end of the piRNA. This cut, together with processing at the 3′-end of the transposon mRNA, creates a short RNA that can associate with another protein, Ago3, which preferentially binds to RNAs that represent parts of transposon mRNAs. The RNA-Ago3 complex can then bind to a piRNA precursor RNA by base-pairing, and the slicer activity of Ago3 cuts just upstream of the U of the A–U base pair. This cut, together with end processing of the piRNA precursor, creates a mature piRNA that can bind to Piwi or Aubergine to start the cycle over.

Note that this mechanism accomplishes two things: It slices up transposon mRNA, thereby blocking transposition, and it amplifies the amount of piRNA available, thus stimulating the process. Because the transcription of piRNA clusters is confined to germ cells, and somatic cells immediately

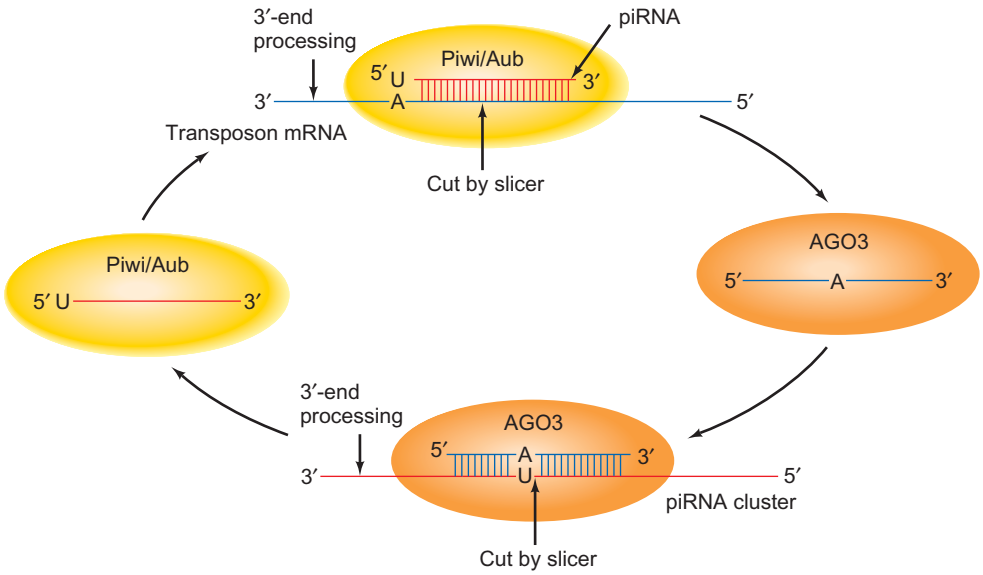


Figure 16.37 Model for a ping-pong amplification loop for piRNAs. Details are in the text.

surrounding the germ cells, transposition is specifically blocked in germ cells, where it would be especially dangerous.

Animal somatic cells do not produce piRNAs, so transposons must be inactivated by another mechanism in these cells. Phillip Zamore and colleagues showed in 2008 that *Drosophila* somatic cells produce endogenous siRNAs complementary to transposon mRNAs (and to some normal cellular mRNAs). These endogenous siRNAs are distinguished from miRNAs, which we will discuss later in this chapter, by two features: They contain a 2′-O-methylation at their 3′-ends; and they have a very narrow size distribution centered on 21 nt. Furthermore, they are not derived from stable stem-loop precursors, as miRNAs are. These endogenous siRNAs are also unlike piRNAs in that they have no tendency to begin with U or to have an A at position 10. Thus, *Drosophila* somatic cells use an endogenous RNAi mechanism, rather than a piRNA-based mechanism, to control transposition. Furthermore, although animal germ cells have the piRNA pathway to inactivate transposons, they also appear to produce endogenous siRNAs directed against at least some transposons, so they can bring at least two different mechanisms to bear on the transposon problem.

Plants lack Piwi proteins, so they must use a different pathway to produce and amplify RNAs complementary to transposon mRNAs. *Arabidopsis* cells produce short RNAs from transposons by an unknown mechanism, and these RNAs bind to the Ago protein Ago4. Without Piwi proteins to produce complementary RNAs by an amplification loop, these complementary RNAs are made by RNA-dependent RNA polymerases (see previous section). The short RNAs complementary to both strands of a transposon can anneal to form a trigger dsRNA that initiates destruction of transposon mRNA by RNAi.

SUMMARY Transposition of transposons is blocked in animal germ cells by a ping-pong amplification and mRNA destruction mechanism involving piRNAs. A piRNA complementary to a transposon mRNA binds to Piwi or Aubergine, and then base-pairs to a transposon mRNA. This initiates cleavage of the transposon mRNA by a slicer activity in the Piwi protein, and the 3′-end of the transposon mRNA is also processed. The resulting small RNA binds to Ago3, where it can base-pair to a piRNA precursor RNA. This initiates cleavage of the precursor RNA at a specific A–U base pair 10 nt from the 5′-end of the transposon mRNA fragment. Together with 3′-end processing of the precursor RNA, this generates a mature piRNA that can participate in a new round of transposon mRNA destruction and piRNA amplification. No piRNAs are produced in animal somatic cells, but transposition can be blocked by an endogenous RNAi mechanism. Plants lack Piwi proteins, so they must rely on an RNAi mechanism to control transposition in somatic and germ cells alike. Plants do have RNA-dependent RNA polymerases, so they can readily amplify siRNAs directed at transposon mRNAs.

16.8 Post-Transcriptional Control of Gene Expression: MicroRNAs

The siRNAs and piRNAs are not the only small RNAs that participate in gene silencing. Another class of small RNAs called **microRNAs** (**miRNAs**) are 22-nt RNAs produced naturally in plant and animal cells by cleavage from a larger, stem-loop precursor. In animals, these miRNAs then base-pair (though imperfectly) with the 3′-untranslated regions of specific mRNAs and silence gene expression primarily by blocking translation of those mRNAs. In plants, miRNAs base-pair perfectly (or almost so) with the interiors of mRNAs and direct the cleavage of those mRNAs. Let us consider the actions of miRNAs, and then their biogenesis.

Silencing of Translation by miRNAs

The first inkling of the importance of miRNAs came from work that began in 1981, which showed that mutations in the *lin-4* gene of the roundworm *(Caenorhabditis elegans)* caused developmental abnormalities. Subsequent genetic work suggested that the *lin-4* gene product acted by suppressing the level of LIN-14, the protein product of the *lin-14* gene. Interestingly, Gary Ruvkun and his colleagues showed that *lin-4* needed the **3′-untranslated region** (**3′-UTR**) of the *lin-14* mRNA in order to exert its LIN-14 suppression. Finally, in 1993, Victor Ambros and colleagues mapped the *lin-4* mutation, and found that it did not map to a protein-encoding gene. Instead, it mapped to the gene encoding the precursor of an miRNA. This suggested that an miRNA played an important role in *C. elegans* development, by reducing the expression of the *lin-14* gene. The sequence of the *C. elegans* genome bolstered this suggestion, showing that the miRNA was partially complementary to sequences within the 3′-UTR of the *lin-14* mRNA—the very sequences that are required for *lin-4* function.

We now know that miRNAs play crucial roles in the regulation of plant and animal genes. There are hundreds of miRNA genes in most plant and animal species examined so far, and each miRNA potentially controls many other genes. Mutations in miRNA genes typically have very deleterious effects, especially on development, underscoring the importance of these mRNAs, and suggesting that

many disease states may be caused by mutations in, or improper regulation of, miRNA genes.

Indeed, miRNAs are so important in regulating genes in normal and diseased cells that they have enormous potential as drug targets in treating diseases such as cancer. Typically, cancer cells have abnormal spectra of miRNA expression, with some miRNAs unusually scarce and others unusually abundant. The trick will be to find which of these are important to the disease state, and then try to use drugs, possibly including the miRNA precursors themselves, to adjust the concentrations of those key miRNAs. However, macromolecules like miRNA precursors are notoriously difficult to use as drugs, and it is not clear how to selectively control the genes that encode miRNAs.

Given the importance of miRNAs, it is important to understand the mechanism by which they control genes. We will examine some of the evidence leading to different conclusions, but we will see that no one mechanism can explain all the data at hand.

In 1999, Philip Olsen and Ambros first demonstrated that the *lin-4* miRNA acts by limiting translation of the *lin-14* mRNA. The LIN-14 protein plays an important role in *C. elegans* development. During the first larval stage (L1), LIN-14 levels are high because this protein helps to specify the fates of cells that develop in that stage. However, at the end of L1, LIN-14 levels must drop so that other proteins can determine cell fate in the second larval stage, L2. This suppression of LIN-14 level depends on the *lin-4* RNA, a 22-nt miRNA that base-pairs to seven imperfect repeats of a sequence partially complementary to *lin-4* in the 3′-UTR of the *lin-14* mRNA.

Olsen and Ambros performed Western blots (Chapter 5) that showed at least a 10-fold decrease in LIN-14 protein between the L1 and L2 stages. On the other hand, their nuclear run-on analysis (Chapter 5) showed that the steady-state level of *lin-14* mRNA decreased less than two-fold between L1 and L2. Thus, control of *lin-14* appears to be at the translational level, not the transcriptional level.

Next, Olsen and Ambros used RT-PCR (Chapter 4) to amplify the 3′-ends, and thereby measure the sizes of the poly(A) tails, of *lin-14* mRNAs from the L1 and L2 stages. This analysis showed that the poly(A) tails of the mRNAs from the two stages were unchanged. Thus, the *lin-14* mRNA is not destabilized by shrinking its poly(A) tail in the L2 stage. In fact, Olsen and Ambros showed that *lin-14* mRNA was associated with polysomes (ribosomes in the act of translating an mRNA [Chapter 19]) just as much in L2 as in L1. Thus, translation initiation on *lin-14* mRNA appeared to be working just as well in stage L2 as in L1.

If appearance of LIN-14 protein is blocked in L2, but initiation of translation of its mRNA is normal, a reasonable conclusion would be that elongation or termination of translation on this mRNA is somehow blocked. Indeed, if *lin-4* miRNA really does bind to its target sites in the 3′-UTR of the *lin-14* mRNA, it would be well positioned to interfere with termination of translation. If so, both *lin-4* miRNA and *lin-14* mRNA should be found together on polysomes.

To test this hypothesis, Olsen and Ambros purified polysomes from L1 and L2 larvae by sucrose gradient ultracentrifugation (Chapter 17), and checked them for the presence of *lin-14* mRNA and *lin-4* miRNA by RNase protection assay (Chapter 5). Figure 16.38 shows the results. The "hump" to the right in each diagram (top) contains the fast-sedimenting polysomes. The polysomes are also contained in the middle two lanes in the electropherograms

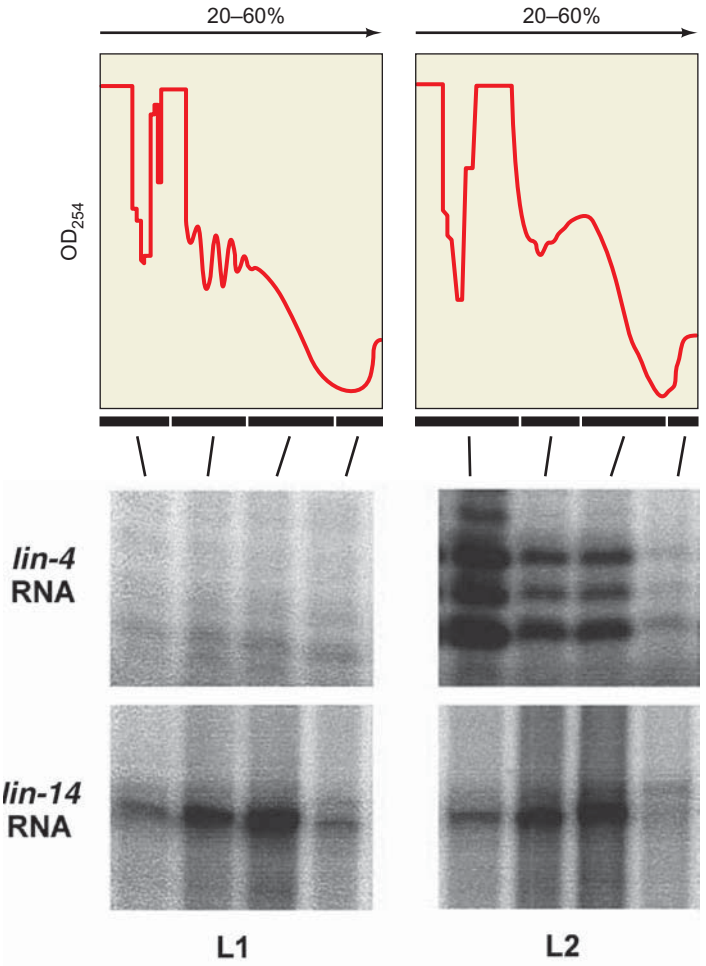


Figure 16.38 Both *lin-4* miRNA and *lin-14* mRNA are associated with polysomes in L1 and L2 larvae. Olsen and Ambros used sucrose gradient ultracentrifugation to display polysomes from *C. elegans* L1 (left) and L2 (right) larvae. They collected four fractions from the gradients, the middle two containing polysomes, and hybridized the RNAs from these fractions to labeled RNA probes for *lin-4* and *lin-14* RNAs. After they treated the RNA hybrids with RNase, they electrophoresed the protected probes on polyacrylamide gels. The results with *lin-4* and *lin-14* probes are at middle and bottom, respectively. The multiple bands represent protected probes differing by one nucleotide, and are presumably caused by "nibbling" at the ends of the hybrids by RNase. (*Source: Developmental Biology,* Volume 216, Philip H. Olsen and Victor Ambros, "The lin-4 Regulatory RNA Controls Developmental Timing in Caenorhabditis elegans by Blocking LIN-14 Protein Synthesis after the Initiation of Translation." fig. 8, p. 671–680, Copyright 1999, with permission from Elsevier.)

below the diagrams, which show the results of the RNase protection assays. We can see that the polysomes from both L1 and L2 larvae appear identical and contain approximately equal amounts of both *lin-4* miRNA (middle) and *lin-14* mRNA (bottom), presumably because the two RNAs are base-paired together.

These results present a difficulty: It is true that *lin-4* miRNA and *lin-14* mRNA are found together on polysomes, suggesting that they are base-paired together. But the polysome profile looks identical in L1 and L2 larvae. If the miRNA blocked translation elongation completely, or nearly completely, polysomes should have accumulated with very few ribosomes attached to the mRNA, so the polysomes would be lighter, and the peak would shift to the left. This was not observed. On the other hand, if the miRNA caused a more moderate inhibition of translation elongation, or if the miRNA blocked termination, polysomes should have accumulated with more ribosomes attached, and the polysome peak would shift to the right. This was not observed, either. Thus, *lin-4* miRNA does not appear to limit *lin-14* protein concentration in L2 embryos by a simple inhibition of translation elongation or termination. It is conceivable that *lin-4* miRNA inhibits both translation initiation and elongation in such a way that the polysome profile does not change. It is also possible that, by binding to the 3′-end of the mRNA, *lin-4* positions itself to capture newly synthesized LIN-14 protein and causes it to be degraded.

At least part of this question about *lin-4* miRNA activity could be explained by work by Amy Pasquinelli and her colleagues, reported in 2005. These workers used Northern blotting of *C. elegans* RNA (Figure 16.39) to show that

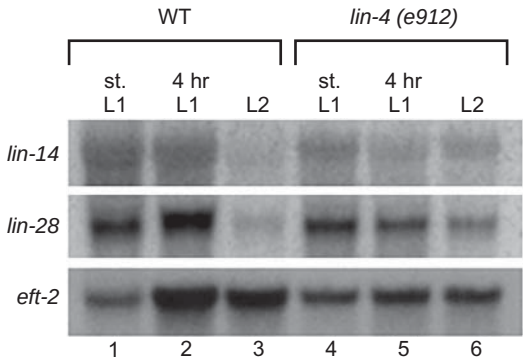


Figure 16.39 Concentrations of various mRNAs during development in *C. elegans*. Pasquinelli and colleagues Northern blotted RNAs from the following time points during *C. elegans* development, as indicated at top: starved L1; 4h L1; and L2. Then they hybridized the blot to probes for *lin-14* and *lin-28* mRNAs, as well as *eft-2* mRNA as a control (an mRNA known not to be influenced by *lin-4*). The concentrations of *lin-14* and *lin-28* mRNAs fell significantly between phases L1 and L2 in wild-type cells, but not in *lin-4(e912)* cells. (*Source*: Reprinted from *Cell,* Vol 122, Shveta Bagga, John Bracht, Shaun Hunter, Katlin Massirer, Janette Holtz, Rachel Eachus, and Amy E. Pasquinelli, "Regulation by let-7 and *lin-4* miRNAs Results in Target mRNA Degradation," p. 553–563, fig. 6a, Copyright 2005, with permission from Elsevier.)

lin-14 (and *lin-28*) mRNA levels actually do decrease about four-fold between stages L1 and L2. This figure also shows that this decrease depends on *lin-4* miRNA: Only modest decreases, at most, occurred in the *lin-4 e912* mutant. Thus, *lin-4* miRNA may exert its control via more than one mechanism.

Another approach to understanding the mechanism of miRNA action has been to use synthetic reporter mRNAs with one or more target sites for a particular miRNA, and then examine the effect of the miRNA (strictly speaking, a transfected siRNA that mimics the miRNA) on the behavior of the reporter mRNA. Phillip Sharp and colleagues tried one such strategy in 2006 and found that, when they inhibited translation initiation, the association of the reporter mRNA with ribosomes decayed more rapidly in the presence of the miRNA than in its absence. This suggested that the miRNA causes premature release of ribosomes from the mRNA (**ribosome drop-off**). These investigators also found that a reporter mRNA lacking a cap, but containing an internal ribosome initiation site (IRES), was also responsive to silencing by an miRNA. As we will learn in Chapter 17, cap recognition is the initiating step in eukaryotic translation, so this again indicated that the miRNA was acting downstream of the initiation step. Thus, the data were consistent with the ribosome drop-off model.

On the other hand, Filipowicz and colleagues presented evidence in 2005 for miRNA action at the translation initiation stage. They performed sucrose gradient ultracentrifugation to separate polysomes (actively translating ribosomes, Chapter 19) from mRNPs (proteins coupled to mRNAs that are not being translated). They found miRNAs and their target mRNAs associated with the mRNPs, rather than with polysomes. This suggested that the target mRNAs were not being translated, and therefore that the miRNAs were preventing translation initiation. Furthermore, if miRNAs act at the initiation step, which we will learn in Chapter 17 involves recognition of the cap at the 5′-end of the mRNA, allowing cap-independent initiation at an IRES should avoid silencing by miRNAs. That is exactly what Filipowicz and colleagues found, thereby reinforcing the hypothesis that miRNAs can block initiation of translation. There is also evidence that miRNAs team up with Argonaute proteins to compete with translation initiation factors for binding to mRNA caps, thereby blocking initiation.

Later in this chapter, we will see evidence that miRNAs can act by helping to degrade mRNAs. Thus, there are at least three major hypotheses for miRNA action: Blocking translation initiation; blocking translation elongation; and degradation of mRNAs. How do we reconcile all these ideas? It is possible that the differences we see reflect the different experimental approaches and the different organisms studied. But there is clear evidence for multiple mechanisms even within the same organism. It is also possible that different miRNAs act in different ways, or that the same miRNA can act in different ways, depending on the cellular

context. Finally, Elisa Izaurralde and her colleagues have suggested that the different mechanisms that have been observed are different manifestations of the same unknown underlying mechanism. We will have to wait for more studies to fully answer this fascinating question.

In animals, at least, it appears that the degree of base-pairing between a small RNA and the target mRNA, not the origin of the small RNA, determines the kind of silencing that occurs. If the base-pairing is perfect, the mRNA tends to be degraded, even if the small RNA is an miRNA, rather than an siRNA. And if the base-pairing is imperfect, translation of the mRNA tends to be blocked, even if the small RNA is an siRNA, rather than an miRNA.

A good example of perfect base-pairing between an miRNA and mRNA, leading to mRNA destruction, is the miR-196 miRNA and the *HOXB8* mRNA in mice. Mammals and other animals possess clusters of **homeobox** (**HOX**) genes, which encode transcription factors that contain homeodomains (Chapter 12). These transcription factors tend to play critical roles in embryonic development. The HOX genes are down-regulated by miRNAs transcribed from genes that reside within the HOX clusters. One of these miRNAs, miR-196, base-pairs perfectly with the *HOXB8* mRNA, except for a single G–U wobble base pair (Chapter 18). In 2004, David Bartel and colleagues used rapid amplification of cDNA ends (RACE, Chapter 4) to detect the 5′-ends of fragments of *HOXB8* mRNA that were cut within the region that base-pairs with miR-196. They focused on mRNA fragments between days 15 and 17 of mouse embryogenesis because they knew that miR-196 miRNA was present during that time period. The RACE assay did indeed produce eight cDNA clones corresponding to broken *HOXB8* mRNA, and seven of these ended within the region of base-pairing with miR-196 miRNA.

These results suggested that the miRNA was causing breakage of the mRNA within the region of base-pairing between the two RNAs. To check this hypothesis, Bartel and colleagues placed the miR-196 complementary sequence into a firefly luciferase reporter gene and transfected this gene into HeLa (human) cells, along with either miR-196 miRNA, or a noncognate miRNA. Then they used their RACE assay to detect cleavage of the reporter gene's mRNA. They found that the miR-196 miRNA, but not the noncognate miRNA, caused cleavage of the luciferase mRNA. Thus, mammalian miRNAs, if they match their target mRNAs perfectly or nearly perfectly, can cause cleavage of the target mRNAs.

Note three important distinctions between the actions of siRNAs and miRNAs in animals:

1. The siRNAs silence genes by inducing degradation of the target mRNAs, while the miRNAs tend to silence genes by interfering with accumulation of the protein products of the target mRNAs. However, if base-pairing between an animal miRNA and its target mRNA is perfect or near perfect, the miRNA can cause cleavage of the target mRNA.
2. The siRNAs are formed by Dicer action on double-stranded RNAs that usually contain at least one strand that is foreign to the cell, or derive from transposons. On the other hand, the miRNAs are formed by Dicer action on the double-stranded part of a stem-loop RNA that is a normal cellular product.
3. The siRNAs base-pair perfectly with the target mRNAs, whereas the miRNAs usually base-pair imperfectly with their target mRNAs.

Silencing with both kinds of small RNA, siRNA and miRNA, depends on a RISC complex. In *Drosophila,* there are two Dicers (Dicer-1 and Dicer-2) and two RISCs, **siRISC** and **miRISC,** but there is no simple one-to-one correspondence. Silencing by siRNAs requires siRISC, and both Dicers, but Dicer-2 is more important in producing siRNAs. Silencing by miRNAs requires miRISC, and only Dicer-1 is required for producing miRNAs. However, this division of labor cannot be a general mechanism because other organisms, including yeast and mammals, have only one RISC. In spite of these complexities, it is becoming increasingly clear that the basic mechanisms of mRNA degradation mediated by siRNAs and miRNAs, at least in plants, are very similar, if not identical. They both require a Dicer to create the double-stranded siRNA or miRNA, and these double-stranded RNAs give rise to single-stranded RNAs that bind to an Argonaute-containing RISC. The single-stranded siRNAs or miRNAs then attract mRNAs with complementary sequences, which are broken by the RISC.

It is important to emphasize that not all animal miRNAs act at the translational level. They can also decrease mRNA concentrations, presumably by destabilizing the mRNAs. We have already seen two examples, including *lin-4,* the founding member of the miRNA class, which can decrease mRNA concentration, as well as inhibit translation. However, such decreases in mRNA concentration caused by miRNAs like *lin-4* cannot operate by an RNAi-like mechanism because RNAi requires perfect complementarity between miRNA and mRNA.

In Chapter 25, we will learn that transfection of human (HeLa) cells with either of two miRNAs caused a reduction in the levels of about 100 mRNAs. In fact, one miRNA, normally expressed in the brain, shifted the HeLa cell mRNA profile to something resembling the profile of mRNAs in the brain. By contrast, the other miRNA, normally expressed in muscle, shifted the mRNA profile closer to that of muscle cells. Moreover, the 3′-untranslated regions (3′-UTRs) of the destabilized mRNAs tended to contain sequences complementary to sequences near the 5′-ends of the respective miRNAs, the miRNA **seed regions** (usually residues 1-7 or 2-8). Thus, base-pairing between the miRNA and target mRNAs appeared to be important

to the mRNA destabilization. The fact that each miRNA seemed to affect, directly or indirectly, the levels of about 100 mRNAs, also suggests that the miRNAs play a very widespread role in controlling gene expression in animals—a role whose importance may even rival that of the protein transcription factors.

The discovery of miRNAs and their function in destabilizing mRNAs has elucidated the role of **AU-rich elements** (**AREs**), which have been known since 1986 to exist in the 3′-UTRs of certain unstable mRNAs. In 2005, Jiahuai Han and colleagues reported that the instability of the *Drosophila* tumor necrosis factor-α mRNA depends on Dicer-1, Ago1 and Ago2, which are all involved in miRNA-mediated mRNA degradation. They went on to show that the instability of human ARE-containing mRNAs also depends on Dicer. Furthermore, a specific human miRNA (mi-R16), which is complementary to the ARE sequence (AAUAUUUA), is required for mRNA instability.

In contrast to the translation blockage model in animals, miRNAs in plants appear to silence by base-pairing perfectly or nearly perfectly with their target mRNAs and sponsoring degradation of those mRNAs. For example, James Carrington and colleagues showed in 2002 that a 21-nt RNA, known as miRNA 39, from *Arabidopsis thaliana* accumulates in flowering tissues and base-pairs to target sites in the middle of the mRNAs from several members of a family of transcription factors known as *Scarecrow-like* (*SCL*). This base pairing results in cleavage of the mRNAs within the region of base-pairing with the miRNA. Relatively little miRNA 39 accumulates in leaf and stem tissues, and no dectectable *SCL* mRNA cleavage occurs in those tissues.

To demonstrate miRNA-directed cleavage of mRNAs, Carrington and colleagues introduced the gene encoding the precursor to miRNA 39 into leaf tissue. They observed a high level of miRNA 39, suggesting that leaf tissue contains a Dicer-like enzyme that can produce miRNA from its precursor. More significantly, they observed active cleavage of *SCL* mRNA to a smaller, inactive product, in the leaf tissue expressing miRNA 39.

On the other hand, some plant miRNAs, although they base-pair very well with their target mRNAs, silence gene expression by interfering with translation. Xuemei Chen presented an example in 2004: miRNA172 of *Arabidopsis* base-pairs almost perfectly with the mRNA from a floral homeotic gene called *APETALA2,* yet it silences that gene by blocking translation, not by mRNA degradation. Thus, plant miRNAs, regardless of the degree of base-pairing with their target mRNAs, can use either mRNA degradation or translation blocking to silence genes.

Figure 16.40 summarizes the actions of miRNAs when base-pairing is imperfect (the typical situation in animals) and when it is perfect or near-perfect (the typical situation in plants; also observed in animals). In the former situation, translation, or at least appearance of protein product, is blocked. In the latter situation, the mRNA is cleaved. However, one should keep in mind that each of these canonical pathways has exceptions. That is, animal miRNAs, though they may base-pair imperfectly with their targets, can cause mRNA degradation, and plant miRNAs, though they may base-pair perfectly with their targets, can cause blockage of translation.

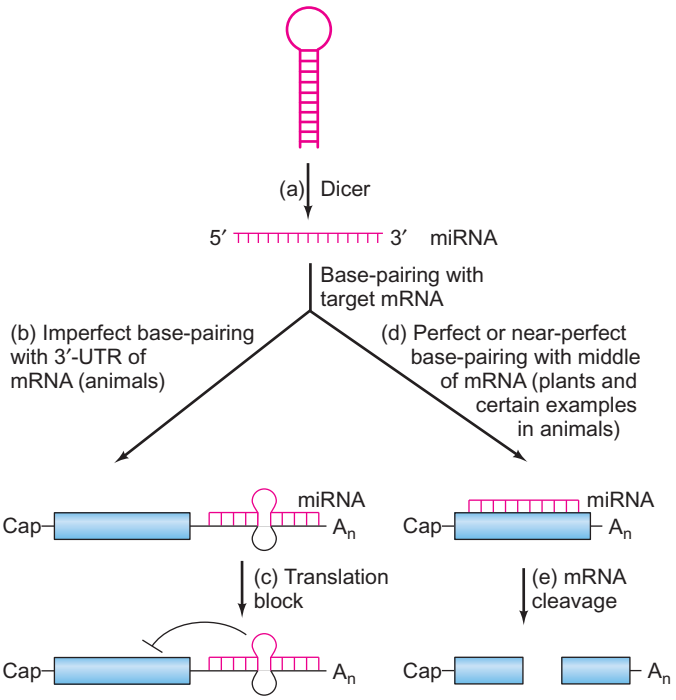


Figure 16.40 Two pathways to gene silencing by miRNAs. (a) A stem-loop miRNA precursor is cleaved by Dicer to yield a short miRNA about 21 nt long. **(b)** If the base-pairing between the miRNA and the 3′-UTR of its target mRNA is imperfect, as usually occurs in animals, the miRNA causes blockage of translation, or at least accumulation of the mRNA's protein product **(c). (d)** If the base-pairing between the miRNA and the middle of its target mRNA is perfect, or nearly so, as usually occurs in plants, and sometimes in animals, the mRNA is cleaved **(e),** which inactivates the mRNA.

MicroRNAs do not serve solely as modulators of cellular gene activity. There is also good evidence that they act as antiviral agents in plants and invertebrates by targeting viral mRNAs. It was widely assumed that vertebrates relied on their potent interferon systems, rather than on miRNAs, to combat viral infections. However, Michael David and colleagues showed in 2007 that miRNAs can also target viral mRNAs, and that these miRNAs are themselves a product of the interferon system.

In particular, David and colleagues demonstrated that interferon-β (IFN-β) stimulates the production of many miRNAs. Among these are eight miRNAs that are complementary to parts of the hepatitis C virus (HCV). These miRNAs appear to be effective in combating HCV because introduction of corresponding synthetic miRNAs mimics the effects of IFN-β on HCV infection and replication.

SUMMARY MicroRNAs (miRNAs) are 18–25-nt RNAs produced from a cellular RNA with a stem-loop structure. In the last step in miRNA synthesis, Dicer cleaves the double-stranded stem part of the precursor to yield the miRNA in double-stranded form. The single-stranded forms of these miRNAs can team up with an Argonaute protein in a RISC to control the expression of other genes by base-pairing to their mRNAs. In animals, miRNAs tend to base-pair imperfectly to the 3′-UTRs of their target mRNAs and inhibit accumulation of the protein products of these mRNAs. However, perfect or perhaps even imperfect base-pairing between an animal miRNA and its target mRNA can result in mRNA cleavage. In plants, miRNAs tend to base-pair perfectly or near-perfectly with their target mRNAs and cause cleavage of these mRNAs, although there are exceptions in which translation blockage can occur.

Stimulation of Translation by miRNAs

MicroRNAs do not always inhibit translation. Joan Steitz and her colleagues first noticed indications of positive action by miRNAs when they found that the ARE of the human tumor necrosis factor-α (TNFα) mRNA activates translation during serum starvation, which arrests the cell cycle in the G_1 phase. They also found that Ago2 and fragile X mental retardation-related protein (FXR1) associate with the ARE during translation activation, and are required for the activation.

This work suggested that miRNAs, which bind along with proteins to AREs, might be capable of directing activation, rather than inactivation, of translation under certain conditions. To test this hypothesis, Steitz and colleagues first used bioinformatics techniques (Chapter 25) to search the human genome for miRNAs with seed sequences complementary to the TNFα ARE. They identified five miRNA candidates, not counting miR16, which is known to reduce TNFα mRNA levels by binding outside the ARE region.

To screen the five miRNAs for effects on TNFα mRNA translation, they attached the TNFα ARE to the firefly luciferase reporter gene and tested this construct for translation efficiency in transfected cells under a variety of conditions. Only one miRNA, miR369-3, had an effect. It stimulated translation, but only in serum-starved cells.

First, Steitz and colleagues tested the effect of serum on miR369-3 levels using an RNase protection assay. Figure 16.41b shows that the level of the miRNA rose under serum starvation conditions, but that this rise was blocked by treatment with an siRNA that targets the loop of the pre-miR369-3. By contrast, serum had no effect on the levels of three control RNAs: miR369-5, which is essentially the complementary strand of miR369-3 in the stem of the pre-miRNA; miR16; or U6 snRNA. As expected, the siRNA also knocked down the level of miR369-5.

Next, Steitz and colleagues tested the effect of serum on reporter mRNA translation in the presence and absence of serum, and in the presence and absence of the siRNA that blocks accumulation of miR369-3. Figure 16.41c shows that translation efficiency increased about five-fold under serum-starved conditions. However, when the siRNA targeting pre-miR369-3 was included, the stimulation of translation disappeared. On the other hand, when the investigators rescued miR369-3 by adding a synthetic miR369-3 immune to the siRNA, translation again rose about five-fold upon serum starvation. Furthermore, serum had no effect on translation when the ARE did not match the seed sequence of the miRNA.

To test the importance of base-pairing between miR369-3 and the ARE, Steitz and colleagues used an intergenic suppression approach. They mutated the ARE to the sequence they called mtARE (Figure 16.41a) and tested the altered gene for activation with the wild-type miR369-3. As Figure 16.41d shows, no activation occurred upon serum starvation. Next, they added a mutant miR369-3 (miRmt369-3, Figure 16.41a) with a sequence complementary to that of mtARE, and re-tested for activation. This time, serum starvation caused activation. As expected, a control miRNA (miRcxcr4) caused no activation. Thus, complementarity between the ARE and the miRNA appears to be important.

To probe the importance of the seed regions in particular, Steitz and colleagues mutated each of the identical regions (seed1 and seed2) in the ARE of the mRNA that are complementary to the seed regions in miR369-3, and then made compensating mutations in the seed region of the miRNA. The mutant AREs are called mtAREseed1 and mtAREseed2, and the compensating mutant miRNA is called miRseedmt369-3. These sequences are all given in Figure 16.41a, and Figure 16.41e shows the results. As predicted, changing the sequences of each of the anti-seed regions in the mRNA eliminated activation by serum starvation, and making compensating mutations in the seed region of the miRNA restored activation. Thus, miR369-3 really is responsible for the activation, and base-pairing between the seed region of the miRNA and the ARE in the mRNA is critical for this activation.

Finally, Steitz and colleagues looked directly for miR369-3 associated with the reporter mRNA. They tagged the reporter mRNA with an S1 aptamer that allowed it to be affinity purified by binding to streptavidin. Then they cross-linked any associated RNAs with formaldehyde, performed streptavidin affinity purification of the reporter mRNA, and detected any miR369-3 associated with it by RNase protection assay. Figure 16.41f shows the results. The miR369-3 was associated with the reporter mRNA in serum-starved cells, but not in cells grown in serum. No association was detected in cells treated with the siRNA that targets the pre-miR369-3, but it was detected when these cells were rescued with miR369-3 and

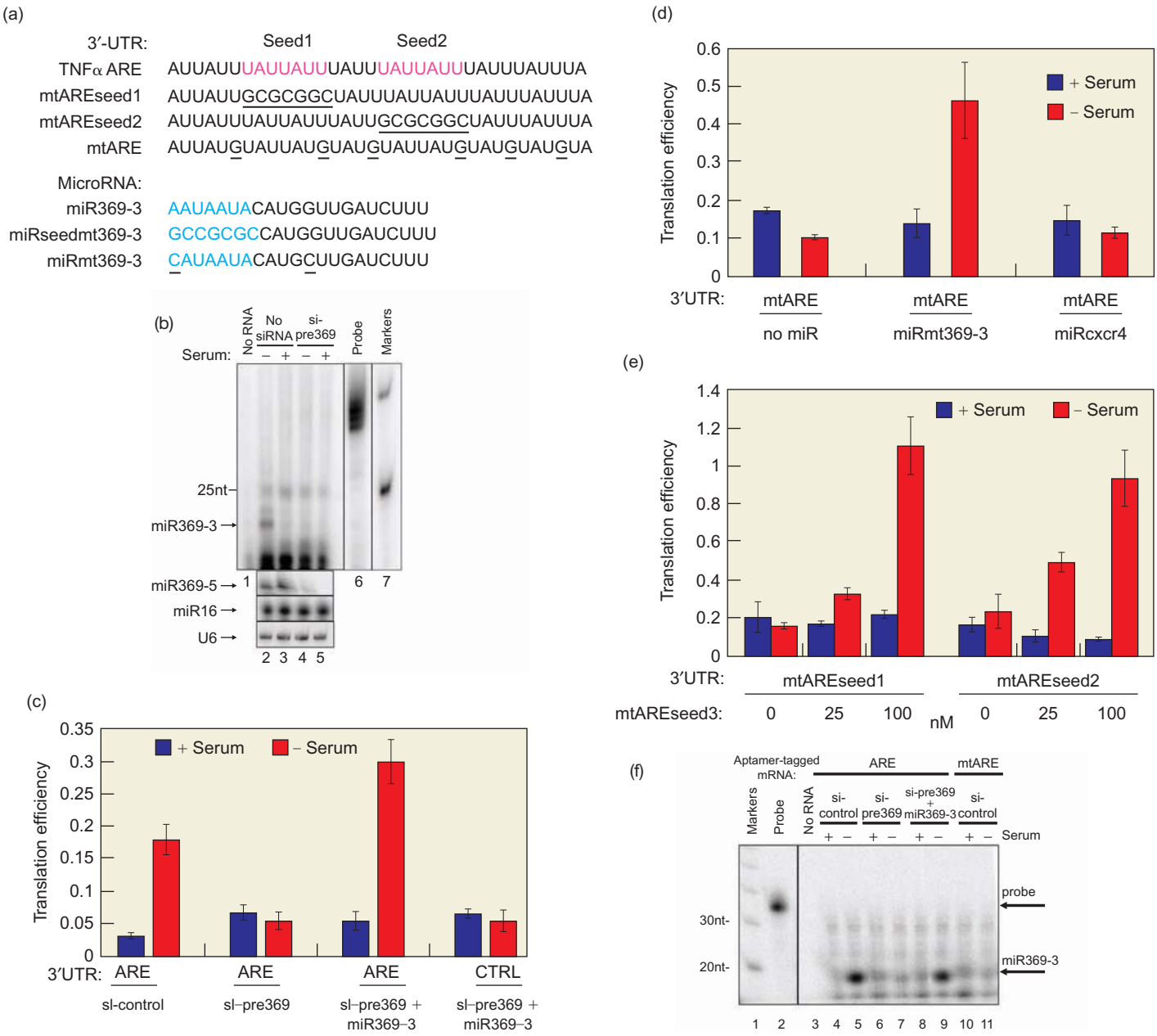


Figure 16.41 Role of MiR369-3 activation of reporter mRNA translation. **(a)** Sequences of wild-type and mutant TNFα 3′-UTRs linked to the luciferase reporter mRNA, and wild-type and mutant miRNAs. All sequences are written 5′→3′, so one must be inverted for complementarity with the other to be obvious. Note that the wild-type ARE has two regions (pink) that are complementary to the seed region (5′-AAUAAUA-3′, blue) in miR369-3. **(b)** Concentration of miR369-3, measured by RNase protection assay. RNA levels were measured with and without serum, as indicated at top, and with without an siRNA that targets the pre-miR369-3. At bottom, concentrations of miR369-5 (the passenger starand of miR369-3), as well as two control RNAs (miR16 and U6 snRNA) were measured. The position of miR369-3 is indicated at left, along with the position of a 25-nt marker RNA. **(c)** Translation efficiencies of mRNAs bearing the wild-type ARE, or a control ARE (CTRL) are shown with and without serum (blue and red, respectively). The experiments were run with no siRNA (si-control), with an siRNA targeting the pre-miR369-3 (si-pre369), or with the siRNA plus a rescuing miR369-3 (si-pre369 + miR369-3), as indicated at bottom. **(d)** Translation efficiencies of mRNAs bearing the mutated ARE (mtARE) are shown with and without a complementary mutated miR369-3 (miR369-3) or with a control miRNA (miRcxcr4). **(e)** Translation efficiencies of mRNAs bearing AREs with mutated anti-seed 1 or anti-seed 2 regions (mtAREseed 1 and mtAREseed 2, respectively indicated at bottom) are shown with and without serum (blue and red, respectively) and with three concentrations of an miRNA with a seed region complementary to the mutated anti-seed region (miRseedmt369-3), as indicated at bottom. **(f)** Detection of association between reporter mRNA and miR369-3. Formaldehyde-cross-linked RNAs were affinity-purified via an S1 aptamer tag on the reporter mRNA, and miR369-3 was delected by RNase protection assay. The experiments were run with no siRNA (si-control), with an siRNA targeting the pre-miR369-3 (si-pre369), or with the siRNA plus a rescuing miR369-3 (si-pre369 + miR369-3), as indicated at top. Also, a tagged control mRNA (mtARE) with a mutated ARE was used (lanes 10 and 11). (*Source:* Reprinted with permission of *Science*, 21 December 2007, Vol. 318, no. 5858, pp. 1931–1934, Vasudevan et al, "Switching from Repression to Activation: MicroRNAs Can Up-Regulate Translation." © 2007 AAAS.)

serum-starved. Also, no miR369-3 associated with a reporter mRNA with a mutated ARE (mtARE). Taken together, the results in Figure 16.41 show that the activation of reporter mRNA translation by serum starvation depends on an association between miR369-3 and the ARE of the mRNA.

Steitz and colleagues extended these studies to two other reporter mRNAs. One (CX) contained four synthetic miRNA (miRcxcr4) target sites; the other (Let-7) contained seven target sites for the endogenous Let-7 miRNA. Translation of both reporter mRNAs was activated by serum starvation in two different cell lines. Thus, all three of the miRNAs in this study can respond to serum starvation by activating translation.

Steitz and colleagues knew from previous experiments that translation activation was cell cycle-dependent, so they reasoned that synchronized cells might show more dramatic effects of serum than the nonsynchronized cells used in Figure 16.41. Accordingly, they synchronized cells by starving them of serum, and then released them to reenter the cell cycle by adding serum. When they measured translation efficiency, they found that synchronized cells growing in serum actually had about a five-fold lower translation efficiency than unsynchronized serum-grown cells. Furthermore, this translation repression depended on miR369-3. Thus, this miRNA can activate translation under some conditions, and repress it under other conditions.

Previous studies had shown that Ago2 and FXR1 are both required for translation activation upon serum starvation, so Steitz and colleagues measured the recruitment of these two proteins to ribonucleoprotein (RNP) complexes on aptamer-tagged mRNAs. They found both Ago2 and FXR1 in the RNP complex associated with the reporter mRNA under serum-starved conditions. However, when miR369-3 was depleted with the siRNA directed against pre-miR369-3, the amount of Ago2 in the RNP complex fell, but it was restored by adding miR369-3. In RNP complexes isolated from synchronized cells growing in serum, Ago2 was prominent, but FXR1 was not, and the amount of Ago2 in the complex dropped when miR369-3 was depleted. Steitz and colleagues concluded that miR369-3 recruits both proteins to the mRNA under serum-starved conditions, and these proteins participate in translation activation. On the other hand, miR369-3 recruits Ago2, but not FXR1, to the mRNA in synchronized proliferating cells, so Ago2, but not FXR1 appears to be involved in translation repression.

SUMMARY MicroRNAs can activate, as well as repress translation. In particular, miR369-3, with the help of AGO2 and FXR1, activates translation of the TNFα mRNA in serum-starved cells. On the other hand, miR369-3, with the help of Ago2, represses translation of the mRNA in synchronized cells growing in serum.

Biogenesis of miRNAs MicroRNAs are synthesized by RNA polymerase II as longer precursors known as **primary miRNAs** (**pri-miRNAs**). We know that RNA polymerase II transcribes the pri-miRNA genes because the pri-miRNAs are capped and polyadenylated, which is characteristic of class II transcripts, because low concentrations of α-amanitin inhibit pri-miRNA synthesis, and because ChIP analysis shows association between polymerase II and chromatin containing pre-miRNA promoters.

A well-studied human pri-miRNA gene contains the coding regions for three miRNAs (miR23a, miR27a, and miR24-2). The pri-miRNA is about 2.2 kb long, including its poly(A) tail, which lies about 1.8 kb downstream of the last miRNA coding region. Although this gene is clearly transcribed by polymerase II, its promoter, which extends as much as 600 nt upstream of the transcription start site, has none of the typical class II core promoter elements we studied in Chapter 10, nor the PSE element characteristic of the class II snRNA promoters.

The pri-miRNAs contain each miRNA coding region as part of a stable stem-loop. The first step in processing this precursor to a mature miRNA occurs in the nucleus and requires an RNase III known as **Drosha**, which cleaves near the base of the stem, releasing a **pre-miRNA** consisting of a 60-70-nt stem-loop with a 5′-phosphate and a 2-nt 3′-overhang. However, Drosha cannot recognize and cleave a pri-miRNA on its own. It needs a double-stranded RNA-binding protein partner. In humans, this partner is called **DGCR8**; in *C. elegans* and *Drosophila* it is called **Pasha**. Together, Drosha and Pasha make up an RNA processing complex called **Microprocessor**. The final processing of a pre-miRNA to a mature miRNA is carried out in the cytoplasm by Dicer, the same RNase III responsible for siRNA production in RNAi. Figure 16.42a illustrates the two-step process of miRNA biogenesis.

Another mode of miRNA biogenesis bypasses the Drosha cleavage step. Many miRNAs are encoded in introns, and some of these, known as **mirtrons** ("mir" from miRNA, and "trons" from introns), take advantage of the splicing mechanism, rather than Drosha, to generate the pre-miRNA. As Figure 16.42b shows, the whole intron is a pre-miRNA. Therefore, the normal splicing machinery will cut it out of the primary transcript as a lariat-shaped intron, which will then be linearized by the debranching enzyme, whereupon it can fold into the stem-loop shape of a pre-miRNA.

Some miRNAs require A → I editing, which we discussed earlier in this chapter. For example, all but one member of the miR-376 RNA cluster in mice and humans undergo A → I editing in certain tissues, including the brain, at specific sites in the pri-miRNA. One of the most commonly edited sites is four bases from the 5′-end of the miRNA, within the seed region that base-pairs to the complementary site in the 3′-UTR of the target mRNA. Thus, this change in base sequence of the miRNAs changes the identity of their targets, with important implications for brain function.

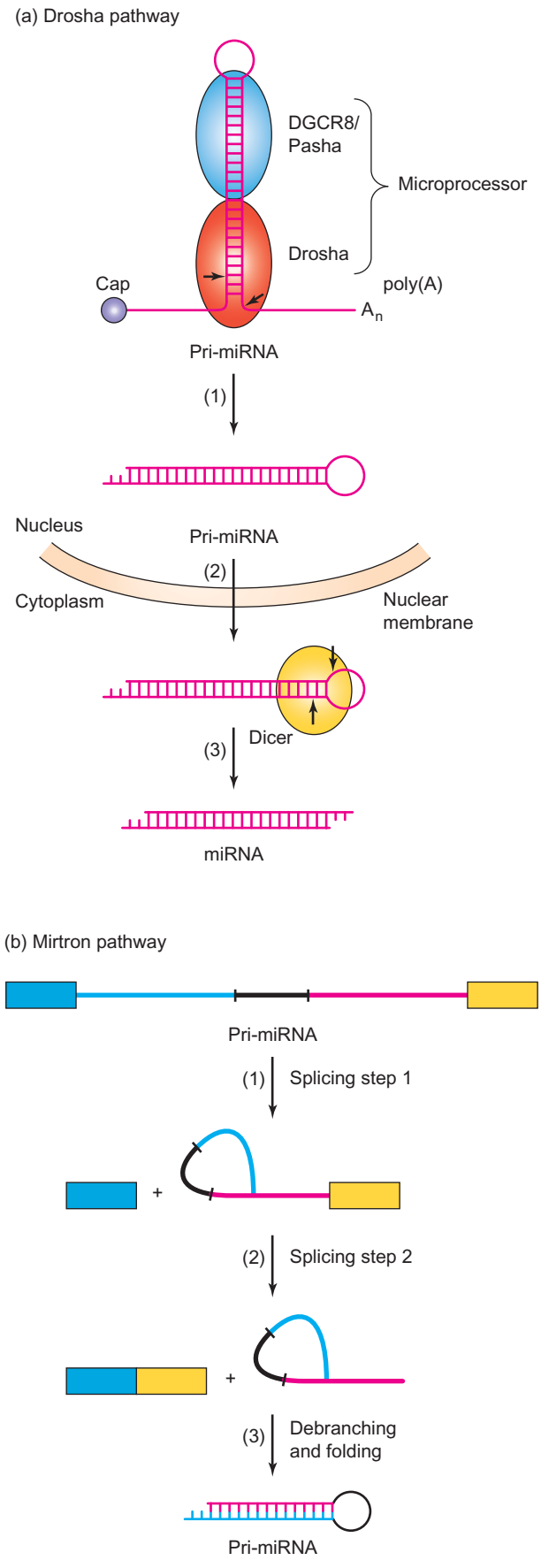


Figure 16.42 Maturation of a human miRNA. The primary transcription product of an miRNA gene is a pri-miRNA. It is made by RNA polymerase II and it may contain more than one miRNA sequence. For simplicity, this one contains just one. **(a)** The Drosha pathway. (1) Microprocessor, which consists of a double-stranded RNA-binding protein (DGCR8, or Pasha) and an RNase III (Drosha), binds to the pri-miRNA and cleaves it at the base of the stem, releasing a 60–70-nt stem-loop pre-miRNA. (2) The pre-miRNA is transported from the nucleus to the cytoplasm. (3) Dicer binds to the pre-miRNA in the cytoplasm and cuts 22 nt from the cut made by Drosha, yielding the mature miRNA. **(b)** The mirtron pathway. (1) The mirtron is color coded cyan, black, and magenta, corresponding to the three parts of the pre-miRNA it will become: the top strand of the stem; the loop; and the bottom strand of the stem, respectively. The first step of splicing separates the mirtron from the first exon and forms it into a lariat that is still attached to the second exon. (2) The second splicing step separates the mirtron from the second exon, still in lariat shape. (3) Debranching of the lariat, and folding (which occurs naturally) yields the mirtron as a pre-miRNA. It has the usual approximately 22 base pairs, but fewer are shown here for simplicity.

SUMMARY RNA polymerase II transcribes the miRNA precursor genes, to produce pri-miRNAs, which may encode more than one miRNA. Processing a pri-mRNA to a mature miRNA is a two-step process. In the first step, a nuclear RNase III known as Drosha cleaves the pri-miRNA to release a 60–70-nt stem-loop RNA known as a pre-miRNA. In the second step, which occurs in the cytoplasm, Dicer cuts the pre-miRNA within the stem to release a mature double-stranded miRNA. A mirtron is an intron that consists of a pre-miRNA. Thus, the spliceosome cuts it out of its pre-mRNA, then it is debranched and folded into a stem-loop pre-miRNA, without any participation by Drosha. Some miRNAs require A → I editing at the pri-miRNA stage, and some of this editing changes the targeting of the miRNAs to different mRNAs.

16.9 Translation Repression, mRNA Degradation, and P-Bodies

Processing bodies

(**P-bodies**, also known as **PBs**) are discrete cytoplasmic collections of RNAs and proteins that are involved in mRNA decay and translational repression. These cellular foci are enriched in enzymes that deadenylate mRNAs (deadenylases); decap mRNAs (the decapping enzyme, which, in *Drospophila,* contains two subunits, Dcp1 and Dcp2); and catalyze 5′→3′ degradation of mRNAs (exonuclease Xrn1). Thus, P-bodies appear to be involved in translational repression and also in degradation of mRNAs by a

non-RNAi-like mechanism that entails deadenylation and decapping prior to 5′→3′ exonucleolytic destruction.

Degradation of mRNAs in P-bodies

One of the important partners for the miRNAs in mRNA silencing in P-bodies, at least in higher eukaryotes, is **GW182**. The "GW" in the name refers to repeats of glycine (G) and tryptophan (W) in the protein. GW182 is required for P-body integrity, but its role extends far beyond a simple structural one: This protein appears to be an essential part of the mRNA silencing machinery. One clue to the importance of GW182 is that it associates with DCP1, Ago1, and Ago2—all key players in mRNA silencing—in human cell P-bodies. Another indication of the importance of GW182 is that RNAi-mediated knockdown experiments in human cells showed that reducing the levels of GW182 impaired both miRNA function and the mRNA decay that is an essential part of RNAi. In *Drosophila* cells, by contrast, knockdown of GW182 impaired miRNA function, which depends on Ago1, but not RNAi, which depends on Ago2.

In 2006, Elisa Izaurralde and colleagues presented the results of their inquiry into the exact role of GW182 in miRNA-mediated silencing of mRNA function in *Drosophila*. Because GW182 and Ago1 both appear to be involved in miRNA-mediated mRNA silencing in *Drosophila* cells, these workers employed high-density oligonucleotide arrays (Chapter 24) to investigate the profiles of RNAs in cells depleted of GW182, Ago1, or Ago2 by knockdown using dsRNAs specific for each of the three genes. They found that there was a high correlation between the mRNAs up-regulated in response to knockdown of GW182 and Ago1 (a rank correlation coefficient r of 0.92). Rank correlation coefficients are computed by arranging two groups of values by rank and then calculating how closely the two ranks compare with each other. In this case, the mRNAs were ranked according to the degree to which they were up-regulated (or down-regulated) in response to knockdown of GW182 (first ranking) or Ago1 (second ranking). So an r of 0.92 indicates that mRNAs strongly up-regulated by a GW182 knockdown are also usually strongly up-regulated by an Ago1 knockdown. By contrast, there was much less correlation between the mRNAs up-regulated in response to knockdown of GW182 and Ago2 ($r = 0.64$).

Figure 16.43a shows the impressive similarity between the profiles of mRNAs regulated in the same way by both GW182 and Ago1. In this figure, 6345 transcripts were analyzed to see if they were up-regulated or down-regulated in response to a given knockdown. Red represents transcripts that are up-regulated at least two-fold, blue represents transcripts down-regulated at least two-fold, and yellow represents all the other transcripts, which were up- or down-regulated less than two-fold. Next, Izaurralde and colleagues focused on the mRNAs that were at least two-fold up- or down-regulated in response to GW182 or Ago1 knockdowns. Figure 16.43b illustrates the very high degree of concordance.

If GW182 and Ago1 knockdowns are up-regulating certain mRNAs because these mRNAs would otherwise be silenced by miRNA-mediated degradation, one should observe that known miRNA target mRNAs are up-regulated by knocking down either GW182 or Ago1. Indeed, when Izaurralde and colleagues did that experiment, they got exactly the predicted results. Figure 16.43c shows that all nine of the known miRNA targets were up-regulated at least two-fold by knockdowns of either GW182 or Ago1. In fact, even the degree of up-regulation of each mRNA correlated well between the two knockdowns. Izaurralde and colleagues also checked the oligonucleotide array data by performing classical Northern blots with selected mRNAs. Figure 16.43d shows that the Northern blot and array data match very well. Thus, GW182 and Ago1 seem to have the same effect: silencing genes by reducing mRNA concentration.

Izaurralde and colleagues wondered if GW182 by itself could silence the expression of target mRNAs. To find out, they physically tethered GW182 to a firefly luciferase reporter mRNA by the following strategy (further illustrated in Chapter 17): They added five λ phage box B coding sequences to the 3′-UTR of the reporter gene. As we learned in Chapter 8, box B sequences in an RNA are binding sites for the λN protein. Accordingly, these workers fused the GW182 gene to a gene fragment encoding the part of λN (the N-peptide) that binds to box B. Then they transfected *Drosophila* cells with the λN-GW182 construct, the reporter gene, and a control plasmid containing the *Renilla* (sea pansy) luciferase gene, whose protein product they could assay as a control for transfection efficiency.

Note that this combination of constructs yields a reporter mRNA containing box B sequences in its 3′-UTR, and a λN-GW182 protein with a natural affinity for box B. Thus, the λN-GW182 protein becomes tethered to the reporter mRNA. When Izaurralde and colleagues assayed for firefly luciferase activity (corrected for transfection efficiency), they found a 16-fold reduction in expression of the reporter mRNA with tethered λN-GW182, compared to a reporter mRNA tethered to λN protein by itself. Thus, GW182 alone is capable of strongly silencing expression of a bound mRNA. Is this silencing due to reduction of mRNA level alone? To answer this question, Izaurralde and colleagues performed Northern blots on RNA from cells expressing λN-GW182, or λN alone. They found only a four-fold decrease in reporter mRNA concentration when it was tethered to λN-GW182. This four-fold loss of mRNA clearly cannot fully explain the 16-fold decrease in expression, so it appears that GW182 also controls translation of at least some mRNAs to which it binds.

Is the silencing observed with tethered λN-GW182 independent of Ago1? To find out, Izaurralde and colleagues repeated the tethering experiment in ordinary cells, and in Ago1 knockdown cells. They found no difference, so

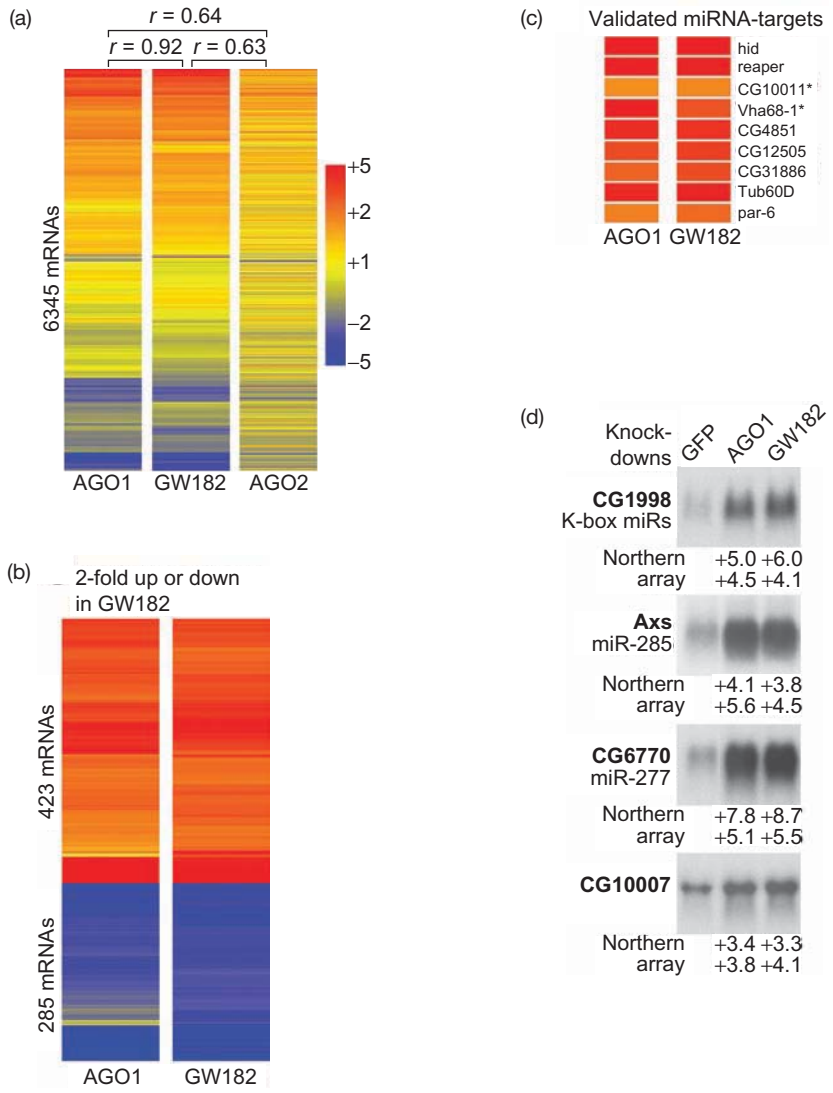


Figure 16.43 Effect of knockdowns of Ago1, GW182, ad Ago2 on abundance of other transcripts. (a) Izaurralde and colleagues isolated transcripts from untreated *Drosophila* cells, and from cells treated with dsRNAs to knock down Ago1, GW182, and Ago2 by RNAi. They hybridized transcripts from each of the three groups of treated cells, and untreated cells, to oligonucleotide arrays and determined the abundance of each of 6345 miRNAs before and after treatment. They coded up-regulation by at least two-fold as red, down-regulation by at least two-fold as blue, and less than two-fold change in either direction as yellow, according to the key at right. Note the similarity between the mRNA profiles form Ago1 and GW182 knockdowns, and the relative dissimilarity between either Ago1 or GW182 and Ago2. **(b)** Results of the same study, but only mRNAs up- or down-regulated by at least two-fold in Ago1 or GW182 knockdowns are presented. **(c)** The results from nine mRNAs that are known miRNA targets are shown for Ago1 and GW182 knockdowns. Note again the great similarity in the effects of knocking down Ago1 and GW182. **(d)** Northern blots of four different mRNAs, identified at left, are shown for Ago1 and GW182 knockdowns, along with a control green fluorescent protein (GFP) knockdown, which should not have any effect on the abundance of any of these mRNAs. The degrees of up-regulation of each mRNA in the Ago1 and GW182 knockdowns were calculated from these Northern blots and from the microarry analysis in panel (a), and are given below the respective blots. Note the similarity in degree of up-regulation determined by Northern blots and microarrays. (*Source*: Reprinted by permission of E. Izaurralde from Behm-Ansmant et al, mRNA degradation by miRNAs and GW182 requires both CCR4: NOT deadenylase and DCP1: DCP2 decapping complexes, *Genes and Development*, V. 20, pp. 1885–1898. Copyright © 2006 Cold Spring Harbor Laboratory Press.)

silencing appeared to work just as well without Ago1. Thus, binding GW182 to an mRNA appears to sidestep the requirement for Ago1, which may mean that Ago1 helps recruit GW182 to mRNAs targeted for silencing.

We have seen that tethering λN-GW182 to a reporter mRNA causes about a 75% degradation of the mRNA. In addition, Izaurralde and colleagues noticed that the remaining mRNA was a little shorter than the same reporter mRNA in cells without λN-GW182. They wondered whether this shortening was due to deadenylation, and whether this deadenylation would occur under normal circumstances. To find out, they isolated RNA from cells at time zero and 15 min

after stopping transcription with actinomycin D. Then they deadenylated the mRNAs by oligo(dT)-targeted RNase H degradation (Chapter 14). Finally, they subjected these RNAs to Northern blot analysis with probes specific for the reporter mRNA and for rp49, an endogenous mRNA (not an miRNA target) that encodes the ribosomal protein L32. They found that the control RNA contained poly(A) at both time points, as it could be shortened by oligo(dT)-directed RNase H destruction of poly(A). On the other hand, the luciferase reporter mRNA contained poly(A) immediately after transcription, at time zero, but it appeared to be deadenylated by 15 min after transcription was halted, as it could not be further shortened by oligo(dT)-directed RNase H treatment. Thus, deadenylation appears to be part of the silencing caused by GW182. Furthermore, knockdown experiments showed that silencing by GW182 depends on the CCR4/NOT deadenylase in *Drosophila*.

Decapping of mRNA is also part of the miRNA-mediated mRNA degradation pathway, so Izaurralde and colleagues examined the effects of knocking down DCP1 and DCP2 in the λN-GW182 reporter mRNA tethering assay. They found that depleting cells of the DCP1/DCP2 decapping complex restores reporter mRNA levels to normal. However, loss of DCP1 and 2 had little effect on the strong silencing of luciferase activity by tethering λN-GW182 to its mRNA. A probable explanation comes from the finding that the reporter mRNA was still deadenylated in the DCP1/DCP2-depleted cells—and deadenylated mRNAs are expected to be poorly translated.

The GW182-mRNA tethering studies not only bypassed the need for Ago1, they also bypassed miRNAs. So we are left with the impression that GW182, along with Ago1, is an important player in miRNA-mediated silencing, but we have so far seen no direct evidence for this hypothesis. Accordingly, Izaurralde and colleagues examined the mechanism of miRNA-mediated mRNA decay and found that it depends on deadenylation by CCR4/NOT, decapping by DCP1/DCP2, as well as on GW182 and Ago1. These workers constructed three luciferase reporter mRNAs that were silenced by two miRNAs. The first contained the 3′-UTR from the *Drosophila* gene CG10011, including a binding site for miR-12. The second contained the 3′-UTR from the Nerfin gene, including a binding site for miR-9b. The third contained the 3′-UTr from the Vha68-1 gene, also including a miR-9b binding site. When these workers measured mRNA levels and luciferase activities in cells co-transfected with each of the reporter genes and their cognate miRNAs, they found the following: (1) Silencing of the luciferase-CG10011 reporter by miR-12 appeared to operate exclusively by reducing the level of the transcript. (2) Silencing of the luciferase-Nerfin reporter by miR-9 involved primarily a reduction in translation efficiency. (3) Silencing of the luciferase-Vha68-1 reporter used a combination of the two mechanisms, mRNA level reduction and translation inhibition.

Next, Izaurralde and colleagues measured luciferase activities and mRNA levels in *Drosophila* S2 cells transfected with each of the reporters and the miRNAs, and also depleted of CAF1, NOT1, DCP1/DCP2, or GW182 by knockdown. Control knockdowns were depleted of the essential Ago1 or the irrelevant green fluorescent protein (GFP). As expected, knockdown of Ago1 or GW182 resulted in normal luciferase activities and mRNA levels from all reporters, even in the presence of cognate miRNAs. That is because silencing by miRNAs depends on both Ago1 and GW182. And because silencing of these reporter mRNAs depends on both translation inhibition and mRNA decay, it appears that both Ago1 and GW182 are involved in both silencing mechanisms.

In miRNA-treated, NOT1-depleted cells, CG10011 and Vha68-1 mRNAs were restored to non-miRNA-treated levels, and luciferase activities were partially restored. Silencing of these two reporters depends wholly or principally on mRNA decay and deadenylation is a key part of that decay. Thus, it is not surprising that removing the deadenylation enzyme NOT1 prevents such mRNA decay. On the other hand, depleting NOT1 in miRNA-treated cells had no effect on the loss of luciferase activity from the luciferase-Nerfin reporter. Because the luciferase-Nerfin reporter responds to miRNA by decreasing translation efficiency, rather than by mRNA decay, this result suggests that, while deadenylation is an essential part of mRNA decay, it is not required for miR-9a-mediated translation silencing of the luciferase-Nerfin reporter.

Depletion of DCP1/DCP2 in miRNA-treated cells restored the levels of all three reporter mRNAs to normal. Although none of the mRNAs presumably suffered decapping in these cells, they all were deadenylated. Taken together, these two findings suggest that deadenylation alone cannot initiate mRNA decay, for example by a 3′→5′ exonuclease. Thus, it is more likely that deadenylation and decapping are followed by mRNA degradation by a 5′→3′ exonuclease. Also, the fact that all three reporter mRNAs were deadenylated helps explain why the luciferase activities from all three reporter mRNAs remained low: Deadenylation presumably inhibited translation of these mRNAs.

SUMMARY P-bodies are cellular foci where mRNAs are destroyed or translationally repressed. GW182 is an essential part of the *Drosophila* miRNA silencing mechanism in P-bodies, whether this mechanism involves translation inhibition or mRNA decay. Ago1 probably recruits GW182 to an mRNA within a P-body, and this marks that mRNA for silencing. GW182 and Ago1-mediated mRNA decay in P-bodies appears to involve both deadenylation and decapping, followed by mRNA degradation by a 5′→3′ exonuclease.

Relief of Repression in P-Bodies

There is a flow of mRNAs back and forth between polysomes and P-bodies. Therefore, the more an mRNA is associated with polysomes, and is therefore being actively translated, the less that mRNA will be found in P-bodies. And conversely, mRNAs that are enriched in P-bodies are poorly represented in polysomes. Although many mRNAs are degraded in P-bodies, many others are merely held and repressed there, and may rejoin polysomes once cellular conditions change.

Witold Filipowicz and colleagues provided good evidence for this dynamic association between repressed mRNAs and P-bodies in their studies on the human cationic amino acid transporter (CAT-1), which transports lysine and arginine into cells. CAT-1 is normally kept at low levels in liver cells to prevent loss of arginine from serum. That loss would occur because liver cells have a high concentration of arginase, which rapidly degrades imported arginine. But, under certain stress conditions, including amino acid starvation, liver cells need to import more arginine, and the CAT-1 level is up-regulated. Filipowicz and colleagues showed that the reason CAT-1 levels are low in liver cells is that a miRNA represses CAT-1 mRNA translation in those cells. Furthermore, the relief of repression of CAT-1 mRNA translation under stress conditions is accompanied by a loss of CAT-1 mRNA from P-bodies.

Filipowicz and colleagues chose to study Huh7 hepatoma cells because evidence suggested that CAT-1 expression in these cells was controlled by an miRNA known as miR-122. First, these workers used a Western blot to show that the CAT-1 concentration was significantly lower in Huh7 cells than in three other human cell lines (Figure 16.44a). Then they used a Northern blot to establish that the CAT-1 mRNA levels were essentially the same in all four human cell lines (Figure 16.44b). Thus, control of CAT-1 levels in Huh7 cells does not occur at the transcriptional level, or even at the level of mRNA stability, but probably at the translational level.

Is this control dependent on miR-122? Possibly, because the Northern blot in Figure 16.44c reveals that, of the four cell lines, only Huh7 expresses miR-122. Furthermore, if miR-122 is really responsible, we would expect that treatment of cells with an anti-miR-122 oligonucleotide would abolish the control, and CAT-1 levels would rise in cells treated with the antisense oligonucleotide. Figure 16.44d shows that this is indeed what happened, whereas irrelevant oligonucleotides had no effect. This increase in CAT-1 protein was not reflected in an increase in CAT-1 mRNA, suggesting again that the regulation was occurring at the translational level.

To investigate further the role of miR-122 in control of CAT-1 production, Filipowicz and colleagues made a series

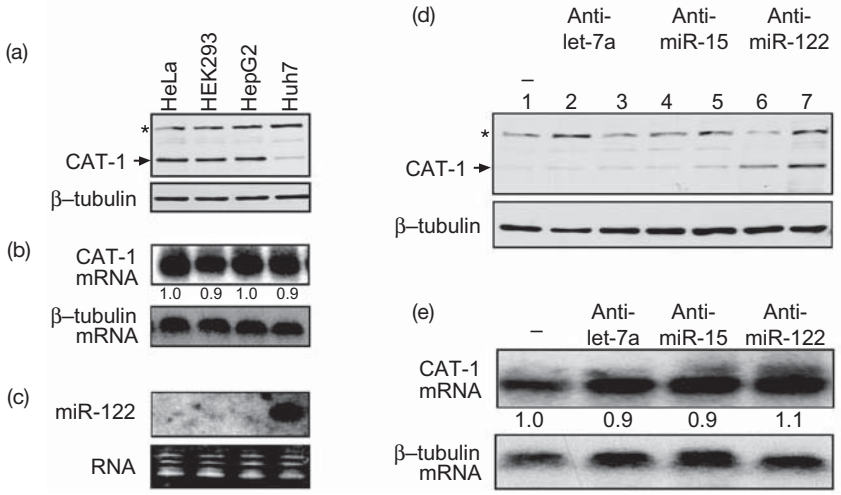


Figure 16.44 Repression of *CAT-1* translation in Huh7 cells. **(a)** Protein levels in four different human cell lines. Filipowicz and colleagues measured CAT-1 and β-tubulin protein levels in the four cell lines by Western blotting, using antibodies against the two proteins. β-tubulin was a control for the consistency of extract preparation, and the fact that the amount of β-tubulin in each extract was about equal means that the differences in CAT-1 content are real, and Huh7 cells really do contain less the protein. **(b)** Measurement of CAT-1 and β-tubulin mRNA concentrations in the four cell lines by Northern blotting. Again, β-tubulin mRNA was a control, and the concentrations of CAT-1 mRNA were normalized to the β-tubulin mRNA concentrations in the same cells. The normalized values for the CAT-1 mRNA levels are given between the two Northern blots. No significant difference was observed between CAT-1 mRNA levels in Huh7 cells and in the other three cell lines. **(c)** Upper panel: Northern blot analysis of miR-122 concentration in the four cells lines. Lower panel: Ethidium bromide staining of the gel used for the Northern blot, showing roughly equal amounts of RNA in all lanes. **(d)** Western blot analysis of the effects of miRNA antisense oligonucleotides on CAT-1 levels in Huh7 cells. Only the anti-miR-122 had a stimulatory effect. **(e)** Northern blot analysis of the effects of miRNA antisense oligonucleotides on CAT-1 and β-tubulin mRNA levels in Huh7 cells. CAT-1 mRNA levels were normalized to β-tubulin levels in the same extracts and the normalized values are presented between the two Northern blots. The anti-miR-122 oligonucleotides had no significant effect on CAT-1 mRNA level.
(*Source:* Reprinted from CELL, Vol. 125, Bhattacharyya et al, Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress, Issue 6, 13 June 2006, pages 1111–1124, © 2006, with permission from Elsevier.)

of reporter constructs containing the *Renilla* luciferase coding region fused to various versions of the CAT-1 mRNA 3′-UTR. Then they tested these constructs in Huh7 and HepG2 cells. In HepG2 cells, in which the CAT-1 gene is not regulated, they found that constructs containing the miR-122 binding sites produced the same amount of luciferase as constructs lacking these sites. However, in Huh7 cells, in which the CAT-1 gene is regulated, reporter constructs lacking the miR-122 binding sites produced about three times more luciferase than constructs that contained these sites. Again, Northern blot analysis showed that mRNA levels did not vary, even though luciferase levels did. These findings support the hypothesis that CAT-1 production is controlled negatively by miR-122.

Based on what we know so far, we would predict that starvation for amino acids should derepress CAT-1 production in Huh7 cells, and this stimulatory effect should depend on miR-122. Accordingly, Filipowicz and colleagues starved Huh7 and HepG2 cells for amino acids and used Western blots to assay the effects on CAT-1 expression. As predicted, they observed a four-fold increase in CAT-1 level upon starvation of Huh7 cells, but not HepG2 cells, and this effect occurred within one hour. On the other hand, Northern blots showed that, while there was a 1.8-fold increase in CAT-1 mRNA level, this effect was undetectable until after three h of starvation. These results indicate that the stimulatory effect of starvation on Huh7 cells occurs via enhanced translation of preexisting CAT-1 mRNA.

The use of luciferase reporter constructs with and without miR-122 binding sites showed that the stimulatory response to starvation in Huh7 cells occurred only with constructs containing these sites. Thus, the derepression appeared to be dependent on miR-122. To check this conclusion, Filipowicz and colleagues turned to HepG2 cells, which do not normally express miR-122, and in which CAT-1 production is not inducible by starvation. To these cells, they added a miR-122 gene construct that would be expressed constitutively. In these engineered cells, a luciferase reporter construct with the CAT-1 mRNA 3′-UTR was activated by starvation, indicating that miR-122 is really involved in the repression observed in Huh7 cells.

Another interesting finding came from these studies in HepG2 cells: A luciferase reporter construct containing just the miR-122 binding sites from the CAT-1 mRNA 3′-UTR was *not* responsive to starvation. This result spurred Filipowicz and colleagues to look more closely at the CAT-1 mRNA 3′-UTR. They focused on a part of the 3′-UTR known as region D, which contains an ARE, which they named ARD. This is not a binding site for miR-122, or any other known miRNA, but it is a binding site for a protein known as HuR. This finding led to the hypothesis that HuR, in addition to miR-122, is required for regulation of CAT-1 production in starved Huh7 cells.

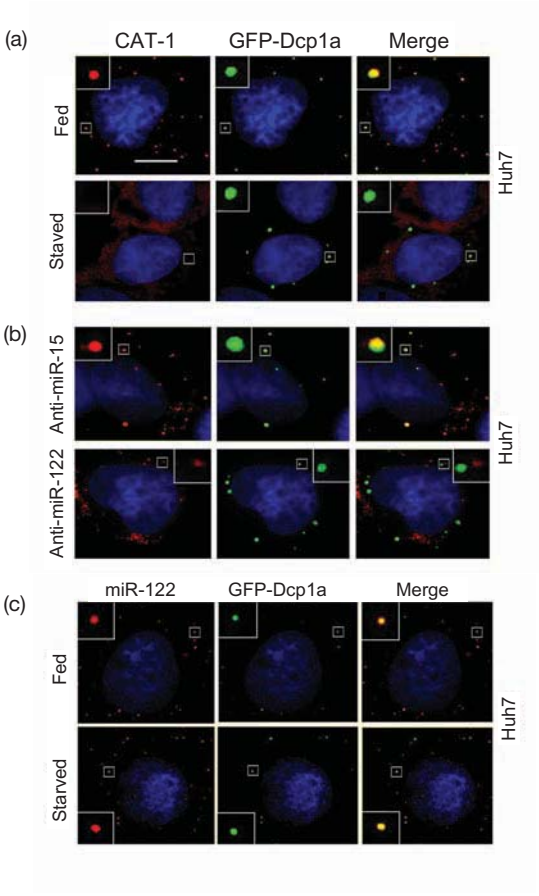
To test this hypothesis, Filipowicz and colleagues first demonstrated that knocking down the cellular level of HuR by RNAi abolished the responsiveness to starvation of luciferase reporters bearing the CAT-1 mRNA 3′-UTR in Huh7 cells. Thus, HuR does seem to be required for CAT-1 regulation. Second, they showed that HuR binds to the CAT-1 mRNA 3′-UTR by immunoprecipitating reporter constructs bearing the CAT-1 mRNA 3′-UTR with an anti-HuR antibody. As expected, the construct containing only the miR-122 binding sites, but not the region D, could not be immunoprecipitated with this antibody. A second set of binding studies using a gel mobility shift assay showed that complexes formed between a labeled region D RNA fragment and a GST-HuR fusion protein. It is significant that reporter constructs containing only a region D, with no miR-122 binding sites, were not subject to regulation in Huh7 cells. Thus, HuR and miR-122 act together to regulate expression of the CAT-1 gene.

Because it was known that repressed mRNAs could be found in P-bodies, while actively translated mRNAs are found in polysomes, Filipowicz and colleagues looked in these compartments for CAT-1 mRNA and luciferase reporters under starved and unstarved conditions. Figure 16.45a shows immunofluorescence data for CAT-1 mRNA (detected by in situ hybridization with a red-fluorescent-tagged CAT-1 antisense probe). In fed cells, the red CAT-1 mRNA was found in discrete cytoplasmic bodies. We know they are P-bodies because a marker for P-bodies, GFP-Dcp1a, which fluoresces green, co-localizes with the red fluorescing CAT-1 mRNA. Together, the red and green fluorescence produce the yellow color seen in the right hand panel. Transfecting the cells with an anti-miR-122 antisense RNA abolished the P-body location of the CAT-1 mRNA in fed cells (Figure 16.45b), demonstrating that this localization is miR-122-dependent.

On the other hand, in starved cells, CAT-1 mRNA was no longer detectable in P-bodies (Figure 16.45a). Was all miR-122 lost from the P-bodies along with the CAT-1 mRNA? Figure 16.45c, in which miR-122 was detected by in situ hybridization with a red-fluorescing probe, shows that it was not. Thus, miR-122 presumably regulates the translation of a large number of mRNAs in liver cell P-bodies, so the loss of one (or perhaps a few) regulated mRNAs during starvation did not significantly lower the miR-122 concentration in these P-bodies.

Did the CAT-1 mRNA in starved cells move from the P-bodies to polysomes? To find out, Filipowicz and colleagues displayed polysomes by sucrose gradient ultracentrifugation and assayed each sample for CAT-1 mRNA by Northern blotting. Figure 16.45d shows a big increase in CAT-1 mRNA in polysomes upon starvation of Huh7 cells, and Figure 16.45e quantifies this effect. This effect is specific to CAT-1 mRNAs. Most mRNAs react to starvation as the control β-tubulin mRNA did in Figure 16.45d and e: They move out of polysomes.

Filipowicz and colleagues also showed that the migration of CAT-1 mRNA from P-bodies to polysomes in



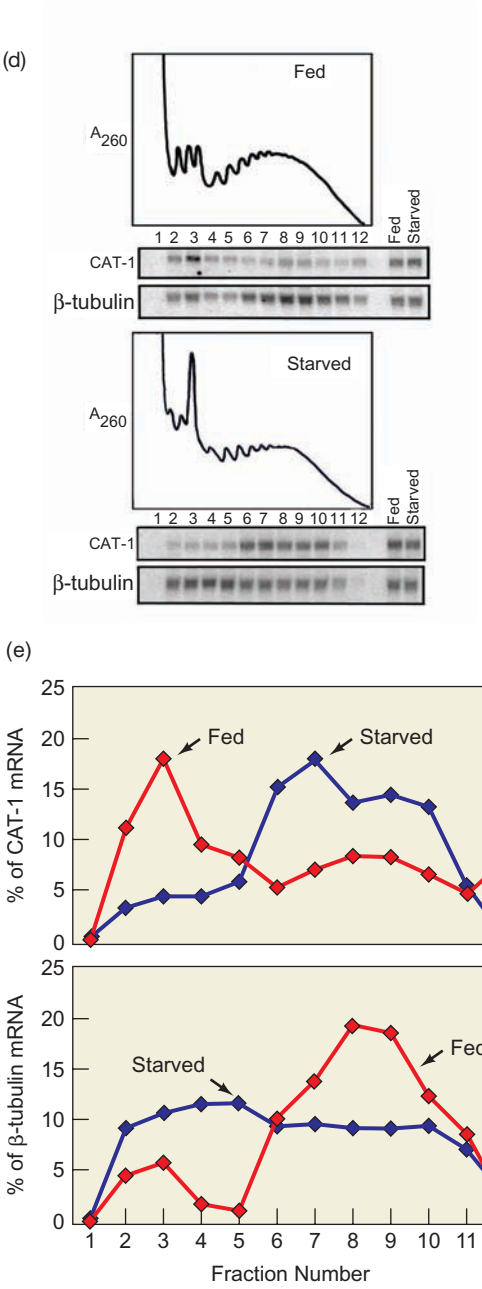


Figure 16.45 Starvation-induced relocation of CAT-1 mRNA from P-bodies to polysomes. (a) Loss of CAT-1 mRNA from P-bodies upon starvation in Huh7 cells. CAT-1 mRNA (left column) was detected by in situ hybridization with a red-fluorescent-tagged probe. The P-body marker, GFP-Dcp1a (middle column) fluoresces green. The right column is a merged view of the other two columns. In each micrograph, a P-body (small square) was selected, enlarged and presented in the large square at the upper left corner. The top row contains fed cells, and the bottom row, starved cells, as indicated at left. In fed cells, the merged view is yellow, reflecting the co-localization of the CAT-1 mRNA (red) and GFP-Dcp1a (green). In starved cells, there is essentially no red fluorescence in the P-bodies, so the merged view is green. **(b)** Effect of two antisense miRNAs on P-body localization of CAT-1 mRNA in fed cells. The irrelevant anti-miR-15 had no effect, but the anti-miR-122 blocked the localization of CAT-1 mRNA to P-bodies. Staining of the cells in the three columns was as in panel (a). **(c)** Presence of miR-122 in P-bodies in fed and starved Huh7 cells. Staining of the cells in the three columns was as in panel (a) except that a red-fluorescing anti-miR-122 oligonucleotide was used in the left-hand coumn. **(d)** Polysome analysis. Polysomes from fed and starved cells were displayed by sucrose gradient ultracentrifugation, and gradient fractions were subjected to Northern blotting and probed for either CAT-1 mRNA or β-tubulin mRNA, as indicated at left. Input RNA from fed and starved cells is probed at right. Starvation caused an increase in CAT-1 mRNA, but a decrease in β-tubulin mRNA, in heavy polysomes. **(e)** Graphic representation of the data from panel (d). The amount of CAT-1 (top) and β-tubulin (bottom) mRNAs are plotted vs. gradient fraction number in polysome profiles from fed (red) and starved (blue) cells. (*Source:* Reprinted from CELL, Vol. 125, Bhattacharyya et al, Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress, Issue 6, 13 June 2006, pages 1111–1124, © 2006, with permission from Elsevier.)

starved cells depended on HuR and region D of the CAT-1 mRNA 3′-UTR. They demonstrated that HuR moved with CAT-1 mRNA from P-bodies to polysomes upon amino acid starvation. Furthermore, when they knocked down HuR in starved Huh7 cells, they found that CAT-1 mRNA no longer relocated from P-bodies to polysomes.

If HuR helps move CAT-1 mRNA out of P-bodies upon starvation, then perhaps endowing another mRNA with the HuR binding site (region D) would enable it to move out of P-bodies under the same conditions. Filipowicz and colleagues tested this prediction by placing region D into another luciferase reporter mRNA (RL-3XBulge) that is responsive to the miRNA *let-7*. Ordinarily, this reporter mRNA is directed to P-bodies in cells, such as HeLa cells, that express *let-7*, and does not move out of P-bodies upon starvation. However, with region D added, the mRNA responded to starvation in HeLa cells by exiting the P-bodies. All this evidence points to an important role for HuR in transporting CAT-1 mRNA out of P-bodies in starved cells. It also suggests that the stress-related reactivation of mRNAs undergoing miRNA-mediated repression may be a general phenomenon that applies to a variety of mRNAs in a variety of cell types.

SUMMARY In a liver cell line (Huh7), translation of the CAT-1 mRNA is repressed by the miRNA miR-122, and the mRNA is sequestered in P-bodies. Upon starvation, the translation repression of the CAT-1 mRNA is relieved and the mRNA migrates from P-bodies to polysomes. This derepression and translocation of the mRNA depends on the mRNA-binding protein HuR, and on its binding site (region D) in the 3′-UTR of the mRNA. Such derepression and translocation in response to stress may be a common response of miRNA-repressed mRNAs.

Other Small RNAs

Since the discoveries of siRNAs, miRNAs, and piRNAs, other small RNAs have been found, although the functions of these RNAs are largely still unknown. One example is the **endo-siRNAs** of *Drosophila*. Like miRNAs, these are made from *Drosophila* genes as double-stranded RNA precursors. However, like siRNAs, these RNA precursors are processed by the Dicer-2 (DCR-2) pathway, and are loaded onto a RISC that contains Ago2. Thus, even though these RNAs are produced endogenously, their processing pathway suggests that they should be called siRNAs, rather than miRNAs. Accordingly, we call them endo-siRNAs, even as we acknowledge that these RNAs blur the line between siRNAs and miRNAs.

It is interesting that fruit flies with defective DCR-2 or Ago2 experience an increased level of transposon expression in somatic cells. This finding suggests that endo-siRNAs may help protect somatic cells against transposition, just as piRNAs protect germ cells.

SUMMARY Endo-siRNAs of *Drosophila* are encoded in the cellular genome, yet they are processed like siRNAs, rather than miRNAs. They may help protect somatic cells against transposons.

SUMMARY

Ribosomal RNAs are made in eukaryotic nucleoli as precursors that must be processed to release the mature rRNAs. The order of RNAs in the precursor is 18S, 5.8S, 28S in all eukaryotes, although the exact sizes of the mature rRNAs vary from one species to another. In human cells, the precursor is 45S, and the processing scheme creates 41S, 32S, and 20S intermediates. The snoRNAs play vital roles in these processing steps.

Extra nucleotides are removed from the 5′-ends of pre-tRNAs in one step by an endonucleolytic cleavage catalyzed by RNase P. RNase P's from bacteria and eukaryotic nuclei have a catalytic RNA subunit called M1 RNA. RNase II and polynucleotide phosphorylase cooperate to remove most of the extra nucleotides at the 3′-end of an *E. coli* tRNA precursor, but stop at the +2 stage. RNases PH and T are most active in removing the last two nucleotides from the RNA. In eukaryotes, a single enzyme, tRNA 3′-processing endoribonuclease (3′-tRNase), processes the 3′-end of a pre-tRNA.

Trypanosome mRNAs are formed by *trans*-splicing between a short leader exon and any one of many independent coding exons.

Trypanosomatid mitochondria (kinetoplastids) encode incomplete mRNAs that must be edited before they can be translated. Editing occurs in the 3′→5′ direction by successive action of one or more guide RNAs. These gRNAs hybridize to the unedited region of the mRNA and provide A's and G's as templates for the incorporation of U's missing from the mRNA or deletion of extra U's.

Some adenosines in mRNAs of higher eukaryotes, including fruit flies and mammals, must be deaminated to inosine post-transcriptionally for the mRNAs to code for the proper proteins. Enzymes known as adenosine deaminases active on RNAs (ADARs) carry out this kind of RNA editing. In addition, some cytidines must be deaminated to uridine for an mRNA to code properly.

A common form of post-transcriptional control of gene expression is control of mRNA stability. For example, the mammalian casein and transferrin receptor (Tfr) genes are

controlled primarily by altering the stabilities of their mRNAs. When cells have abundant iron, the level of tranferrin receptor is reduced to avoid accumulation of too much iron in cells. Conversely, when cells are starved for iron, they increase the concentration of transferrin receptor to transport as much iron as possible into the cells. The transferrin receptor (TfR) mRNA stability is controlled as follows: The 3′-UTR of the TfR mRNA contains five stem-loops called iron response elements (IREs), which render the mRNA susceptible to degradation by RNase. When iron concentration is low, aconitase exists as an apoprotein that lacks iron. This protein binds to the IREs in the TfR mRNA and protects the RNA against attack by RNases. But when iron concentration is high, the aconitase apoprotein binds to iron and therefore cannot bind to the mRNA IREs. This leaves the RNA vulnerable to degradation.

RNA interference occurs when a cell encounters dsRNA from a virus, a transposon, or a transgene (or experimentally added dsRNA). This trigger dsRNA is degraded into 21–23-nt fragments (siRNAs) by an RNase III-like enzyme called Dicer. The double-stranded siRNA, with Dicer and the Dicer-associated protein R2D2, recruit Ago2 to form a pre-RISC complex that can separate the siRNA into its two component strands: the guide strand, which will base-pair with the target mRNA in the RNA-induced silencing complex (RISC) and guide cleavage of the mRNA, and the passenger strand, which will be discarded. Ago2 cleaves the passenger strand, which then falls off the pre-RISC complex. The guide strand of the siRNA then base-pairs with the target mRNA in the active site in the PIWI domain of Ago2, which is an RNase H-like enzyme, also known as slicer. Slicer cleaves the target mRNA in the middle of the region of its base-pairing with the siRNA. In an ATP-dependent step, the cleaved mRNA is ejected from the RISC, which can then accept a new molecule of mRNA to be degraded. In certain species, the siRNA is amplified during RNAi when antisense siRNAs hybridize to target mRNA and prime synthesis of full-length antisense RNA by an RNA-dependent RNA polymerase. This new dsRNA is then digested by Dicer into new pieces of siRNA.

The RNAi machinery is involved in heterochromatization at yeast centromeres and silent mating-type regions, and is also involved in heterochromatization in other organisms. At the outermost regions of centromeres of fission yeast, active transcription of the reverse strand occurs. Occasional forward transcripts, or forward transcripts made by RdRP, base-pair with the reverse transcripts to kick off RNAi, which in turn recruits a histone methyltransferase, which methylates lysine 9 of histone H3, which recruits Swi6, which causes heterochromatization. In plants and mammals, this process is abetted by DNA methylation, which can also attract the heterochromatization machinery. Individual genes in mammals can also be silenced by RNAi, which targets the control region, rather than the coding region, of the gene. This silencing process involves DNA methylation, rather than mRNA destruction.

MicroRNAs (miRNAs) are 18–25-nt RNAs produced from a cellular RNA with a stem-loop structure. In the last step in miRNA synthesis, Dicer cleaves the double-stranded stem part of the precursor to yield the miRNA in double-stranded form. The single-stranded forms of these miRNAs can team up with an Argonaute protein in a RISC to control the expression of other genes by base-pairing to their mRNAs. In animals, miRNAs tend to base-pair imperfectly to the 3′-UTRs of their target mRNAs and inhibit accumulation of the protein products of these mRNAs. However, perfect or perhaps even imperfect base-pairing between an animal miRNA and its target mRNA can result in mRNA cleavage. In plants, miRNAs tend to base-pair perfectly or near-perfectly with their target mRNAs and cause cleavage of these mRNAs, although there are exceptions in which translation blockage can occur.

MicroRNAs can activate, as well as repress translation. In particular, miR369-3, with the help of Ago2 and FXR1, activates translation of the TNFα mRNA in serum-starved cells. On the other hand, miR369-3, with the help of Ago2, represses translation of the mRNA in synchronized cells growing in serum.

RNA polymerase II transcribes the miRNA precursor genes, to produce pri-miRNAs, which may encode more than one miRNA. Processing a pri-mRNA to a mature miRNA is a two-step process. In the first step, a nuclear RNase III known as Drosha cleaves the pri-miRNA to release a 60–70-nt stem-loop RNA known as a pre-miRNA. In the second step, which occurs in the cytoplasm, Dicer cuts the pre-miRNA within the stem to release a mature double-stranded miRNA. A mirtron is an intron that consists of a pre-miRNA. Thus, the spliceosome cuts it out of its pre-mRNA, then it is debranched and folded into a stem-loop pre-miRNA, without any participation by Drosha.

P-bodies are cellular foci where mRNAs are stored, destroyed, and translationally repressed. GW182 is an essential part of the *Drosophila* miRNA silencing mechanism in P-bodies, whether this mechanism involves translation inhibition or mRNA decay. AGO1 probably recruits GW182 to an mRNA within a P-body, and this marks that mRNA for silencing. GW182 and AGO1-mediated mRNA decay in P-bodies appears to involve both deadenylation and decapping, followed by mRNA degradation by a 5′→3′ exonuclease.

In a liver cell line (Huh7), translation of the CAT-1 mRNA is repressed by the miRNA miR-122, and the mRNA is sequestered in P-bodies. Upon starvation, the translation repression of the CAT-1 mRNA is relieved and

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Endo-siRNAs of *Drosophila* are encoded in the cellular genome, yet they are processed like siRNAs, rather than miRNAs. They may help protect somatic cells against transposons.

REVIEW QUESTIONS

1. Draw the structure of a mammalian rRNA precursor, showing the locations of all three mature rRNAs.
2. What is the function of RNase P? What is unusual about this enzyme (at least the bacterial and eukaryotic nuclear forms of the enzyme)?
3. Illustrate the difference between *cis*- and *trans*-splicing.
4. Describe and give the results of an experiment that shows that a Y-shaped intermediate exists in the splicing of a trypanosome pre-mRNA. Show how this result is compatible with *trans*-splicing, but not with *cis*-splicing.
5. Describe what we mean by RNA editing. What is a cryptogene?
6. Describe and give the results of an experiment that shows that editing of kinetoplast mRNA goes in the 3′→5′ direction.
7. Draw a diagram of a model of RNA editing that fits the data at hand. What enzymes are involved?
8. Present direct evidence for guide RNAs.
9. Outline the evidence that shows that editing of the mouse *GluR-B* transcript by ADAR2 is essential, and that this transcript is the only critical target of ADAR2.
10. Describe and give the results of an experiment that shows that prolactin controls the casein gene primarily at the post-transcriptional level.
11. What two proteins are most directly involved in iron homeostasis in mammalian cells? How do their levels respond to changes in iron concentration?
12. How do we know that a protein binds to the iron response elements (IREs) of the TfR mRNA?
13. Describe and give the results of an experiment that shows that one kind of mutation in the TfR IRE region results in an iron-unresponsive and stable mRNA, and another kind of mutation results in an iron-unresponsive and unstable mRNA. Interpret these results in terms of the rapid turnover determinant and interaction with IRE-binding protein(s).
14. Present a model for the involvement of aconitase in determining the stability of TfR mRNA.
15. What evidence suggests that RNA interference depends on mRNA degradation?
16. Present a model for the mechanism of RNA interference.
17. Describe and give the results of an experiment that shows that Argonaute2 has slicer activity.
18. What roles do R2D2 and Ago2 play in formation of the RISC? What happens if R2D2 is absent?
19. Diagram the ping-pong mechanism whereby piRNAs are thought to amplify themselves and inactivate transposons at the same time.
20. Present a model for the involvement of the RNAi machinery in heterochromatization in fission yeast. How would this model have to be modified to describe the situation in mammals?
21. Present a model for gene silencing and heterochromatization in flowering plants. In what major ways does this differ from the model in fission yeast?
22. What is the evidence for the importance of non-siRNA transcripts in gene silencing in fission yeast and in flowering plants?
23. Chromatin targets for heterochromatization in dividing cells must be transcribed in order to be silenced. How is this problem resolved in fission yeast and in flowering plants?
24. Describe and give the results of experiments showing: (1) that a mammalian gene can be silenced by a mechanism involving an siRNA directed at the gene's control region; and (2) that DNA methylation is involved in the silencing.
25. Outline the processes by which siRNAs and miRNAs are produced. List the key players in these processes. Be sure to include two different ways to produce pre-miRNAs.
26. How can siRNAs that target the promoter region of a gene be made? Present evidence to support your hypothesis.
27. Compare and contrast the typical actions of siRNAs and miRNAs in animals.
28. MicroRNAs in animals typically base-pair imperfectly to their targets in the 3′-UTRs of mRNAs. How does their activity change if they base-pair perfectly, or near-perfectly? Present evidence.
29. Describe an example in which an miRNA activates translation of a gene. How was this activation assayed? Present evidence that base-pairing between this miRNA and the mRNA's ARE is important in activation.
30. Describe and present the results of an experiment that shows that the protein GW182 can reduce translation of an mRNA in P-bodies. Include a description of how the protein can be physically tethered to the mRNA. How much of the loss of protein product is due to mRNA destruction, and how much is due to translation repression? How can these two effects be experimentally separated?
31. Describe and give the results of experiments that show that:
    (a) translation of an mRNA is repressed by an miRNA in P-bodies.
    (b) this repression can be overcome in stressed cells.
    (c) an mRNA-binding protein is also required for relief of repression.
    (d) relief of repression is accompanied by the translocation of the mRNA from P-bodies to polysomes.

ANALYTICAL QUESTIONS

1. Why can *dicer* dsRNA never completely block RNAi?
2. Predict the effects of the following mutations on the abundance of the TfR mRNA. That is, would the mutations result in a constitutively low or high level of the TfR mRNA regardless of iron concentration, or would they have no effect on the mRNA level?
   a. A mutation that blocks the production of aconitase.
   b. A mutation that prevents aconitase from binding iron.
   c. A mutation that prevents aconitase from binding to the IREs.
3. Discuss the conflicting evidence about the effect of *lin-4* miRNA on expression of the *lin-14* gene in *C. elegans*.

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