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# Translation repression by antisense sequences

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**Abstract.** Antisense sequences that hybridize to messenger RNA can inhibit target gene expression in a variety of ways. The best-known antisense mechanisms trigger messenger RNA (mRNA) degradation; however, translation repression by ribosome interference is a common natural antisense mechanism. In bacteria, there are fascinating examples of cis-encoded and trans-encoded antisense sequences that reversibly repress translation. In eukaryotes, microRNAs that bind 3′UTR target sequences also repress translation, although the mechanism is unclear. An important feature of translation repression is that the mRNA can remain intact during periods of repression, and rapid expression switching can occur in response to cellular and environmental signals. Recent genome analyses indicate many new short noncoding RNAs with predicted antisense activities. Therefore, translation repression by antisense sequences is likely to be a common and important form of posttranscriptional gene control, and such natural mechanisms provide a basis for the development of synthetic antisense gene control in research and drug development.

**Key words.** Antisense; translation; repression; RNA; ribosome.

## Introduction

Antisense is usually considered as a mechanism for sequence-specific messenger RNA (mRNA) recognition that leads to transcript degradation. However, the antisense mechanism, by definition, includes all forms of sequence recognition that reduce or alter expression of a particular gene transcript. In nature, antisense gene control is widespread, and several distinct new mechanisms have recently been discovered. The variety of effects observed include mRNA destruction, repression and activation, and even altered RNA processing and effects on transcription [1]. A similar wide variety of effects are possible using synthetic antisense sequences. Therefore, antisense should be seen with a wide perspective.

This review focuses on translation repression by antisense sequences. Translation repression by antisense is part of posttranscriptional gene control. While transcription is clearly important in gene expression control, several studies have shown that translation is also extensively regulated. Translation control can elaborate expression beyond what is possible with transcription control, and translation repression by antisense offers possibilities beyond what is possible with mRNA degradation. This review covers examples of translation repression by antisense sequences in prokaryotes and eukaryotes, and both natural and synthetic antisense sequences are discussed.

## Translation repression by steric hindrance

Proteins that bind DNA to repress transcription are well known, whereas factors that repress translation are less appreciated. Translation repression can involve inhibition of ribosome elongation; however, the ribosome is highly processive, and only very tight binding antisense agents can block progression of an assembled ribosome [2]. Translation initiation, on the other hand, can be inhibited by the mRNA structure itself or by antisense sequences that cover or mask recognition signals. It is easy to envision how this can occur in bacteria, where the ribosome binds to mRNA at the well-characterized ribosome binding site (RBS) [3]. In eukaryotes, mRNA recognition and assembly is more complex, and it is difficult to design inhibitory antisense agents. Nevertheless, there are many successful examples of antisense inhibition of translation

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initiation in eukaryotes. The 5′cap structure and start codon regions, and other regions involved in translation initiation appear to be the most susceptible targets [4, 5] (fig. 1). Certain eukaryotic mRNAs are translated through ribosomal initiation at an internal ribosomal entry site (IRES), and this process can be hindered by antisense [6]. Finally, the 3′UTR can influence translation initiation, and antisense sequences can repress the process [7]. Therefore, regions susceptible to translation repression by antisense sequences mainly lie upstream and downstream of the open reading frame and are involved in initiation (fig. 1).

Like transcription repression, translation repression can be a reversible process where ribosome initiation or elongation is inhibited. Although mRNA that is not actively translated can be degraded in cells, there are many examples where the message remains intact, and this provides a basis for reversible translation repression. This a interesting feature of translation repression by antisense sequences, and it discussed further below.

## Translation repression by trans-encoded antisense transcripts

Many natural antisense sequences are trans-acting regulatory RNAs, where the antisense RNA is transcribed from a distant locus. There may be some bias towards the discovery of such trans-encoded RNAs, as this is the expected nature of antisense; however, it seems likely that this is indeed the most common mechanism for natural antisense. Examples of trans-encoded antisense RNAs are listed in table 1.

Table 1. Examples of antisense-mediated translation repression.

| Species | Gene/RNA | Cell process affected | Locus | Ref. |
|---|---|---|---|---|
| *E. coli* | *cat* | antibiotic resistance | cis | [16] |
| *E. coli* | *rpoS* | growth | cis | [18] |
| *E. coli* | *ryhB* | iron metabolism | trans | [46] |
| *E. coli* | OxyS | global regulator | trans | [9] |
| *E. coli* | *micF* | stress response | trans | [8, 47] |
| *E. coli* | *spf*/Spot42 | carbon utilization | trans | [10] |
| *Bacillus subtilis* | *spo0B* | sporulation | cis | [19] |
| *S. aureus* | RNAIII | virulence | trans | [21] |
| *C. elegans* | *lin-4-7* | embryonic development | trans | [5] |
| Human | *CTGF* | growth | cis | [20] |

In prokaryotes, trans-encoded antisense sequences typically work by binding to the start codon region of mRNA. For example, MicF RNA represses the translation of the outer membrane protein gene *ompF* [8], and the OxyS

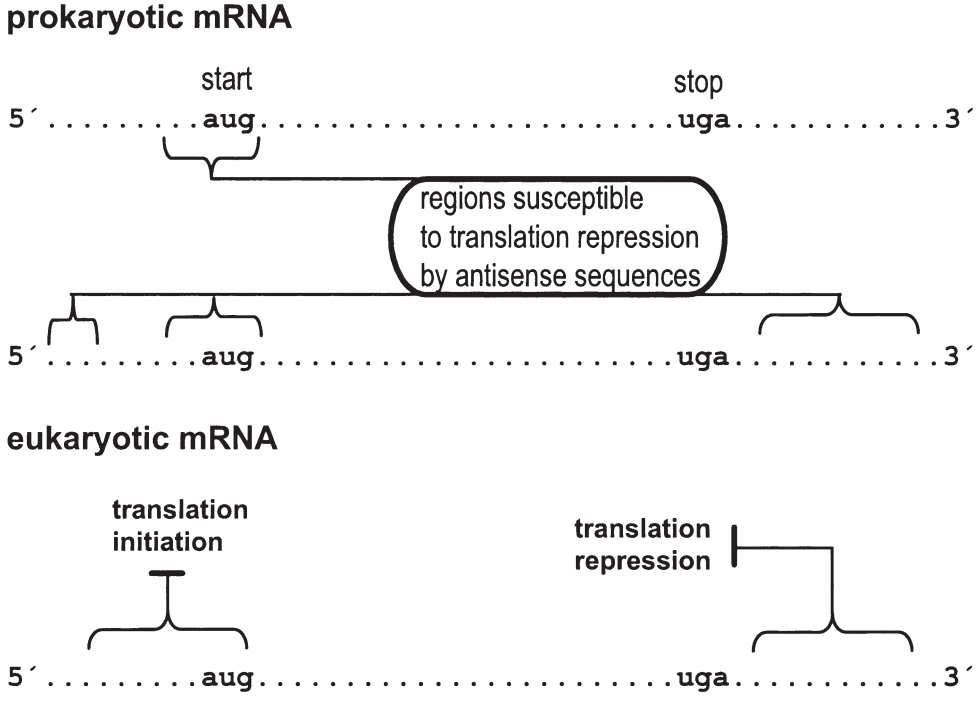


Figure 1. mRNA regions susceptible to translation repression by antisense. The translation start codon region of prokaryotic mRNAs and the 3′UTR of eukaryotic mRNA are sites where antisense sequences either in cis or in trans anneal and repress translation. Antisense sequence binding within the upstream or downstream untranslated regions can repress translation. For target sequences within the 3′UTR, the mechanism of translation repression is uncertain.

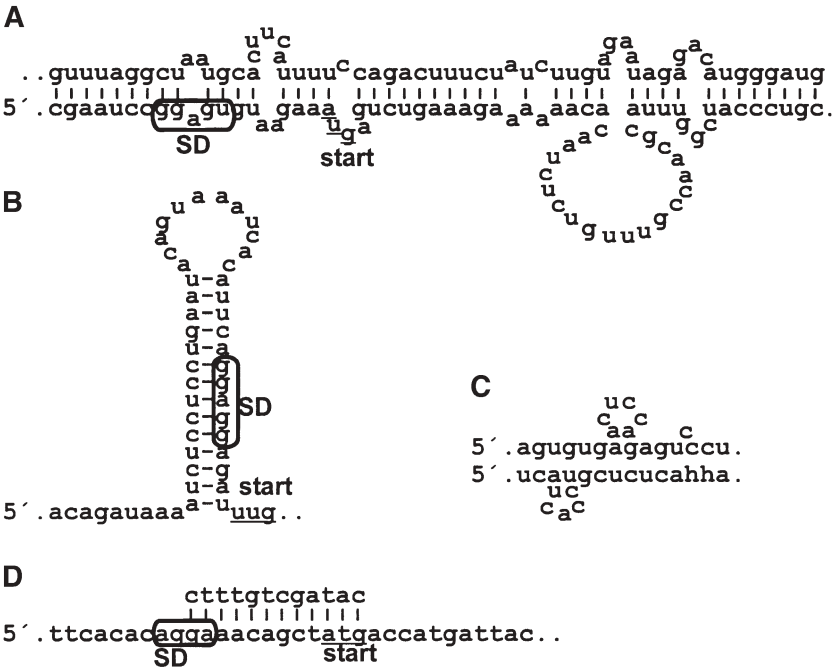


Figure 2. Examples of antisense interactions that repress translation. (*A*) The trans-encoded Spot 42 RNA annealed to its target *galK* transcript [46]. (*B*) The cis-encoded attenuating structure of the *catA86* mRNA [16]. (*C*) The trans-encoded *lin*-4 miRNA annealed to its target within the 3′UTR of *lin*-14 mRNA. (*D*) Example of a synthetic antisense agent sequence targeting the *E. coli lacZ* mRNA [25]. For the prokaryotic examples the ribosome binding site and start codon for the transcripts are indicated, and the antisense sequence sequesters the RBS [5].

RNA inhibits translation of two target genes, *fhlA* and *rpoS* [9]. There are many other examples involved in a range of cellular processes (table 1) [1]. An especially interesting case is *Escherichia coli* Spot 42 RNA repression of *galK* within the *galETKM* operon transcript [10]. Figure 2 illustrates Spot 42 RNA interaction with the *galK* transcript; note that the antisense interaction masks the ribosome binding site (RBS) used for translation initiation. This example shows that antisense RNA can repress translation initiation and even discoordinate expression of an otherwise coordinated expression system. Note also that such discoordinated expression of operon-encoded genes would be difficult for the cell to achieve through transcription control.

In eukaryotes, most natural antisense RNAs repress translation by binding sequences within the 3′UTR [7]. In *Caenorhabditis elegans*, the *lin*-4 and *let*-7 RNAs display partial complementarity to multiple sites that lie within the 3′UTRs of *lin*-14 and *lin*-28 transcripts, and the antisense interaction represses translation to influence the time of postembryonic development [5, 11]. The sequence of *let*-7 is conserved, and a similar antisense-sense pairing may regulate temporal development in higher eukaryotes [12]. Therefore, this is a well-known and possibly widespread antisense interaction in eukaryotes, yet the mechanism of repression is unclear. Beyond evidence for antisense-sense pairing, there is speculation that trans-acting protein factors are involved [13]; also mismatches between the antisense and target RNAs appear to be important (see below). In human cells, antisense sequences may hybridise to the 3′UTR of several genes to control translation [14].

Trans-acting small antisense RNAs are typically transcribed from a distant locus; however, this need not be the case. There is at least one example of a cis-encoded, trans-acting antisense RNA. The *ldrD* locus encodes a short toxic peptide, and like other toxic peptides its expression is negatively regulated by an antisense RNA, which masks the translation start region [15]. Interestingly, the *rdlD* encoded antisense RNA, the antitoxin, is perfectly complementary to the *ldrD* transcript that encodes the LdrD toxin. Therefore, a single locus encodes both the target mRNA and the antisense regulator, where the target is a toxin encoding mRNA with a long half-life, and the antisense RNA is a short half-life antitoxin. It is not clear how the cell might benefit from a cis-encoded antisense RNA, except that this would ensure correct pairing between the toxin and antitoxin in the event of mutation within the region of overlap.

### Translation attenuation by cis-encoded antisense transcripts

The regulatory regions within a single mRNA can contain neighbouring cis-encoded antisense sequences that form an intramolecular antisense-sense fold. Although, this arrangement is less obvious as a mechanism for antisense control, it is has been associated with many genes and was first described in 1985, soon after the description of trans-encoded antisense RNAs (table 1). As with trans-encoded antisense RNAs, the folded structure masks or sequesters the RBS, and this prevents initiation.

Well-known examples of cis-encoded antisense sequences lie within the chloramphenicol and erythromycin resistance genes in *E. coli* [16]. Antibiotic exposure is an example of a mechanism that requires a rapid response to ensure cell survival, and it seems reasonable for cells to constitutively transcribe antibiotic resistance genes and then control expression at the translation level. For both the chloramphenicol and erythromycin resistance genes, rapid expression control appears to be provided by antisense sequences that lie within a short open reading frame just upstream of the start codon region. In the absence of antibiotics that inhibit translational elongation, the leader peptide is rapidly translated, and the signals for the downstream open reading frame are masked. When an antibiotic slows translation elongation, poor translation of the upstream gene leads to extended unmasking of the downstream initiation region of the resistance gene and expression. In other words, leader peptide translation slows in the presence of the antibiotic, and the retarded ribosome unmasks the RBS of the downstream antibiotic resistance gene.

The key feature in these examples of antisense gene regulation is the adjacent cis-encoded antisense sequence. This proximity should favour rapid binding and gene-specific inhibition. In prokaryotes, cis-encoded antisense elements control growth and pathogenesis-related genes. For example, a temperature-responsive cis-encoded antisense sequence that represses virulence gene expression in *Listeria monocytogenes* responds quickly to temperature changes encountered during pathogenesis [17]. Also, as *E. coli* enters stationary phase growth, the majority of posttranscriptional gene control of *rpoS* expression is provided by a cis-encoded antisense element [18]. Similarly, as *Bacillus subtilis* encounters nutritional depravation, several sporulation-specific genes are expressed after a release from antisense repression of translation [19]. In eukaryotes, regulation of several growth factor receptors is controlled by cis-encoded antisense sequences that repress translation. These examples from diverse species suggest that translation repression by cis-encoded antisense sequences is appropriate where rapid expression level changes are needed [20].

### Anti-antisense mechanisms

The importance of antisense regulatory sequences is well established. Therefore, it is worth asking, What regulates the action of antisense RNAs? This question has yet to be addressed systematically; however, there are now two examples of anti-antisense RNAs, where the action of a trans-encoded antisense RNA releases translation repression caused by a cis-encoded antisense sequence. In other words, the anti-antisense RNA acts as a competitive inhibitor of a primary antisense sequence. One example involves activation of haemolysis production in *Staphylococcus aureus* by the trans-encoded antisense RNA RNAIII. The haemolysin (*hla*) transcript can be repressed by cis-encoded antisense sequences, and RNAIII can prevent this interaction and activate toxin expression during infection [21]. A second example involves regulation of rpoS translation, where the DsrA RNA positively regulates translation of *rpoS* mRNA by binding to the antisense sequence that masks the *rpoS* RBS [22]. Therefore, DsrA and RNAIII effectively unmask or prevent masking of translation signals to act as anti-antisense sequences.

### Structural features of short antisense RNAs and target binding

Two typical features of natural antisense features in bacteria are a short length and a stable secondary structural fold. A secondary structural fold can contribute nuclease resistance and separable functional domains [22]. Also, the structure itself may aid recognition and binding. In many cases folded domains improve binding either directly through loop interactions, or indirectly through binding with a trans-acting factor. Among bacterial antisense RNAs, comparative sequence analysis revealed the 'U-turn' structure, which consists of a short hairpin with a tetraloop containing the YUNR motif (pyrimidine, uracil, any nucleoside, purine). This motif is thought to aid antisense interactions by lowering the local net negative charge to speed binding [23, 24]).

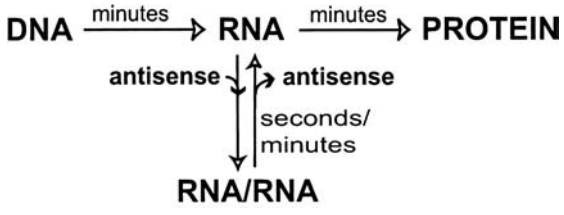


Figure 3. Kinetics of genetic switching at the translational level. The flow of genetic information is illustrated with time intervals indicated for each process. Time estimates are based on rates for transcription and translation elongation and measurements of the kinetics of short nucleic acid interactions [53].

The problem of sequence-specific target recognition must be considered with respect to genome complexity. Simple sequence uniqueness calculations suggest that gene or sequence-specific inhibitory effects in bacteria require a target sequence of approximately 12 bases [25], and in mammalian cells 15 or more bases are needed. While these are only very rough approximations, they are supported by the structure and activity of natural and synthetic antisense sequences. Natural antisense RNAs typically form 12–30 bp with target mRNA, but binding is often complicated by the presence of mismatches and gapped structures. Designed antisense agents range in size from 6 to 25 residues, and are often near 20 residues for mammalian cell applications. Therefore, antisense sequences tend to be just long enough to provide stable binding properties and gene-specific recognition. There are examples of rather long natural antisense RNAs; however, only a portion of these RNAs are involved in target hybridization, with much of the molecule folded and not directly involved in target binding.

The rate-limiting step in RNA/RNA interactions is sequence recognition. Diffusion can be a factor particularly for large RNA molecules; however, RNA/RNA hybridizations appear inherently constrained, possibly by electrostatic repulsion [23, 24]). The U-turn motif described above provides one way to reduce such constraints to binding. A prototype example of antisense RNA/mRNA recognition and binding that involves U-turn structures is the *copA/copT* system involved in plasmid replication control. Known as the 'kissing-complex' interaction, hairpin loop heads on both the antisense and target sequence speed initial binding [26]. After initial loop interactions, kissing loops provide a metastable interaction that can extend to provide the binding stability needed to inhibit the target RNA. Another feature of the *copA/copT* system is that mismatches between the antisense and target RNAs ease loop extension and formation of the final stable binding complex [27, 28]. Therefore, successful antisense interactions and stabilized binding can depend on sequence composition, higher-order structural motifs and in some cases complex RNA dynamics during binding.

Antisense RNAs have the capacity to work alone as gene regulators through RNA/RNA interactions; however, protein factors may also play a role by binding to antisense and target RNAs. Double-stranded RNA binding domains are well known as factors in RNA processing, and these domains may mediate RNA interactions. Also, a eukaryotic Sm-like sequence motif ($GAU_3GGA$-$AUAU_4AG$ AND $GU_6A$) is present in many antisense RNAs, and the *E. coli* Sm homologue Hfq recognizes this motif both within the antisense RNA and target mRNA, and Hfq can stabilize an interaction [29, 30]. Sm and Sm-like proteins are involved in a number of RNA metabolism/processing steps in eukaryotes, and this general role is conserved in bacteria, where Hfq mediates antisense-sense RNA pairing.

## The abundance of short regulatory RNAs

The first natural antisense mechanisms identified were associated with accessory elements in bacteria and viewed as peculiarities within the area of gene expression control. It is now clear that antisense mechanisms are abundant in *E. coli* and many other species. Furthermore, natural antisense is involved in core cellular processes as well as the control of accessory elements (table 1). Estimates for the abundance of short noncoding RNAs in several species are shown in table 2.

The importance of coding and structural RNAs is well recognized, but regulatory RNAs have been overlooked [31]. We are only beginning to appreciate the abundance and importance of these transcripts. Short nontranslated RNAs are widespread in *E. coli* and higher organisms. Estimates range into the hundreds for the number of short expressed RNAs in *E. coli*, and a similar large number seems likely in *C. elegans*. Bioinformatics analyses suggest that hundreds of small noncoding RNAs are expressed in *E. coli* [32] and higher organisms (table 2). Experimental evidence also indicates large numbers of expressed micro-RNAs (miRNAs) in *Caenorhabditis elegans* [33] and *Drosophila melanogaster* [34]. In the mouse, over 2000 putative sense-antisense RNA pairs are expressed [35], and tissue-specific miRNAs have been identified [36].

## miRNAs

Many of the short RNAs discovered in eukaryotes are classified as miRNAs or precursors of miRNAs (see table 2). Interestingly, these miRNAs resemble several short

Table 2. The widespread abundance of short noncoding RNAs.

| Species | No. of new short RNAs predicted and detected |
|---|---|
| *E. coli* | 144 predicted in silico [32]<br>275 predicted in silico [48]<br>17 confirmed experimentally [49]<br>24 predicted in silico and 14 confirmed experimentally [50] |
| *C. elegans* | 15 new miRNA genes [33] |
| *D. melanogaster* | 17 new miRNA genes confirmed experimentally [51] |
| Mouse | 78 miRNA genes confirmed experimentally [36]<br>2431 sense-antisense pairs [35] |
| Human | 31 miRNA genes confirmed experimentally [51]<br>217 predicted in silico [52] |

regulatory RNAs in *C. elegans*, which are well-established antisense RNAs [5]. Similar to short inhibitory sequences (siRNAs), which are part of the RNAi pathway, miRNAs are ~22 nucleotides long. However, unlike siRNAs, miRNAs appear to be restricted to binding target sequences within the 3′UTR with several mismatches in their target complementarity [37]. Also, miRNAs repress translation rather than trigger mRNA degradation. Destruction of mRNA may be avoided by the presence of mismatched bases [37], as this is a common structure for stable double-stranded RNAs in cells.

The presence of antisense/target sequence mismatches and repressor effects on translation resemble natural antisense structures in bacteria, and this similarity may reflect an ancient origin for imperfectly matched antisense-sense RNA pairs in translation control. Whatever the relationships between these RNAs, they are clearly widespread and important regulators. Also, the mechanism can be exploited in the laboratory using delivered or expressed sequences [37, 38]. It may seem surprising that binding to sites within the 3′UTR can repress translation; however, this region interacts with the 5′UTR where translation begins, and there are many examples of translation repression by elements contained within the 3′UTR. In some cases repression involves factors that bind to 3′UTR elements [7, 39]. The 3′UTR region can affect translation initiation and elongation, and there is increasing evidence for its widespread importance in posttranscriptional gene control [7].

## Translation repression by expressed antisense transcripts

Soon after the discovery of natural antisense RNAs, researchers aimed to engineer and express antisense transcripts as a means to control gene expression. While this approach can work, most attempts fail, presumably due to complexities in RNA sequence and structure. To overcome this problem, several research groups used libraries of genomic fragments to express large numbers of diverse antisense RNAs. This method was developed in the bacterial pathogen *Staphylococcus aureus* and the fungal pathogen *Candida albicans*. The objective was to reveal genes needed for growth [40]. A caveat with the approach is that many inhibitory RNAs uncovered appear to operate through nonantisense mechanisms [41], and this raises doubt over the authenticity of antisense inhibition. Nevertheless, the approach has provided new leads on genes needed for growth. In eukaryotes, there are now several examples where expressed miRNAs can repress translation in an RNA interference (RNAi)-independent manner [37, 38]. Hopefully, more success with expressed RNAs will follow improved understanding of antisense RNA structure and activity.

## Translation repression by antisense agents

Antisense agents are short DNA analogues or DNA mimics that can modulate gene expression. Again, researchers typically aim to degrade the target mRNA through activation of RNase H. As with natural antisense, there are also many interesting opportunities to repress translation using antisense agents. An important point in this regard is that translation repression may provide more specific inhibition than is typical for RNase-H-mediated degradation, which is a rather promiscuous enzyme that cleaves efficiently within stretches of only six base pairs. Also, only a few sites within mRNAs are accessible to antisense agent binding, and only certain segments of the mRNA are susceptible to translation repression control by antisense (fig. 1). Therefore, antisense agents that repress translation can provide gene specific effects, even where there are mismatches within the interaction.

In bacteria, antisense inhibition of translation is conceptually straightforward. As described above, it is possible to block translation initiation at the well-characterized ribosome binding site. The main challenge with bacteria is to overcome the cell barriers that protect bacteria from foreign compounds. Antisense agents are inherently rather large for efficient cell uptake by diffusion, so delivery strategies are needed. Ethereint antisense effects in bacteria have been achieved using peptide nucleic acids (PNAs) with attached cell-permeating peptides. Peptide-PNAs can provide potent and specific gene inhibition. Also, the effects are sufficient to limit growth when essential genes are targeted, and this has opened attractive new possibilities for antisense antibacterial development [25, 42].

In eukaryotes, translation initiation is more complex, and usually begins with small subunit RNA binding to the cap structure of mRNA. Despite these differences, there is a similar potential to interfere with ribosomal recognition of mRNA near the cap structure, or during ribosome assembly at the start codon. There are several reports of steric hindrance of translation initiation in eukaryotic cells using antisense agents that do not activate RNase H [2, 4]. Also, miRNAs can be designed and applied as synthetic antisense agents [38], and it will be interesting to learn more about the specificity and half-life of short antisense RNAs and whether they can be developed as effective antisense agents for in vivo use. Thus, there are several ways to repress translation in eukaryotes using antisense agents.

## Antisense-mediated translation repression as a genetic switch

Translation repression by antisense and other mechanisms has been associated mainly with early embryonic

development, when transcriptional quiescence demands translational control. However, there are now examples showing that translation repression by antisense is widespread. The reversible or pseudo-reversible nature of translation repression by antisense holds many possibilities for genetic switching during development and in response to environmental signals. Indeed, there is evidence for reversible antisense-mediated translation repression in response to at least four types of signals. First, when translation-slowing antibiotics are present, antisense leader sequences unmask antibiotic resistance genes [16]. Second, end products of biosynthetic pathways can stabilize inhibitory antisense structures within mRNAs that encode enzymes of the same biosynthetic pathway; in this way, an antisense-sense duplex provides a mechanism for feedback inhibition of gene expression [43, 44]. Third, genes involved in the response to temperature change are controlled by translation repression by cis-encoded antisense sequences [17]. Fourth, antisense interactions can switch on apoptosis pathways in response to a loss of genetic material [45]. Therefore, translation repression by antisense sequences can respond to a variety of cellular and environmental signals.

## Summary

Translation repression is a form of posttranscriptional gene control, where gene-specific inhibitory effects are possible and the mRNA can be preserved intact for reversible effects on gene expression. The examples of cis-encoded and trans-encoded antisense sequences described above show how translation repression can hinder the ribosome when hybridized to mRNA sequences that mainly lie outside of the open reading frame. With the discovery of large numbers of expressed small RNAs (table 2), it is clear that many new antisense RNAs will be described. Of particular interest are antisense sequences that mediate genetic switching in response to environmental signals, and there are already a variety of examples of this type of control. Finally, cloned and expressed antisense RNAs and synthetic antisense agents can efficiently repress translation, and these approaches can be very valuable in research and drug development.

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