Perspective

The Ribosome Filter Redux

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ABSTRACT

The ribosome filter hypothesis postulates that ribosomes are not simply translation machines but also function as regulatory elements that differentially affect or filter the translation of particular mRNAs. On the basis of new information, we take the opportunity here to review the ribosome filter hypothesis, suggest specific mechanisms of action, and discuss recent examples from the literature that support it.

INTRODUCTION

In eukaryotes, the translation of mRNA into protein is an important component of gene expression, with significant implications for normal cellular function and development as well as for the ability of cells to respond to stress and disease. The process of translation involves the decoding of mRNA templates into peptide sequences on ribosomes. In eukaryotes, this process begins with recruitment of a 40S ribosomal subunit at either the 5' m7G cap-structure or an internal ribosome entry site (IRES). In most cases, the ribosomal subunit is then relocated to an initiation codon. Initiation is an important site of regulation that may affect protein synthesis globally or at the level of individual mRNAs. Translation initiation is affected by mRNA-binding factors[1-11] as well as features of the mRNA itself.

A number of observations from our own and other laboratories hinted at yet another level of regulation mediated by the ribosomal subunits themselves and these observations formed the basis for proposing the ribosome filter hypothesis.[12] At that time, we made an argument for ribosomal filtering based largely on circumstantial evidence. We suggested that the ribosomal subunits affect the translation of particular mRNAs through mechanisms involving differential mRNA-capture (Fig. 1). We now revisit this hypothesis in light of new evidence. Many of the recent examples supporting this notion involve alterations by disease. Additional investigations will be required to determine whether ribosomal filtering is disrupted in these diseases or whether ribosomal filtering occurs predominantly in the disease state.

THE RIBOSOME FILTER HYPOTHESIS

A key observation pointing to the hypothesis was the finding that large numbers of mRNAs contain segments that are either similar or complementary to sequences within 18S or 28S rRNAs.[12-21] This observation suggested a potential mechanism by which mRNAs might interact directly with ribosomal subunits, e.g., by mRNA-rRNA base pairing between complementary nucleotide sequences. Moreover, several reports suggested that within an organism, eukaryotic ribosomes in various cell types or during different stages of development (e.g., see ref. 22) vary in both ribosomal protein composition and rRNA sequences. These findings raised the possibility that structurally different populations of ribosomes might vary in their ability to translate specific subsets of mRNAs.

Based on these and other observations, we proposed four hypothetical tenets:

(1) **The ribosome is a regulatory structure that embodies mechanisms for preferentially translating different subsets of the message population.** It was suggested that the ability of ribosomes to filter translation depends on specific interactions in which segments of mRNAs bind to ribosomal proteins or to rRNA. Several of our earlier studies[14-16,23,24] focused on demonstrating base pairing between complementary segments of mRNA and rRNA. We suggested that, during initiation of translation, most such interactions would
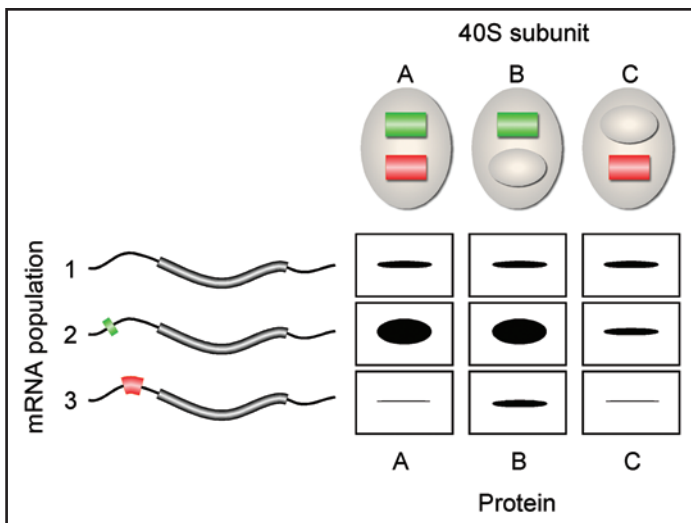
Figure 1. Schematic representation of ribosomal filtering. 40S ribosomal subunits are indicated as large ovals. Subunit A contains two mRNA-binding sites indicated as green and red bars. The red binding site is masked in subunit B and the green binding site is masked in subunit C. The mRNA population shows three types of mRNAs: (1) lacks mRNA-elements that function as ribosomal binding sites, (2) contains an mRNA-element (green bar) that enhances translation initiation when it binds to the green binding site in 40S subunits, and (3) contains an mRNA-element (red bar) that can block translation initiation when it binds to the red binding site in the 40S subunits. The amounts of protein expressed by the various mRNA-ribosomal subunit combinations are represented by the size of the black bands. Translation of mRNA 1 occurs by a cap-dependent mechanism and is translated with the same relative efficiency by ribosomal subunits A, B, or C. Translation of mRNA 2 is enhanced when translation involves ribosomal subunits A or B, in which the green binding site is accessible. Translation of mRNA 3 is inhibited when translation involves ribosomal subunits A or C, in which the red binding site is accessible.

occur with the 40S subunit, but we did not exclude the possibility of interactions with the 60S subunit or with 80S ribosomal complexes.

(2) **Ribosomes may display a continuum of regulatory effects.** Some interactions may enhance the translation of particular mRNAs while other interactions may sequester certain mRNAs and diminish their rates of translation.

(3) **Competition for binding sites in ribosomal subunits may affect the rate of translation of different mRNAs.** It was hypothesized that segments of mRNAs that bind to ribosomal subunits may act as competitive modules or domains. This competition for particular binding sites in ribosomal subunits might therefore affect the rate of translation of other mRNAs or groups of mRNAs that are able to bind to these same sites.

(4) **The filter may also be modulated as a result of altering or masking particular binding sites on ribosomes.** Subsets of mRNA may be translated with different relative efficiency by populations of ribosomes that differ in the accessibility of ribosomal binding sites, for example as a result of ribosome heterogeneity due to differences in ribosomal protein composition, or by interaction with extraribosomal factors, which may include proteins or noncoding RNAs.

The first tenet of the hypothesis suggests two main mechanisms of ribosomal filtering, one involving complementarity between mRNA and rRNA sequences, and the other involving interactions between mRNA sequences and ribosomal proteins. We consider these mechanisms and then discuss recent supporting evidence for them.

MECHANISMS OF FILTER ACTION

mRNA-rRNA interactions: evidence for base pairing. A critical feature of the filter hypothesis concerns the ability of different members of a population of ribosomes to interact specifically and selectively with particular mRNAs. In earlier studies we investigated potential mRNA-rRNA base-pairing as an important mechanism underlying this interaction. Our most extensive studies in this regard focused on a 9-nucleotide element located in the mRNA of the *Gtx* homeodomain protein.[16,23]

The 9-nt *Gtx* element was shown to be an IRES module in studies performed using dicistronic mRNAs.[16] We found that linking together individual *Gtx* elements could lead to an exponential increase in the expression of a reporter protein. Various control experiments indicated that the *Gtx* elements enhanced expression by increasing the efficiency of translation. To avoid potential limitations associated with the use of dicistronic mRNAs, we also tested *Gtx* elements in the 5' leader of a monocistronic mRNA.[23] In this context, multiple linked copies of the *Gtx* element enhanced translation up to eight-fold compared to the activity mediated by the cap-structure alone. RNA analyses showed that this increased activity could not be accounted for by alterations in mRNA levels.

Sequence comparisons revealed a perfect complementary match between the 9-nt *Gtx* element and nucleotides contained within helix 26 of the 18S rRNA.[15] It is notable that these nucleotides in the rRNA are located in the platform of the 40S subunit, in close proximity to the predicted mRNA-binding tract.

These results suggested that the *Gtx* element affects translation by a mechanism that involves base pairing to complementary nucleotides in the 18S rRNA. To test this notion, we performed a number of functional and biochemical experiments.[16,23,24] In one set of studies, we altered the length of the complementary match.[23] The data showed that translation was maximally enhanced by a specific 7-nt sequence in the 9-nt element and that the degree of enhancement declined progressively as the sequences involved in the complementary interactions became longer or shorter. These observations suggested that the shorter complementary interactions were insufficiently stable for efficient translation initiation and that the longer complementary interactions were too stable. This conclusion was corroborated by biochemical studies which showed that the length of the complementary match was directly correlated with the ability of RNA probes to bind to ribosomes.

To assess the requirement for complementarity in the match, we developed a novel yeast system that enabled us to alter both the mRNA and rRNA sequences.[24] The 18S rRNA of the yeast *Saccharomyces cerevisiae* has a poor complementary match to the *Gtx* element and we found that the presence of the *Gtx* elements did not enhance translation of mRNA in these cells.[24] To determine whether the *Gtx* elements were inactive because they could not base-pair to yeast 18S rRNA, we introduced complementary sequences into the yeast 18S rRNA either in the form of mouse-yeast hybrid 18S rRNAs, or directly by introducing appropriate point mutations of the yeast 18S rRNA itself. When constructs containing the *Gtx* elements were tested in yeast whose rRNAs contained the complementary match, translation efficiencies were dramatically increased over that in cells lacking the complementary match. Any of five different single point mutations within the 7-nt *Gtx* elements were shown to disrupt translation rates. In each case, activity was restored by a

corresponding mutation of the hybrid 18S rRNA designed to restore complementarity.

By showing that the activity of the *Gtx* elements required an intact complementary match to the 18S rRNA, these studies provided the first evidence in eukaryotes for an mRNA-rRNA base-pairing interaction analogous to the Shine-Dalgarno interaction in bacteria.[25-30] This example supports a key mechanism proposed to underlie instances of the ribosomal filter. Furthermore, this example served to demonstrate the principle of ribosomal filtering, albeit synthetically, by showing that an mRNA containing *Gtx*-elements is translated more efficiently by ribosomes that have a complementary match to the *Gtx*-element (mouse-yeast hybrid) than by ribosomes lacking such a match (yeast). We expect that in addition to the *Gtx*-element, there are other mRNA elements that affect translation initiation in eukaryotes by base pairing to 18S rRNA.

Ribosomal protein heterogeneity. Heterogeneity in a ribosomal protein may affect the translation of specific mRNAs that bind to that ribosomal protein. Alternatively, such heterogeneity may modulate the translation of specific mRNAs by altering the accessibility of binding sites contained within the rRNA. Examples of ribosomal heterogeneity were discussed in our earlier publication as a possible mechanism by which different populations of ribosomes differentially regulate the translation of subsets of mRNAs.[12] More recent evidence for ribosomal heterogeneity comes from a global survey of protein expression in mice, which was performed using mass spectrometric multi-dimensional protein identification technology (MudPIT).[31] We searched the data from this study using a web-browser interface and found that some ribosomal proteins, including S3, S4 and S7, were detected in all tissues examined while other ribosomal proteins were detected at high levels in some tissues but were not detected in others. For example, ribosomal protein S6 was detected at high levels in brain, kidney, and liver, but was detected at only a low level in lung tissue and not at all in the heart. These results complement in situ hybridization results obtained in our laboratory (Prieto A, Mauro V, Edelman G, unpublished data), which showed that the levels of mRNAs for ribosomal proteins had patterns of expression that were dramatically different in different tissues. In a different study, Angelastro et al.[32] found that the relative abundance of individual ribosomal protein mRNAs varied by over a hundredfold in rat pheochromocytoma (PC12) cells and that the levels of approximately half of these ribosomal protein mRNAs changed (up to five-fold) when cells were treated with nerve growth factor (NGF).

These findings, together with various other observations (see ref. 12), suggest that the ribosomal subunits in different tissues, as well as in different subcellular locations are likely to be heterogeneous with respect to their constituent ribosomal proteins. However, additional studies are required to determine to what degree this heterogeneity affects filtering.

RECENT EVIDENCE SUPPORTING THE FILTER HYPOTHESIS

Recent observations over a wide range of biology, including different species and various diseases (see refs. 33–42) have been specifically suggested by their investigators to be consistent with the function of a ribosome filter. We choose here to discuss a number of examples to illustrate the widespread applicability of filter mechanisms. Some of these examples involve modification of the 18S rRNA or ribosome binding proteins. Other examples involve ribosomal protein heterogeneity.

Pseudouridinylation of rRNA. Strong evidence for a ribosome filter comes from Yoon et al.,[43] who addressed the possibility of altered ribosomal function in the X-linked *dyskeratosis congenita* syndrome. This disease results in bone marrow failure and is characterized by premature aging, nail and skin abnormalities, and a predisposition to cancer. The disease is caused by a mutation in the *DKC1* gene that disrupts dyskerin, a protein associated with ribonucleoprotein (RNP) complexes, including the H/ACA family of small nucleolar (sno) RNP particles. In these snoRNPs, dyskerin functions as a pseudouridine synthase that modifies specific uridine residues in rRNA. Using a mouse model, the authors showed that global protein synthesis was generally unaffected in cells with a hypomorphic mutation in the *DKC1* gene. However, a limited microarray analysis identified three mRNAs with a decreased representation in polysomes of such cells when compared to polysomes of wild type cells: the p27 tumor suppressor, the X-linked inhibitor of apoptosis protein (XIAP), and the mitochondrial ion channel Bcl-xL. All three mRNAs were shown to contain an IRES: *Bcl-xL* in the Yoon et al., study;[43] *XIAP* and *p27* in other studies (see refs. 44 and 45, respectively).

To assess whether the observed translational effects were directly mediated by the ribosomal subunits, an mRNA containing the cricket paralysis virus (CrPV) IRES was tested. This IRES binds directly to the 40S ribosomal subunit and facilitates translation initiation even in the absence of initiation factors and the initiator Met-tRNA.[46] The experiments revealed that translation mediated by the CrPV IRES was impaired in cells with the *DKC1* mutation. The data makes accounting for the results by indirect effects unlikely, particularly since pseudouridine does not appear to be found in mRNA.[47] It is more likely that ribosomes lacking pseudouridine modifications were impaired in their ability to translate specific IRES-containing mRNAs, even though polysome analyses suggested that these ribosomes were generally unaffected in their ability to translate most other mRNAs. These observations are consistent with the notion that the ribosome is a regulatory structure that embodies mechanisms for preferentially translating particular subsets of the mRNA population.

Methylation of ribosomal protein S24. Studies performed in the slime mold *Dictyostelium discoideum* showed that the translation of ribosomal protein mRNAs is altered during development by a subset of structurally distinct ribosomes.[48,49] During vegetative growth, it was found that ribosomal protein mRNAs were almost completely associated with polysomes. In contrast, at the start of development, ribosomal protein mRNAs were specifically excluded from polysomes. Mangiarotti (2002)[49] reported that these excluded mRNAs were associated with a class of 40S subunits in which ribosomal protein S24 was methylated. This ribosomal protein was not methylated in 40S subunits obtained from later developing cells or growing cells. In vitro experiments using eight ribosomal protein mRNAs showed that methylated ribosomal protein S24 was actually required for the selective binding of these mRNAs to the ribosomal subunits. These results suggest that during slime mold development a ribosomal filtering mechanism regulates the translation of ribosomal protein mRNAs themselves.

Ribosomal proteins S2, S5 and S10. The notion that the ribosome is a regulatory element showing mRNA selectivity and that it is not simply a tape head that converts ribonucleotide sequences into amino acid sequences is illustrated by the translation properties of different eukaryotic ribosomes. For instance, ribosomes from yeast

and mammalian cells show differences in both rRNAs and ribosomal proteins. Nevertheless both can translate similar mRNAs with high efficiency, e.g., simple cap-dependent mRNAs with a short 5' leader. However, not all mRNAs are translated with similar relative efficiencies by yeast and mammalian ribosomes. Indeed high selectivity has been observed in translation mediated by an IRES from hepatitis C virus (HCV).

HCV causes hepatitis C disease, characterized by liver inflammation that can lead to cirrhosis and cancer. Translation of HCV mRNA requires an IRES that binds directly to 40S ribosomal subunits.[50] This binding occurs via a small number of ribosomal proteins as revealed by UV cross-linking studies.[51] This IRES is inactive in *Saccharomyces cerevisiae*, apparently because it fails to bind to yeast 40S ribosomal subunits.[50,51] This failure was attributed to large differences between the mammalian and yeast ribosomal subunits in three ribosomal proteins: S2, S5 and S10, which bind the IRES in mammalian cells.[51] Together, these observations suggest that the ability of 40S ribosomal subunits to initiate translation via the HCV IRES depends on their ability to bind to these ribosomal proteins.

The coevolution of mRNA and ribosomal binding sites may explain why the HCV IRES functions in its mammalian hosts, but not in yeast. We expect that other examples of ribosomal filtering, both of cellular and viral mRNAs, will also involve coevolution of mRNA and ribosomal binding sites.

OTHER INDICATIONS OF RIBOSOMAL FILTERING

The studies discussed above provide strong support for the notion of a ribosomal filter. Numerous other studies are consistent with this notion but require further analysis to know whether they represent instances of ribosomal filtering. We briefly identify some of the most promising examples below.

Binding of Reaper to 40S ribosomal subunits. The activity of the Reaper protein provides another example consistent with ribosomal filtering.[52] In Drosophila, Reaper induces apoptosis through multiple cellular targets, both during normal development and in response to DNA stress.[53] One of Reaper's effects is to inhibit protein synthesis, which is consistent with the results of a recent study reporting that Reaper can bind to the 40S ribosomal subunit.[52] This study further showed that the addition of Reaper to a cell-free lysate blocked the translation of two cap-dependent mRNA constructs containing 5' leader sequences from either *HSP-70* or *globin* mRNAs. However, the translation of a reporter mRNA containing the CrPV IRES in its 5' leader was less sensitive to the presence of this protein. These results suggest that the differential effects of Reaper on mRNA translation reside with the ribosomes and thus are consistent with a ribosomal filtering mechanism.

Methylation of rRNA. As discussed above, the differential pseudouridinylation of rRNA provides a means for generating ribosomal heterogeneity that affects the initiation of particular mRNAs. We raise the possibility that the differential methylation of specific nucleotides in rRNA may also provide a comparable basis for ribosomal filtering. This notion is strengthened by recent reports indicating that a defect in 18S rRNA-methylation underlies Treacher-Collins syndrome, a disease affecting the development of bones and other tissues in the head and face.[54] This disease results from mutations in the nucleolar phosphoprotein treacle, which

appears to affect both transcription and 2'-O-methylation of pre-rRNA. To assess whether methylation of ribosomal nucleotides affects ribosomal filtering will require additional studies, for example of the type used to analyze ribosomal function in *dyskeratosis congenita*.

Mutations in ribosomal proteins S19 and S24. Another potential example of ribosomal filtering comes from Diamond-Blackfan anemia.[55] This disease shares some symptoms with *dyskeratosis congenita*, including bone marrow failure and increased incidence of cancer. Additional symptoms may include short stature as well as other physical defects. Mutations in two ribosomal protein genes, S19 and S24, account for approximately 27% of cases; the remaining 73% of cases are thought to involve mutations in at least two other genes. Ribosomal protein S19 has been found be important in 18S rRNA processing and in the assembly of the 40S subunit. Moreover, when various mutations of the S19 protein associated with the disease (eleven missense mutations and a trinucleotide insertion) were tested in transfected cells, none of the mutated S19 proteins were assembled into mature ribosomes.[56] Theories regarding the effects of S19 mutations suggest a non-specific global effect on ribosomal function,[57] with the possibility that S19 may have extraribosomal functions.[58] However, mutations affecting ribosomal protein S24 give rise to the same disease. Indeed, this ribosomal protein shows high heterogeneity within normal mammalian tissues, suggesting that it is not essential for ribosomal function.[59] Interestingly, S24 is the same ribosomal protein which appears to facilitate ribosomal filtering in *Dictyostelium discoideum* (see above). The fact that mutations affecting two different proteins of the 40S ribosomal subunit give rise to the same disease suggests that Diamond-Blackfan anemia is a ribosomal disorder. However, additional studies are required to determine whether this is a disease of the ribosome filter that differentially affects the translation of mRNAs important for hematopoiesis.

RACK1. RACK1 was recently identified as a ribosomal protein of the 40S subunit (reviewed in ref. 60), and various studies have shown that this protein can affect the translation of subsets of mRNAs. For example, in *Saccharomyces pombe*, cells with a disrupted RACK1 gene showed a small overall reduction in protein synthesis, but a dramatic reduction in the expression of several proteins, including ribosomal protein L25.[61] Additional analysis showed that the levels of the *L25* mRNA were not altered in cells lacking RACK1, suggesting that the reduced levels of ribosomal protein L25 were caused by a decrease in the translation efficiency of its mRNA. In other experiments performed in *Saccharomyces cerevisiae*, RACK1 was deleted. Its absence resulted in a general enhancement of protein synthesis with specific increases in the expression of several proteins.[62] To determine whether RACK1 affects ribosomal filtering will require showing that the observed effects on translation are mediated by the ribosomal subunits themselves.

New lines of investigation. A recent report on the 2006 Translational Control meeting at Cold Spring Harbor indicated that an emerging line of inquiry involved looking for ribosomal modifications as control points for regulating mRNA translation.[63] Two studies were highlighted: the first showed that various manipulations affecting the *Saccharomyces cerevisiae* 60S ribosomal subunit, including deletion of several ribosomal protein genes or genes of proteins involved in 60S processing or maturation, led to cells with significantly longer life spans. In the second study, ribosomes from poliovirus-infected and uninfected HeLa cells were compared using mass spectrometry. In infected cells, the ribosomal proteins showed

increased di-methylation as well as changes in ribosomal protein acetylation. If differences in the composition of the 60S ribosomal subunit or in post-translational modifications of ribosomal proteins can be shown to affect the translation of specific mRNAs these effects may provide examples of ribosomal filtering.

CONCLUSION

This brief review has identified some studies that provide support for the notion that ribosomes can mediate selective mRNA translation. Other studies are consistent with the filter hypothesis and we look forward to further investigations along these lines. The range of examples reflects the variety of means by which ribosomal filtering may be modulated. We expect that future studies will reveal additional filtering mechanisms. Such knowledge will be valuable for understanding normal cellular function and development. In addition, it should clarify the mechanisms used by some viruses to usurp the host translation, as well as those involved in various ribosomal diseases.

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