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Some Thoughts About Translational Regulation: Forward and Backward Glances

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Abstract This review discusses the need to re-examine some popular but unproven ideas about regulation of translation in eukaryotes. Translational control is invoked often on superficial grounds, such as a discrepancy between mRNA and protein levels which could be explained instead by rapid turnover of the protein. It is essential to verify that there is translational control (i.e., essential to rule out alternative mechanisms) before asking how translation is regulated. Many of the postulated control mechanisms are dubious. It is easy to create artifactual regulation (a slight increase or decrease in translation) by over-expressing recombinant RNA-binding proteins. The internal-initiation hypothesis is the source of other misunderstandings. Recent claims about the involvement of internal ribosome entry sequences (IRESs) in cancer and other diseases are discussed. The scanning model for initiation provides a more credible framework for understanding many aspects of translation, including ways to restrict the production of potent regulatory proteins which would be harmful if over-expressed. The rare production in eukaryotes of dicistronic mRNAs (e.g., from retrotransposons) raises questions about how the 3′ cistron gets translated. Some proposed mechanisms are discussed, but the available evidence does not allow resolution of the issue. J. Cell. Biochem. 102: 280–290, 2007.

Key words: RNA-binding protein; IRES; dicistronic mRNA; LINE-1; scanning model

This essay is not about what might be discovered next, in the field of translational regulation, but about how to get there. Progress in understanding translational regulation necessitates clearer formulation of questions and stricter interpretation of experimental results.

"Clearer" and "stricter" imply that progress has been limited by problems. Some readers might protest that the voluminous literature on translational regulation indicates remarkable progress in recent years; but many of the answers that have emerged are speculations based on soft evidence, and some proposed answers are outright wrong. Popular ideas about translational regulation for which there is little hard evidence constitute barriers to finding real answers. Thus, going forward requires stepping back to re-examine and rethink some points.

The common belief that translation is usually regulated at the level of initiation might or might not be true. Nevertheless, this analysis is limited to the initiation step of translation in eukaryotes because that is what most investigators have chosen to study and because I have some knowledge of the issues.

LOOK AGAIN

Clear formulation of questions about translational regulation requires looking again at the underlying biological phenomena. There are many cases in which a phenomenon was not examined carefully before attempting to reconstruct the mechanism in vitro. The most basic question is whether the apparent regulation—for example, turning off expression of a protein—really is at the level of translation.

The first requirement is to be certain about the structure of the mRNA. A common error is equating cDNA with "functional mRNA," that is, forgetting that cDNAs sometimes derive from incompletely spliced transcripts. Introns

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often contain spurious AUG codons, and therefore translation is impeded when an intron is retained in the 5′ untranslated region (UTR). Some phenomena, initially interpreted simply as translational regulation, turned out to involve a change in splicing near the 5′ end of the mRNA [Xie et al., 1991; Leskov et al., 2003; Son et al., 2003; Diernfellner et al., 2005]. Translation is affected only secondarily.

Ascertaining the structure of the mRNA includes searching for additional transcripts. This is especially important in rare cases where a dicistronic mRNA is produced, as with cricket paralysis virus (CrPV) and the long interspersed nucleotide element (LINE)-1 retrotransposon. The common belief is that CrPV coat protein and LINE-1 reverse transcriptase are translated from the 3′ end of their respective dicistronic mRNAs, but the existence of additional (monocistronic) forms of mRNA has not been ruled out. An hypothetical monocistronic mRNA might have escaped detection because it is scarce, in the case of LINE-1 (discussed below), and because no one has looked properly in CrPV-infected cells. Wilson et al. [2000] published a Northern blot wherein the only detectable CrPV transcript was the dicistronic RNA; but if that analysis was performed late in the infection (a critical issue which is not revealed), the dicistronic RNA in progeny virions would have predominated over mRNA. It might be useful for young investigators to re-read some classic old papers to learn the proper way to search for subgenomic viral mRNAs [Hunter et al., 1976; Siddell and Smith, 1978].

Translational regulation is often claimed without having ruled out effects on mRNA stability. The well known ability of poly(A) binding protein (PABP) to protect against degradation of mRNAs complicates attempts to determine whether PABP also promotes translation. In the early days, investigators acknowledged that polyadenylation might help by stabilizing actively-translated mRNAs rather than by directly promoting translation [Sheets et al., 1994]. That cautious interpretation is no longer discussed, but it should be; it requires checking the stability of the small fraction of mRNA that actually supports translation. Most of the RNA delivered via liposome-mediated transfection does not enter cells in a usable way [Barreau et al., 2006]; and therefore, the question of mRNA stability is not settled simply by demonstrating that undegraded mRNAs can be recovered following transfection.

Use of cell-free translation systems does not eliminate concerns about mRNA stability. The early literature harbors a lesson about the danger of hoping that a crude translation system will "work well enough." A big question back then was the direction of ribosome movement, and several investigators—using easily available cell-free translation systems—came up with the answer that ribosomes traverse mRNA in the 3′–5′ direction [Eikenberry and Rich, 1965; Williamson and Schweet, 1965]. The correct answer emerged only when Severo Ochoa undertook the hard work required to eliminate contaminating RNases from the translation system [Salas et al., 1965]. The story is worth telling, not only as an historical note, but because modern-day reagents have not eliminated the problem. An extract derived from HeLa or Krebs cells (or fly embryos or yeast) has as much RNase today as 40 years ago.

Ruling out effects on mRNA stability is necessary but not sufficient. The delayed production of lipoxygenase in differentiating reticulocytes [Thiele et al., 1982] is a classic example wherein a stable mRNA accumulates in the cytoplasm without detectable accumulation of the encoded protein, but the evidence is not sufficient to declare regulation at the level of translation. An alternative possibility—that the protein is synthesized and rapidly degraded in immature cells—has never been examined.

In some other cases, pulse-labeling experiments undertaken to rule out rapid turnover of the protein failed to settle the question because the duration of labeling ($\geq$20 min) was too long. Monitoring nascent polypeptide chains requires use of a short (e.g., 2 min) labeling period [van Daalen Wetters et al., 1989]. Some authors argue that labeling for 20 min is justified in cases where the half-life of the completed protein is several hours [Kawai et al., 2006], but that reasoning is faulty because completed proteins might not be degraded via the same pathway as nascent polypeptides. This criticism applies also to experiments in which the stability of completed proteins is monitored after adding cycloheximide to inhibit new translation.

Instead of beginning with an unbiased look at the biological phenomenon—allowing the accumulated clues to point to the mechanism—investigators sometimes begin with an answer.

They ask not "how does regulation of the inhibitor of apoptosis (c-IAP) change during apoptosis" but "is c-IAP translated via an IRES during apoptosis?" [Van Eden et al., 2004a]. Like stepsisters trying to claim the glass slipper, they try too hard to make the evidence fit their favorite answer. They ignore other mechanisms; for example, that translation of c-IAP might be controlled at the level of splicing (removing a long, AUG-containing intron from the 5′ UTR) or that accumulation of the protein might be regulated by proteolysis.

POPULAR BUT UNPROVEN IDEAS ABOUT TRANSLATIONAL REGULATION

Three ideas underlie much of the current thinking (and contribute to the confusion) about translational regulation.

One is the closed-loop model, which postulates that eIF4G circularizes the mRNA by interacting with eIF4E at the 5′ end and with poly(A)-binding protein (PABP) at the 3′ end. Those interactions indeed can be demonstrated when purified proteins are tested in vitro, but the functional significance vis-à-vis stimulation of translation is questionable. Numerous problems with the experimental evidence for the closed-loop hypothesis are explained in other reviews [Kozak, 2004, 2006].

Another popular idea is the internal initiation hypothesis, which somehow grows more dubious (see below) the more it is studied. Textbooks tell us this alternative mechanism is useful because "it allows selected mRNAs to be translated at a high rate despite a general decline in the cell's capacity to initiate protein synthesis" [Alberts et al., 2002]. But the remarkably low efficiency of most IRESs (if they are IRESs) belies that rationale.

Formation of mRNA-protein (mRNP) complexes is a third concept frequently invoked to explain regulation of translation.

mRNA-Binding Proteins

Lots of proteins associate with eukaryotic mRNAs, and therefore, lots of proteins track with polysomes when cell extracts are analyzed by sucrose gradient centrifugation. That is not sufficient reason to postulate involvement of a protein in regulating translation. RNA-binding proteins that really are implicated in regulation (via genetic evidence) might work in unexpected ways, for example, by exposing binding sites for microRNAs. Thus, it is essential to identify all the functionally important components before attempting to reconstruct the regulation.

Finding all the functionally important components cannot be accomplished simply by identifying a bunch of proteins that co-exist in one or another complex, which is the approach used by Polesskaya et al. [2007] to study Lin-28. The authors decided that IGF-2 mRNA is a likely target of Lin-28 because, among the dozens of Lin-28-interacting proteins, they found certain proteins known to bind IGF-2 mRNA. The accompanying in vitro translation experiment is unconvincing. Instead of first identifying a region of IGF-2 mRNA to which Lin-28 binds (if it does), the authors inserted 1,164 nt from IGF-2 mRNA into the 5′ UTR of an Fluc reporter gene and then showed that addition of recombinant Lin-28 protein (of undetermined purity) increased the yield of Fluc threefold, using a coupled transcription/translation system. Simplistic experiments like this prove nothing, and they distract from finding out how RNA-binding proteins actually do regulate gene expression.

Many misunderstandings involve 3′ UTR-binding proteins. The importance of these proteins in localization and stabilization of mRNAs is indisputable. Whether they also directly regulate translation is less certain. It is too easy to show that over-expression of one or another protein effects a slight increase or decrease in translation of a reporter mRNA; that is, too easy to create the appearance of "translational regulation." Some examples are credible, such as the shutoff of translation by a 3′ UTR-binding protein that interacts with a 5′-UTR binding protein, thereby circularizing the mRNA [Cho et al., 2005]. Many other claims of translational regulation by 3′ UTR-binding proteins are based on flimsy evidence [reviewed in Kozak, 2004, 2006] which sometimes cannot be reproduced [Kong et al., 2006].

A corollary hypothesis is that RNA-binding proteins might coordinate translation of specific sets of mRNAs, analogous to the way specific sets of genes are turned on by transcription factors. Keene and Lager [2005] say this idea of "post-transcriptional operons" is supported by experiments showing that mRNAs encoding classes of functionally related proteins are bound to one or another regulatory protein. That assertion overstates the evidence in two ways: (i) "functionally related" is exaggerated;

and (ii) the regulatory effects of the mRNA-binding proteins are unproven. The most promising finding is the association of Puf proteins with different sets of mRNAs in yeast [Gerber et al., 2004]; but here "functionally related" refers only to where the encoded proteins function, for example, in mitochondria or in membranes. (In direct tests, Puf6p was found to mediate localization of an mRNA but it did not convincingly repress translation; for example, a 64-fold molar excess of Puf6 protein was required to effect a slight reduction in translation in vitro [Gu et al., 2004].) Another example cited by Keene and Lager [2005] is fragile-X mental retardation protein (FMRP), which they describe as a translational repressor of mRNAs involved in Fragile X syndrome. Here the argument is circular ("functionally related" apparently means "mRNAs bound by FMRP"), and the ability of FMRP to repress translation remains unproven. A thoughtful review by Bagni and Greenough [2005] summarizes the complicated findings, for example, that FMRP-bound mRNAs are polysome-associated or non-polysomal, depending on how the experiment is done.

The complexity of mRNP biology should not be used as an excuse to claim, as evidence for the operon hypothesis, fragmentary findings which could be explained in many ways. The lack of correlation between mRNA and protein levels, for example, which is cited by Keene and Lager [2005] as evidence of translational regulation, could be explained instead by rapid turnover of proteins. Clear thinking is needed even (especially) when the subject is complex.

Problems With the Internal Initiation Hypothesis

A big problem with the internal initiation hypothesis is the variety of sequences that purportedly function as IRESs. A well known textbook says there are four types of IRESs [Flint et al., 2004], one of which is an intricate base-paired structure from CrPV. However, no particular sequence or structure was found to be required for IRES activity with a virus closely related to CrPV [Terenin et al., 2005], so perhaps there are five types. Or six if you count a remarkably small (12 nt) sequence from herpes simplex virus mRNA [Griffiths and Coen, 2005]. Or seven if you count an IRES that purportedly functions from a downstream position [Herbreteau et al., 2005]. When you add to the list several dozen putative IRESs from cellular mRNAs, *among which there is no common sequence or structure* [Baird et al., 2006], the problem is undeniable: the variety of structures makes no sense.

Studies undertaken to test one or another version of the internal initiation hypothesis invariably fall short, either because alternative mechanisms (cryptic promoters, splicing, cleavage of the dicistronic mRNA) are not ruled out or because the putative IRES activity is too weak to be credible. These points are documented in other reviews [Kozak, 2003, 2005]. A big problem is the widespread use of the pRF vector, which has *Renilla* luciferase (Rluc) as the 5′ cistron and firefly luciferase (Fluc) as the 3′ cistron. The problem is that this vector facilitates production of spliced, monocistronic mRNAs along with the intended dicistronic transcript [Van Eden et al., 2004b]. Taking into account the deficiencies in these functional tests, the obvious solution to the perplexing structural variety is that not all (perhaps none) of these sequences are IRESs.

A popular belief is that, under various stress conditions, "IRES-mediated translation provides a means for escaping the global decline in protein synthesis and allows the selective translation of specific mRNAs" [Holcik and Sonenberg, 2005]. This idea is faulty for two reasons. (i) The decline in translation, albeit significant, leaves a substantial residual capacity. In HeLa cells, translation persists at ~30% of the normal rate during mitosis [Fan and Penman, 1970] and at ~50% of the normal rate during hypoxia [Koritzinsky et al., 2006]. Thus, the implied need for an alternative cap-independent mechanism is not real. (ii) If the need for an escape mechanism were real, the weak IRES activity in cellular mRNAs would be of little help. The remarkably low efficiency of translation via putative IRESs is documented in other reviews [Kozak, 2001, 2005].

The continued translation of certain mRNAs in cells depleted of eIF4E or eIF4G is commonly cited as evidence for cap-independent translation, but that simplistic interpretation is not justified. In one study where eIF4G was depleted by cleavage, the resulting suppression of translation was found to affect mRNAs in the cytosol while translation of a wide variety of mRNAs persisted in the endoplasmic reticulum [Lerner and Nicchitta, 2006]. (It's not that translation of these mRNAs is independent of eIF4G but that depletion of initiation factors

does not occur uniformly throughout the cell.) Experiments involving manipulation of eIF4E levels are even trickier to interpret, inasmuch as translation is not the sole function of eIF4E (e.g., it also mediates mRNA export) and the reagents employed to reduce eIF4E activity (e.g., rapamycin) have broader effects.

There has been no real progress in understanding how putative IRESs function. Direct binding of eIF4G to an IRES is the exception rather than the rule; for example, eIF4G binds strongly to the 5′ UTR from EMCV, but binding to poliovirus RNA is so weak as to be almost invisible in an autoradiogram of crosslinked proteins [Ochs et al., 2003, Fig. 5]. Other proteins that purportedly augment IRES function—so-called IRES trans-acting factors (ITAFs)—have only small effects [reviewed in Kozak, 2003]. Thus, ITAFs do not solve the problem that the activity of most putative IRESs is too weak to be credible.

A recent study claiming IRES-mediated control of circadian melatonin production [Kim et al., 2007] illustrates these problems: IRES-mediated translation of Fluc was only 2% as efficient as cap-mediated translation of Rluc (Fig. 3B), and over-expression of the putative ITAF increased Fluc production only twofold (Fig. S6). The editor could not have been impressed by such numbers; and therefore, I suspect her reason for accepting the paper was that *it would be interesting if this important gene were translated via a special mechanism*. That sort of reasoning, repeated over and over, is what propels the IRES hypothesis. The belief that "critical regulatory genes need a special mechanism of translation" prompts investigators to test critical genes for IRES activity. And because the criteria for recognizing IRESs are so weak, almost every test uncovers gold.

Claims About Involvement of IRESs in Disease

Casual readers might think the importance of internal initiation was firmly established by finding connections between IRES-mediated translation and diseases such as dyskeratosis congenita (DC) and cancer. But the postulated translational defect in DC cells is based only on studies in mice—no ribosomal defect was detected in human DC cells [Wong and Collins, 2006]—and the experiments conducted with mouse $Dkc1^{m}$ cells were seriously flawed [Yoon et al., 2006]. The claim that "$Dkc1^{m}$ cells are specifically impaired in translation of IRESs" requires strong evidence that the three affected mRNAs (XIAP, $p27^{Kip1}$, and Bcl-xL) are IRESs, but tests of that key point had no controls. (The Bcl-xL "IRES" was compared only to itself in normal versus mutant cells, that is, the Fluc/Rluc ratio in normal unirradiated cells was set at 1.0 without revealing how the Fluc yield from the Bcl-xL dicistronic construct compares to a monocistronic mRNA, or to a dicistronic mRNA lacking an IRES, or to a dicistronic mRNA containing a proven IRES, if such there is.) Yoon et al. [2006] invoke prior reports of IRES activity in XIAP and $p27^{Kip1}$ mRNAs, but those claims were subsequently challenged by the discovery of cryptic promoters and splicing [Van Eden et al., 2004b; Liu et al., 2005].

A similar problem—use of "IRESs" that are not really IRESs—undermines a recent study which postulates a defect in internal initiation as a step in tumorigenesis [Wilker et al., 2007]. The hypothesis is that a protein called 14-3-3σ, which is frequently lost in tumors, normally stimulates IRES-mediated translation of the cyclin-dependent kinase PITSLRE. (Although initial tests for IRES activity in PITSLRE mRNA looked convincing [Cornelis et al., 2000], the activity was much lower in a followup study [Tinton et al., 2005]. *The new study by* Wilker et al. [2007] *assumes, without retesting, that PITSLRE is translated via internal initiation*.) The claim that "IRES-dependent translation is impaired in cells lacking 14-3-3σ" rests on experiments with the dubious $p27^{Kip1}$ IRES (discussed above) and with a putative IRES from human immunodeficiency virus (HIV) which barely supported translation of the 3′ cistron when tested in vitro [Brasey et al., 2003]. (The HIV sequence was more active when tested in vivo, but there were no RNA analyses to rule out splicing, and the putative IRES activity inexplicably depended on using Fluc as the reporter.) Wilker et al. [2007] describe their results with the $p27^{Kip1}$ and HIV IRESs in a way that *hides the inefficiency*: the Fluc/Rluc ratio is simply set at 1.0 in control cells prior to onset of mitosis. The bottom line is that it might be true that the aberrant phenotype of 14-3-3σ-depleted cells results from absence of a short form (p58) of PITSLRE, but p58 is probably translated from a spliced mRNA [Xiang et al., 1994] rather than via internal initiation.

Claims of IRES activity in other genes important in tumorigenesis, such as p53 and

ornithine decarboxylase (ODC), rest on very weak evidence. The ODC sequence scored poorly when IRES activity was tested via a dicistronic vector (<3-fold stimulation of the 3′ cistron); and because the duration of labeling with [^{35}S]Met was too long (20 min), the pulse-labeling experiment failed to rule out the possibility that endogenous ODC is regulated at the level of protein turnover rather than at the level of translation [Pyronnet et al., 2000]. The 5′ UTR from p53 mRNA scored strongly when tested via a dicistronic vector (200-fold stimulation), but the experiment undertaken to rule out cryptic promoter activity was not done correctly: instead of deleting the 5′ promoter from the dicistronic construct, Yang et al. [2006] moved the p53 sequence to an unrelated monocistronic vector. Along with these flawed experiments, the reasoning is flawed. It makes no sense to argue both that ODC is translated via an IRES and that ODC is up-regulated by overexpression of eIF4E in tumor cells; the IRES is supposed to make translation independent of eIF4E. It is nonsense to write about the "importance of ITAFs in p53 translation" while admitting that "no p53 ITAFs were identified yet" [Halaby and Yang, 2007]. (The authors argue that several proteins are known to *bind* the p53 5′ UTR, and such proteins *could be* ITAFs.) They go on to suggest that "p53 ITAFs could become promising therapeutic targets for the treatment of cancer."

Many years ago, when I pointed out flaws in early papers about internal initiation, people complained that I was too demanding. They saw no harm in allowing a fledgling model to get a toehold before asking for stringent tests. But it was harmful. The early inconclusive experiments invited copycat experiments (in large numbers), and the resulting nonsense is now used to propose cancer therapies.

THE SCANNING MODEL EXPLAINS A LOT (BUT NOT EVERYTHING)

Overview: Things Explained

The scanning mechanism explains a great deal of biology, starting with the remarkable difference between prokaryotes and eukaryotes in the pattern of transcription. In eukaryotic cells, each gene has its own promoter; there are usually no polycistronic mRNAs. (In odd cases where the transcription mechanism does produce polycistronic transcripts, they undergo processing to generate monocistronic mRNAs [Blumenthal et al., 2002; Zhang et al., 2007].) The polycistronic mRNAs produced by some plant and animal viruses adhere to the eukaryotic rule in that they are functionally monocistronic; that is, only the 5′ cistron gets translated [Table 1 in Kozak, 2002].

The scanning mechanism predicts that ribosomes initiate translation at the AUG codon nearest the 5′ end of the mRNA, thus rationalizing the monocistronic character of eukaryotic mRNAs. The scanning model specifies two circumstances under which initiation might occur also from sites farther downstream; namely (i) when the first AUG is in a suboptimal context (e.g. lacking A or G in position-3), or (ii) when the first open reading frame (ORF) is small, allowing ribosomes to reinitiate. These rules are explained in full, with a synopsis of the supporting evidence, in another review [Kozak, 2002].

Although the mechanism of scanning (i.e., the driving force) has not yet been elucidated, the hypothesis that the 40 S ribosomal subunit migrates linearly from the 5′ end of the mRNA is strongly supported by the observed effects of adding or removing an AUG codon [Kozak, 2002] and by the effects of edeine on formation of initiation complexes [Kozak and Shatkin, 1978]. There is growing understanding of how initiation factors participate in the ribosome-entry step at the 5′ end of the mRNA [Passmore et al., 2007] and the stop-scanning step at the AUG codon [Cheung et al., 2007; Huang et al., 1997].

The scanning model rationalizes two types of translational regulation. One mechanism involves proteins that bind near the 5′ end of the mRNA, thereby blocking ribosome entry [Rouault, 2002; Cho et al., 2005; Tsai et al., 2007]. The second type of regulation involves *cis*-acting elements in the 5′ UTR. In GC-rich mRNAs, base-paired structures can form near the 5′ end in a way that blocks ribosome entry or slows scanning. The 5′ UTR of other mRNAs has small upstream ORFs which reduce protein synthesis by forcing ribosomes to use the inefficient reinitiation mechanism [reviewed in Kozak, 2002].

Studies of disease-causing mutations in the human thrombopoietin (TPO) gene illustrate the importance of down-regulating translation in this way. Normally, TPO is translated from mRNAs that harbor small upstream ORFs, ensuring a low yield of the protein. Patients

with hereditary thrombocythaemia were found to have point mutations that remove or restructure a small upstream ORF, and the resulting increased production of TPO causes the disease [Ghilardi et al., 1998]. Although other examples have not been tested as thoroughly, the 5′ UTRs of many mRNAs appear to be structured in a way that limits the production of potent proteins which would be detrimental if over-produced [Kozak, 1991]. The long half-life of most mammalian mRNAs necessitates these constraints on translation.

The scanning model cannot explain translation of the LINE-1 retrotransposon, perhaps because the wrong question is being asked.

What About Rare Dicistronic mRNAs?

The LINE-1 element, which is widely distributed in mammalian genomes, gives rise to an abundant dicistronic mRNA wherein the 5′ proximal cistron (ORF1) encodes an RNA-binding protein that promotes retrotransposition [Basame et al., 2006]. This protein is translated very efficiently from the dicistronic mRNA. In contrast, *the notoriously low-level expression of the ORF2 protein* [Goodier et al., 2004] makes it hard to determine whether it is translated from the dicistronic transcript or from a scarce monocistronic mRNA produced by splicing. The problem of detecting low-level translation of ORF2 is solved sometimes by substituting a reporter gene (e.g., Fluc) or using a sensitive genetic assay [Alisch et al., 2006]. While this facilitates detection of the protein product, it does not help vis-à-vis detecting a possible second form of mRNA. Indeed, when Belancio et al. [2006] devised and used sensitive assays to study transcription, they found that LINE-1 elements contain numerous functional splice sites, some of which produce mRNAs that contain only ORF2.

Two other recent studies ignore the possibility that ORF2 might be translated from an alternative form of mRNA, citing as evidence against that idea the *cis*-dependent function of LINE-1 proteins in promoting transposition [Wei et al. 2001]. Li et al. [2006] and Alisch et al. [2006] attempted to define mechanisms whereby ORF2 might be translated from the 3′ end of the dicistronic mRNA.

Li et al. [2006] postulate that the mouse LINE-1 retrotransposon harbors an IRES which mediates translation of ORF2. Their experiments used a dicistronic vector in which the intercistronic spacer (and nearby sequences) from the LINE-1 element were inserted between Rluc and Fluc reporter genes. With this synthetic dicistronic construct, the possibility of splicing was said to be ruled out by using an RNA transfection assay wherein the activity of the LINE-1 IRES was equal to that of CrPV. The problem is that the CrPV sequence barely supported translation of the 3′ Fluc cistron: the Fluc/Rluc ratio was elevated only threefold above background expression from the empty vector. *Comparison to CrPV, which does not itself show convincing IRES activity, does not justify calling the LINE-1 sequence an IRES*. When the synthetic dicistronic construct containing the putative ORF2 IRES was tested by DNA transfection, the Fluc/Rluc ratio was elevated ~30-fold above background [Li et al., 2006, Fig. 1]. Although this is a more convincing level of expression than in the aforementioned RNA transfection experiment, with DNA transfection there is a possibility of splicing. Indeed, the putative IRES activity was mapped to a 53 nt sequence near the 3′ end of ORF1 which includes a motif that resembles a 3′ splice site.

The mechanism of translation of ORF2 was studied independently by Alisch et al. [2006] using a human LINE-1 element. They concluded that ORF2 is *not* translated via an IRES. They postulate a novel reinitiation mechanism to explain unusual findings, such as the continued expression of ORF2 when the AUG codon at the start of that ORF is mutated; but the convoluted mechanism involves lots of speculation. Production of a spliced mRNA that brings in an AUG codon from ORF1 could explain why the AUG codon at the start of ORF2 is dispensable.

The contradictions between these two reports—one claiming and the other denying IRES activity—underscore the need for further study of how LINE-1 proteins are translated.

Reinitiation is invoked sometimes to explain the translation of other dicistronic transcripts, for example, translation of the major (VP1) and minor (VP2) capsid proteins of calicivirus. Again, the possible production in infected cells of a small amount of monocistronic mRNA has not really been ruled out. Glass et al. [2000] show a helpful experiment in which translation in vitro of a synthetic monocistronic mRNA was tested alongside the dicistronic mRNA. In contrast with the high yield of the 23-kDa VP2 from the monocistronic mRNA, there was no

detectable translation from the 3′ end of the dicistronic mRNA. Luttermann and Meyers [2007] disagree: they say VP2 can be translated from the dicistronic mRNA, but they do not show the in vitro translation results, and their in vivo experiments are hard to interpret because they used the T7/vaccinia virus expression system. (In other studies where T7 polymerase was used to drive transcription in eukaryotic cells, only a small percentage of the transcripts actually supported translation [Kong and Sarnow, 2002], making it impossible to verify the structure of the functional form of mRNA.) The bottom line is that, although eukaryotic ribosomes can reinitiate following translation of a small upstream ORF, there is no convincing evidence for reinitiation following translation of a full-length cistron.

WHAT NEXT?

Enough evidence has accrued to outline three important emerging stories about translation: (i) regulation by microRNAs [Kloosterman and Plasterk, 2006]; (ii) regulation in the cytoplasm by mRNA-binding proteins acquired in the nucleus [Chang et al., 2007; Giorgi and Moore, 2007]; and (iii) regulation of gene expression early in development (frog oogenesis; fly embryogenesis). Importantly, all three of these overlapping stories about translational regulation also involve mRNA turnover.

Progress on some of these topics has been slowed by moving too quickly; that is, attempting to reconstruct the regulation before all the components have been identified, and attempting to reconstruct before knowing *what it is* that happens naturally. I do not think experiments with artificial small RNAs [Petersen et al., 2006] are a good way to get answers about how microRNAs work. The effects mediated by natural microRNAs have not been examined carefully enough to justify claiming that artificial RNAs mimic the natural mechanisms.

Lack of a reliable cell-free translation system from *Drosophila* embryos might be one reason for the limited success in understanding how translation is controlled early in development. The cell-free translation system developed by Gebauer et al. [1999] does not work reliably [as reported by Chagnovich and Lehmann, 2001]. The link between mRNA localization and translational repression (in vivo) suggests that expression of maternal mRNAs might be dependent on cellular structures and organization, and that could make it difficult to reproduce the regulation in vitro. Whatever the reason, the bottom line is that elegant genetic experiments have revealed the framework (i.e., which maternal mRNAs are activated or repressed by which proteins), but biochemical experiments have not yet revealed the mechanisms. Recent proposals about how Bruno and Reaper might regulate translation [reviewed in Kozak, 2006] underscore the need for clearer thinking and stricter interpretation of results.

Powerful new tools promise new insights into old questions. Single-molecule technology will make it possible to see how ribosomes really work. I hope someone adapts this technology to look at the scanning step of initiation and how it is affected by edeine [Kozak and Shatkin, 1978].

X-ray crystallography has already transformed our understanding of how ribosomes work. It is important to remember, however, that pretty structures cannot be interpreted without careful functional tests. Structural models of ribosomes bound to the CrPV IRES [Schuler et al., 2006] are of doubtful significance, given the poor ability of this RNA to support translation. The unnatural conditions under which the complexes were formed (using yeast ribosomes and a high concentration of magnesium) raise further doubts about what the structural models mean.

Microarray technology is another tool that is not always used wisely. Identifying the full spectrum of mRNAs that are subject to translational control during early embryogenesis was a good idea [Qin et al., 2007]. The proposal to search for new IRESs via microarray analysis of mRNAs bound to ITAFs [Baird et al., 2006] is a bad idea. ITAFs barely stimulate translation of RNA sequences that barely score as IRESs; and the proteins identified as ITAFs (e.g., La, HuR, pyrimidine-tract binding protein) are involved in many other aspects of RNA metabolism. Microarray experiments would only add another layer of nonsense to the IRES story.

More than new tools, I think what's needed are old-fashioned scientists like Severo Ochoa who understand the importance of solving uninteresting problems (e.g., RNase contamination) before tackling the interesting questions.

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