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Review

Regulation of translation via mRNA structure in prokaryotes and eukaryotes

Marilyn Kozak *

Department of Biochemistry, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA

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Abstract

The mechanism of initiation of translation differs between prokaryotes and eukaryotes, and the strategies used for regulation differ accordingly. Translation in prokaryotes is usually regulated by blocking access to the initiation site. This is accomplished via base-paired structures (within the mRNA itself, or between the mRNA and a small *trans*-acting RNA) or via mRNA-binding proteins. Classic examples of each mechanism are described. The polycistronic structure of mRNAs is an important aspect of translational control in prokaryotes, but polycistronic mRNAs are not usable (and usually not produced) in eukaryotes. Four structural elements in eukaryotic mRNAs are important for regulating translation: (i) the m7G cap; (ii) sequences flanking the AUG start codon; (iii) the position of the AUG codon relative to the 5′ end of the mRNA; and (iv) secondary structure within the mRNA leader sequence. The scanning model provides a framework for understanding these effects. The scanning mechanism also explains how small open reading frames near the 5′ end of the mRNA can down-regulate translation. This constraint is sometimes abrogated by changing the structure of the mRNA, sometimes with clinical consequences. Examples are described. Some mistaken ideas about regulation of translation that have found their way into textbooks are pointed out and corrected.

Keywords: Ribosome binding site; Protein synthesis; Scanning model; AUG; Repressor protein; Reinitiation

1. Introduction

This review focuses on the initiation phase of protein synthesis—in particular, on regulatory mechanisms built into the structure of the mRNA.

Initiation of translation in prokaryotes is mediated by three protein factors, designated IF1, IF2 and IF3. Eukaryotic initiation factors are more numerous (eIF1, eIF1A, eIF2, eIF2B, eIF3, eIF4A, eIF4E, eIF4G, eIF5, eIF5B) and some of these play important regulatory roles (Harding et al., 2001; van der Knaap et al., 2002). One essay cannot cover everything, however, and the initiation factors will be discussed herein only incidentally. Other reviews do an adequate job of explaining the functions of these proteins (Dever, 2002; Kapp and Lorsch, 2004; Laursen et al., 2005; von der Haar et al., 2004).

Other reviews might be consulted also regarding some important emerging stories, such as temporal control of translation during embryonic development (Kuersten and Goodwin, 2003), translation-linked degradation of defective mRNAs (Baker and Parker, 2004), and regulation of translation by microRNAs (Bartel, 2004; Yang et al., 2005). Here, I have focused on mechanisms that are more fully defined.

Regulation of translation is not limited to the initiation step, of course. At the level of elongation, the most common regulatory device involves frameshifting (Márquez et al., 2004; Matsufuji et al., 1995; Namy et al., 2004). Other interesting regulatory mechanisms are built around the pausing of ribosomes at a particular point in elongation (Chartrand et al., 2002; Mason et al., 2000; Murakami et al., 2004; Rüegsegger et al., 2001; Snyder et al., 2003).

With those acknowledgments concerning what the review omits, here is a preview of what it includes. Section 2 discusses aspects of prokaryotic mRNA structure that are important for initiation in general. Section 3 describes specific examples of translational regulation in bacteria and bacteriophage. The unit

Abbreviations: Csr, carbon storage regulator; IF, initiation factor; eIF, eukaryotic IF; IRE, iron-response element; IRES, internal ribosome entry site; IRP, iron-response protein; LEF-1, lymphoid enhancer factor-1; ORF, open reading frame; RBS, ribosome binding site; SD, Shine–Dalgarno sequence; TMV, tobacco mosaic virus; TPO, thrombopoietin; TRAP, *trp* RNA-binding attenuation protein; upORF, upstream regulatory ORF; UTR, untranslated region.

* Tel.: +1 732 235 5355; fax: +1 732 235 5356.
E-mail address: kozakma@umdnj.edu.

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on eukaryotes begins with an overview of mRNA structures relevant to initiation (Section 4), followed by examples of regulation via reinitiation (Section 5) and mRNA binding proteins (Section 6). Section 7 addresses some common questions and misunderstandings about initiation of translation in eukaryotes. Section 8 traces some of the misunderstandings to recurrent problems in the design and execution of experiments.

2. Structural elements in prokaryotic mRNAs that control initiation of translation

mRNA sequences are numbered by designating the A of the AUG codon as +1. The preceding base is position −1 and negative numbering proceeds upstream.

2.1. AUG (or other) start codon

Selection of the AUG or alternative start codon by the 30S ribosomal subunit sets the reading frame for the rest of the translation process. AUG is recognized via pairing with the anticodon (3′-UAC-5′) in fMet-tRNA (Mayer et al., 2003). Structural analyses of initiation complexes help to explain why other tRNAs cannot be used in this step (Allen et al., 2005).

Weaker pairing (two rather than three base pairs) with fMet-tRNA is part of the reason that translation is less efficient when an alternative start codon replaces AUG. In one study, translation was reduced ~8-fold when AUG was replaced by GUG or UUG (Sussman et al., 1996, Table 3). Notwithstanding this reduction in efficiency, 14% of *Escherichia coli* genes use GUG as the start codon and another 3% use UUG (Blattner et al., 1997). Use of UUG as a start codon is more common in Gram-positive bacteria and some bacteriophage (Kunst et al., 1997;Łobocka et al., 2004).

AUU functions even less efficiently than UUG in experimental tests (Sussman et al., 1996), and AUU is the natural start codon in only two *E. coli* genes. One of these encodes a potentially toxic protein, which explains why translation must be restrained (Binns and Masters, 2002). The other encodes initiation factor IF3. This factor has the interesting function of proofreading initiation complexes; i.e. IF3 disfavors initiation at nonstandard start codons, as evidenced by increased initiation at AUU, AUA and CUG codons when IF3 is mutated (O'Connor et al., 2001; Sussman et al., 1996). This leads to the prediction that IF3 mRNA should be translated better when IF3 protein levels are low, which is indeed the case (Butler et al., 1987). Evolutionary conservation of this autoregulatory mechanism underscores its importance (Hu et al., 1993). In addition to functioning as a fidelity factor for selection of the start codon, IF3 also promotes dissociation of 70S ribosomes, generating the pool of free 30S subunits required for initiation.

2.2. SD element and nearby sequences

The RNA component (16S rRNA) in the 30S ribosomal subunit plays a major role in selecting the translational start site. Authentic start codons are preceded by a purine-rich sequence which is complementary to, and base pairs with, a sequence near the 3′ end of 16S rRNA (Jacob et al., 1987; Steitz and Jakes, 1975). This so-called Shine–Dalgarno (SD) sequence in mRNA is typically 4 or 5 nt in length. (It can be as long as 8 nt or as short as 3 nt, if two of the three base pairs are G–C. The mRNA/rRNA complementarity must not be interrupted by unpaired bases.) An exhaustive analysis of *E. coli* genes documents the existence of an SD sequence in all but a few exceptional cases (Shultzaberger et al., 2001).

The SD is usually positioned some 5–8 nt upstream from the start codon.[1] The optimal spacing depends on exactly which bases at the 3′ end of 16S rRNA (3′-AUUCCUCCAC…5′) participate in the interaction (Chen et al., 1994a). Spacing is clearly important, as evidenced by cases in which unused AUG codons occur between the SD and the actual start codon. The spacing requirement can be rationalized by structural models of the ribosome which show the P site, where AUG binds, on the interface side of the 30S subunit while the "anti-SD sequence" in 16S rRNA is around the corner, on the solvent side (Yusupova et al., 2001).

In most mRNAs, the standard 4 or 5 base pair SD interaction is strong enough to mediate efficient translation. Thus, experimentally lengthening the SD sometimes produces no increase in translation (Munson et al., 1984) or only a modest increase (Chen et al., 1994b, construct IF6) or even a diminishment (de Smit and van Duin, 1994a; Komarova et al., 2002). A stronger-than-normal SD interaction does help, however, when the start codon is not AUG (Weyens et al., 1988) or when the initiation site is masked by secondary structure (de Smit and van Duin, 1994a; Munson et al., 1984). On the latter point, the clearest evidence comes from an evolutionary study with coliphage MS2 in which expansion or abbreviation of the SD provoked compensatory changes in the strength of a hairpin structure that encompasses the ribosome binding site (Olsthoorn et al., 1995).

Whereas the presence of secondary structure within the initiation region can be offset by a stronger-than-normal SD sequence, an A/U-rich initiation site that forms no stable secondary structure might require no SD interaction at all (Fargo et al., 1998). Thus, an A-rich, G-poor leader sequence derived from tobacco mosaic virus (TMV) which augments initiation when transposed to bacterial mRNAs (Gallie and Kado, 1989) might do nothing more than preclude secondary structure. The unusually weak SD in ribosomal protein S1

[1] Feltens et al. (2003) describe an unusual case in which a single SD (GGAGG) precedes two functional AUG codons. The sequence is cagGGAGGgagAUGgAUG, wherein the first AUG initiates RNase P and the second AUG initiates ribosomal protein L34. The postulated dual use of an SD is not certain, however, as an upstream AGG sequence (underlined) is better positioned to function as the SD for the first AUG. Thus, the hypothesis requires testing. Another deviation-from-the-norm was postulated for ribosomal protein S1 mRNA (Boni et al., 2001). Here, an SD located far upstream is supposedly brought close to the AUG codon by an array of hairpin structures. The authors invoke phylogenetic conservation as evidence for the model, but in some species the predicted hairpins are very weak (mostly A–U and G–U base pairs). The model was actually tested only with *E. coli* S1 mRNA, where some but not all mutations produced the expected effects.

mRNA might suffice because the A/U-richness of the initiation site allows only weak secondary structures to form. (A far-upstream hairpin in S1 mRNA which appears to augment translation might do so simply by directing secondary structure away from the initiation site, rather than via the complicated mechanism postulated by Boni et al. (2001).)[1]

The ribosome binding site (RBS), defined as the segment of mRNA protected against RNase digestion, consists of ~15 nt on each side of the AUG codon (Steitz and Jakes, 1975). The RBS thus extends slightly 5′ of the SD sequence, but *no required sequence has been defined upstream of the SD*. The stimulatory effect of upstream U-rich or A-rich or A/U-rich sequences (Olins and Rangwala, 1989; Zhang and Deutscher, 1992; Zhelyabovskaya et al., 2004) might be attributed simply to minimizing secondary structure.[2]

The sequence between the SD and AUG codon also plays no defined role. Although mutations in this domain sometimes reduce translation of one or another mRNA (Gross et al., 1990), efficient translation can be restored by a variety of sequences. Again, the primary requirement might be exclusion of secondary structure (Schauder and McCarthy, 1989), which could explain the general A-richness of *E. coli* mRNAs in positions −1 to −6.

Downstream from the initiation codon, A-rich or A/U-rich sequences probably stimulate translation by precluding secondary structure (Chen et al., 1994b; Qing et al., 2003). The inhibition of translation by certain G-rich codons (e.g. AGG, CGG), however, cannot be attributed simply to augmentation of secondary structure. The inhibition is relieved upon inserting or deleting one base in a way that shifts the reading frame, demonstrating that translation of these particular codons in the vicinity of the start site is deleterious, for reasons unknown (Gonzalez de Valdivia and Isaksson, 2004).

2.3. Polycistronic mRNA structure: coupled translation

The expression of prokaryotic genes via polycistronic transcripts makes possible a type of regulation in which translation of a downstream cistron is coupled to that of the preceding cistron. This is achieved via a conformational constraint which is relieved as ribosomes translate the upstream cistron. In the simplest cases, movement of ribosomes through the upstream cistron (e.g. *mok* in Fig. 1B) is sufficient to disrupt the base pairing that constrains translation of the next cistron (*hok*). (This example will be explained below.) A more sophisticated control mechanism requires that ribosomes pause at a particular point during translation of the upstream cistron (Butkus et al., 2003; Chen and Yanofsky, 2004; Gu et al., 1994; Mayford and Weisblum, 1989). In these examples, as in most cases of coupled translation, *the downstream cistron has a usable RBS which is temporarily obscured by secondary structure*.

In rare cases, coupling allows a downstream cistron to be translated even when it lacks a competent RBS. Ribosomes are delivered to the downstream cistron upon completing translation of the preceding cistron. This inefficient *reinitiation* mechanism ensures that certain bacteriophage proteins, needed in only trace amounts, are translated at appropriately low levels (Adhin and van Duin, 1989; Inokuchi et al., 2000; Ivey-Hoyle and Steege, 1992). The efficiency of translation increases when the downstream cistron has an SD, and genes thus configured enabled testing of the hypothesis that reinitiation indeed involves retention and reuse of ribosomes. This was done by mutating the SD sequences of the upstream and downstream cistrons and measuring translation in the presence of ribosomes that carry appropriate compensatory mutations (Rex et al., 1994).

The bacterial genome does not waste space: the terminator codon of one gene often overlaps the start codon of the next (e.g. UGAUG) and this proximity facilitates reinitiation of translation (Sprengel et al., 1985).[3] The expression of foreign genes in *E. coli* can be augmented by copying this arrangement (Ishida and Oshima, 2002; Schoner et al., 1986).

Coupled translation is used sometimes to coordinate gene expression–e.g. allowing production of several ribosomal proteins to be turned on or off via a single control point in the mRNA (Section 3.2)–but coupling does not necessarily ensure equimolar protein yields. On the contrary, the efficiency of translation of downstream cistrons varies widely and is sometimes controlled in unusual ways by the upstream cistron (Praszkier and Pittard, 2002; Yu et al., 2001). In one case, coupled translation has the unexpected advantage of enhancing folding of the protein derived from the downstream cistron (Basu et al., 2004).

2.4. Unusual mRNA structures and alternative initiation mechanisms

Leaderless mRNAs, albeit rare, are interesting because the SD interaction is clearly precluded when the mRNA begins directly with the AUG codon. (AUG is the only start codon able to function in vivo in the absence of a leader sequence; Van Etten and Janssen, 1998.) Unlike initiation sites in the interior of the mRNA, an AUG codon positioned exactly at the 5′ end might be able to thread into the groove between the 30S and 50S subunits, rationalizing the observation that a leaderless mRNA binds more stably to 70S ribosomes than to the 30S subunit (O'Donnell and Janssen, 2002).

A growing body of evidence indeed supports the idea that leaderless mRNAs are translated via a novel pathway which begins with the 70S ribosome rather than with a free 30S subunit. This was demonstrated in vitro, using chemically crosslinked 70S ribosomes, and in vivo via a temperature-sensitive termination factor which promotes accumulation of 70S ribosomes (Moll et

[2] An alternative hypothesis is that ribosomal protein S1 interacts with these so-called enhancer elements, thereby promoting initiation. Although S1 occupies an appropriate position on the ribosome (Sengupta et al., 2001), other evidence undermines the hypothesis: stimulation of translation by S1 is not mRNA-specific (Sørensen et al., 1998), and the isolated S1 protein binds promiscuously to RNAs (McGinness and Sauer, 2004).

[3] Early studies with the *E. coli lacI* gene appeared to show reinitiation occurring far downstream from a stop codon, but the interpretation of those experiments was later revised (Matteson et al., 1991). A rare case involving extensively overlapping genes, wherein the start codon of the second cistron lies far upstream from the stop codon of the first cistron, raises interesting mechanistic questions which require further testing. This very unusual restart site functions very inefficiently (Adhin and van Duin, 1990).

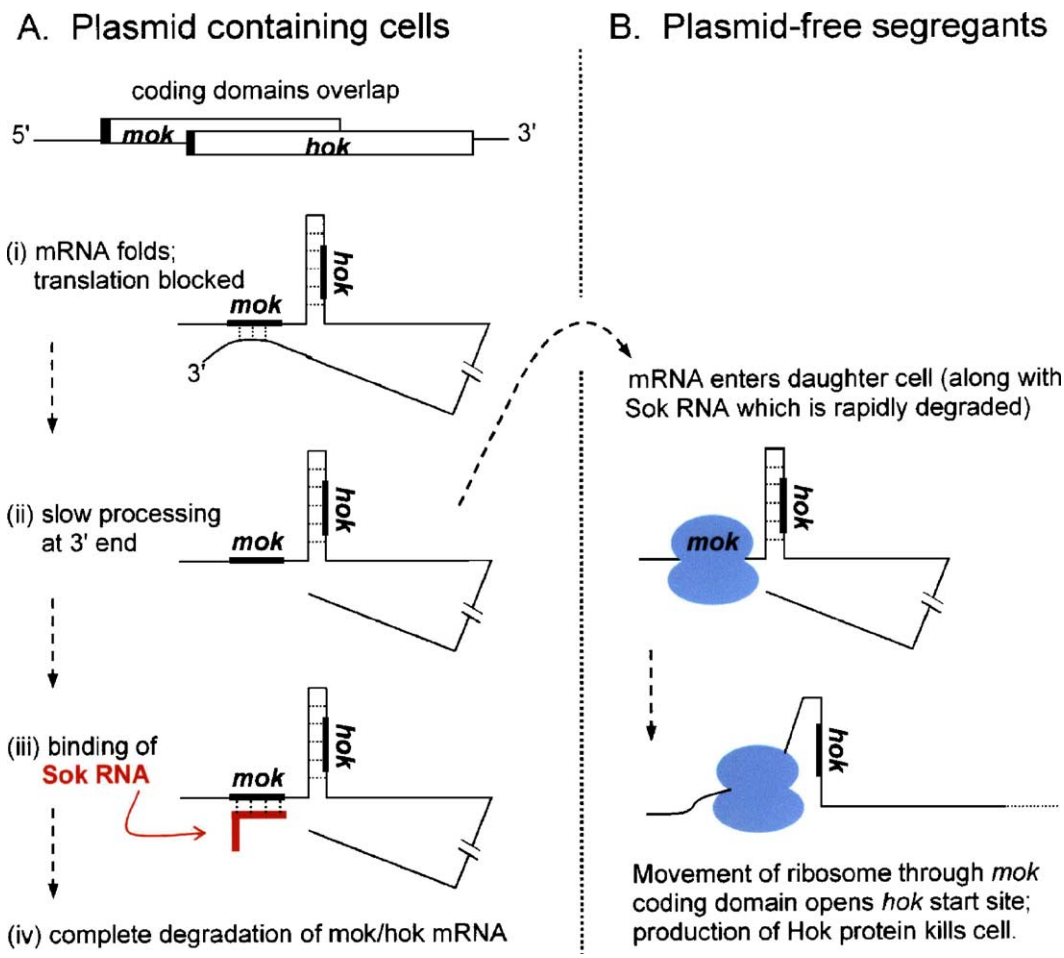

Fig. 1. Translational control of plasmid R1 *hok* gene. Retention of the R1 plasmid in a bacterial population is ensured by killing plasmid-free segregants. The mRNA that encodes the host-killing protein (Hok) is silent in plasmid-containing cells; its translation gets turned on only when the mRNA enters a daughter cell unaccompanied by plasmid DNA. (A) Translation of *hok* is prevented in plasmid-containing cells via coupling to an upstream, overlapping cistron (*mok*). (i) *mok* is untranslatable because its SD sequence (black bar) is blocked by base-pairing to a sequence near the 3′ end of the mRNA. (ii) Slow constitutive processing of the mRNA by 3′ exonuclease exposes the *mok* start site which is quickly blocked by a small, plasmid-encoded antisense RNA (Sok). Sok RNA is very abundant in plasmid-containing cells (despite its short half life) because it is transcribed from a very strong promoter. (iii) Binding of Sok RNA creates a base-paired structure recognized by RNase III. The result is degradation of the entire mRNA. (B) In plasmid-free daughter cells, rapid decay of Sok RNA allows translation of *mok*. As ribosomes advance through the *mok* coding domain, the base-paired structure which had prevented translation of *hok* is disrupted. Production of the toxic Hok protein kills the cell. The RNA structures here depicted diagrammatically are presented in full by Franch et al. (1997) and evidence for the overall scheme is reviewed by Gerdes et al. (1997). Recent studies explain how translation of *mok/hok* is prevented during transcription of the mRNA, i.e. during the interval before the complementary element near the 3′ end of the mRNA is synthesized: the 5′ end of the nascent transcript forms a different base-paired structure which also blocks the *mok* initiation region. (Møller-Jensen et al., 2001).

al., 2004). (Control transcripts containing internal initiation sites were not translated by 70S ribosomes in these experiments, consistent with classic experiments wherein the free 30S subunit was shown to be required for initiation; Guthrie and Nomura, 1968). The postulated mechanism rationalizes the effects of initiation factors: translation of leaderless mRNAs is inhibited by IF3, which promotes dissociation of 70S ribosomes, and augmented by IF2, which stabilizes binding of fMet-tRNA (Grill et al., 2000; Tedin et al., 1999).

This 70S ribosome-mediated pathway, limited to leaderless mRNAs, is the only credible alternative to the standard SD-mediated initiation mechanism. *Other pairings between mRNA and 16S rRNA have been postulated*–e.g. to explain the stimulatory effect of an mRNA sequence located upstream from the SD (the so-called “epsilon” sequence; Olins and Rangwala, 1989) or a sequence located downstream from the AUG codon (Sprengart et al., 1996)–but these ideas were ruled out by the *lack of an effect when the complementary rRNA sequences were mutated* (O'Connor and Dahlberg, 2001; O'Connor et al., 1999).

3. Examples of translational regulation in prokaryotes

In the upcoming examples, a key point is the ease whereby the initiation step of translation can be blocked by base-paired structures in the mRNA. Once ribosomes enter the elongation phase, in contrast, they have a remarkable ability to disrupt base-pairing (Takyar et al., 2005). Structured elements in the coding domain of an mRNA might transiently slow elongation, but ribosomes eventually get through. This is the key to

understanding the aforementioned coupled translation. It also explains why biotechnologists, looking for ways to improve the expression of foreign genes in *E. coli*, sometimes use the simple trick of diverting a base-paired structure from the RBS to the coding domain (Paulus et al., 2004; Satchidanandam and Shivashankar, 1997).

3.1. Conformational masking of the initiation site

Translation is often regulated by base-paired structures in the mRNA which undergo rearrangement, alternately sequestering and exposing the RBS. Many classic examples are described in an earlier review (de Smit and van Duin, 1990). In some recently discovered cases, a conformational change in the mRNA is induced by small metabolites or a change in temperature (Chowdhury et al., 2003; Johansson et al., 2002; Nahvi et al., 2004; Nou and Kadner, 2000; Ravnum and Andersson, 2001; Winkler et al., 2002).[4] The examples discussed in the next few paragraphs illustrate the basic point–that translation can be turned on and off by refolding of the mRNA–and show how refolding is brought about by mRNA-binding proteins, small *trans*-acting RNAs, or movement of the ribosome itself.

The first example (Fig. 1) involves short-range intramolecular base pairing (between *hok* and *mok* cistrons), long-range intramolecular base pairing (between the *mok* initiation site and the 3′ end of the mRNA), and intermolecular pairing (between *mok* and a small antisense RNA called Sok). All these constraints on translation are required because the protein encoded by the *hok* gene is so toxic that it must be produced only in plasmid-free segregants. The mechanism of inhibition is unambiguous here and in other cases where an antisense RNA binds directly across the RBS (Ma and Simons, 1990). A small RNA that binds just upstream from the SD can also inhibit translation (Malmgren et al., 1996).

Whereas small, plasmid-encoded antisense RNAs have a single mRNA target (e.g. Sok regulates only *mok/hok*), some small RNAs encoded on the bacterial chromosome are multifunctional. OxyS RNA, for example, has two well-defined targets (Altuvia et al., 1998); and DsrA RNA can potentially regulate five mRNAs (Lease et al., 1998). The regulatory importance of these small RNAs results from their being turned on (at the level of transcription) in response to environmental cues, such as low temperature or low iron levels. Some small RNAs *inhibit* translation by blocking the initiation site (Altuvia et al., 1998); others *activate* translation by inducing the mRNA to refold in a way that exposes the initiation site (Repoila et al., 2003). In many cases, the primary effect of small RNAs is on mRNA stability rather than translation (Kawamoto et al., 2005; Lenz et al., 2004; Vogel et al., 2004). These and other aspects of the story are reviewed by Gottesman (2002, 2004). A helper protein called Hfq facilitates the refolding required for the small RNAs to bind their mRNA targets (Lease and Woodson, 2004; Mikulecky et al., 2004; Valentin-Hansen et al., 2004).

Other small RNAs control translation indirectly: rather than binding to an mRNA, the small RNA binds to and sequesters a regulatory protein. It is the protein that binds to mRNA and directly blocks translation. Carbon storage regulator A (CsrA) is an example of this rather unusual type of repressor protein (Baker et al., 2002). Most repressor proteins function without involvement of small RNAs, however, as described next.

3.2. Repressor proteins (and more about conformational constraints)

mRNA-specific repressor proteins usually inhibit translation by competing with ribosomes for binding to mRNA. In most cases, the protein binds directly across the RBS (Table 1) or close enough to it to sterically impede ribosome entry (Jenner et al., 2005). The mechanism is more complicated, but still understandable, when the protein binds far upstream from the RBS in a way that causes the mRNA to refold: the refolded conformation sequesters the SD and blocks ribosome entry (Du and Babitzke, 1998). There are a few cases in which, despite binding of a repressor protein near the RBS, the ribosome can still bind to the mRNA, but only in a nonproductive way that does not allow fMet-tRNA to pair with the AUG codon (Philippe et al., 1993; Schlax et al., 2001).

Most of the proteins listed in Table 1 have as their primary function something other than regulating translation. This is important because regulation of translation requires controlled binding of the repressor protein, and control is sometimes achieved via competition between the mRNA and another substrate, such as tRNA or rRNA.

This idea underlies the remarkable feedback mechanism whereby the production of ribosomal proteins is coordinated with the availability of rRNA (Nomura et al., 1984; Zengel and Lindahl, 1994). When rRNA is saturated, certain ribosomal proteins bind to their own mRNAs and shut off further unnecessary translation. In many but not all cases, *the binding site for the protein on mRNA resembles its binding site on rRNA* (Guillier et al., 2005; Merianos et al., 2004; Said et al., 1988; Serganov et al., 2003). The resemblance is imperfect, however, and the protein usually has a higher affinity for rRNA (Nevskaya et al., 2005; Serganov et al., 2003; Wu et al., 1994). (In cases where the protein binds mRNA and rRNA with equal affinity, other mechanisms–e.g. the high cooperativity of ribosome assembly (Deckman and Draper, 1985; Robert and Brakier-Gingras, 2001)–might explain why the protein shuts off translation of mRNA only after all available rRNA is saturated.) Long-distance base-pairing within the polycistronic mRNA (Lesage et al., 1992; Petersen, 1989) probably explains how binding of a repressor protein to one site can turn off translation of all the downstream cistrons. Studies of ribosomal protein synthesis in organisms other than *E. coli* underscore both the importance of feedback control–the basic phenomenon is conserved over a wide range of organisms–and its flexibility vis-à-vis molecular details (Serganov et al., 2003).

[4] The experimental results in some of these studies are best described as "suggestive"; additional tests are needed to confirm the interpretation. Regulation of transcription by binding of small metabolites to mRNA is much better documented than is regulation of translation.

Table 1
Translational repressor proteins in prokaryotes[a]

Organism	Repressor protein	Targeted mRNA	Binding site on mRNA	References
Coliphage MS2/R17	Coat protein	Replicase cistron	Hairpin encompasses RBS.	Bernardi and Spahr, 1972; Carey et al., 1983
T4 phage	DNA polymerase (gene 43)	Gene 43 mRNA	Extends across SD (dependent on upstream hairpin).	Pavlov and Karam, 2000
T4 phage	DNA binding protein (gene 32)	Gene 32 mRNA	Begins at upstream pseudoknot and extends across RBS.	Shamoo et al., 1993
T4 phage	RegA	Numerous	Unstructured domain includes AUG.	Brown et al., 1997
Bacillus subtilis	TRAP[b]	*trpE, trpG, trpP*	Protein binds far upstream in *trpE* mRNA which refolds and blocks SD; binds directly to SD in *trpG* and *trpP.*	Du and Babitzke, 1998; Du et al., 1997; Yakhnin et al., 2004
Lactococcus lactis	Intron-encoded protein LtrA	*LtrA*	Stem-loop structure includes RBS.	Singh et al., 2002
E. coli	CsrA[c]	*glgC, pgaA*	Binding site includes SD.	Baker et al., 2002; Wang et al., 2005a
E. coli	Thr-tRNA synthetase	*thrS*	Binding to hairpin (just 5′ of SD) occludes RBS.	Jenner et al., 2005
E. coli	Ribosomal protein L1	L11 cistron (in same operon as L1)	Protein binds just 5' of SD.	Said et al., 1988
E. coli	Ribosomal protein S7	S7 cistron	Protein binds adjacent to SD.	Robert and Brakier-Gingras, 2001
E. coli	Ribosomal protein S8	L5 cistron (in same operon as S8)	Hairpin[d] includes AUG codon.	Merianos et al., 2004
E. coli	Ribosomal protein S4[e]	S13 cistron (in same operon as S4)	Pseudoknot spans RBS; mRNA refolds into inactive conformation.	Schlax et al., 2001
E. coli	Ribosomal protein S15[e]	S15 cistron	Pseudoknot spans RBS.	Philippe et al., 1993

[a] This is not a complete list; some additional examples are mentioned in the text. The binding site for each protein was determined by mutational analysis, biochemical tests (e.g. protection against RNase or chemical reagents), or iterative in vitro selection.
[b] Along with inhibiting translation, the *trp* RNA-binding attenuation protein (TRAP) causes attenuation of transcription of the trpEDCFBA operon in response to changes in the intracellular concentration of tryptophan. TRAP is neutralized by interacting with another protein which is also translationally regulated (Chen and Yanofsky, 2004).
[c] The function of CsrA is antagonized by small RNAs (CsrB, CsrC) which sequester the protein. CsrA affects mRNA stability as well as translation.
[d] The base-paired element in L5 mRNA looks strong enough to inhibit translation on its own, but it does not; repression requires binding of ribosomal protein S8.
[e] Ribosomal proteins S4 and S15 inhibit translation by trapping rather than competing with ribosomes; see text.

Control of translation by repressor proteins is sometimes regulated by, and other times works in conjunction with, cleavage of the mRNA. In the case of the *E. coli spc* operon, the repressor protein binds at the start of the third cistron, shutting off translation of all downstream cistrons, while the two cistrons upstream from the repressor binding site are silenced via degradation of the 5′ fragment (Mattheakis et al., 1989). Cleavage of coliphage λ N mRNA by RNase III, on the other hand, is part of a mechanism for activating translation (Wilson et al., 2002). In the uncut mRNA, N protein represses translation by binding upstream from, but close to, the SD sequence. Cleavage by RNase III separates the N protein binding site from the RBS, and thus elevates translation.

Use of repressor proteins in conjunction with other mechanisms allows fine tuning of gene expression. In the case of coliphage MS2, a single mRNA encodes four proteins, one of which–the major coat protein–is required in much larger amounts than the other three. Fig. 2 outlines how repressor proteins, conformational constraints, and coupled translation work together to ensure that each viral protein is produced at the correct time and in the correct amount.

The MS2 story helps us recognize the limits and complications of regulation via base-paired structures. We know from other examples that a remarkably small amount of base-pairing in or near the initiation region can block translation (de Smit and van Duin, 1994b; Hall et al., 1982). In the case of MS2, however, the hairpin structure at the start of the R cistron (Fig. 2C) is *not stable enough on its own* to block ribosome entry (Berkhout and van Duin, 1985); only when the repressor protein binds is translation inhibited. At the other extreme, the base-paired structure that sequesters the M initiation site (Fig. 2B) is *so stable* that one might think there could be no way around it. This complicated structure apparently folds slowly, however; and that provides a narrow window for translation of M protein (Poot et al., 1997). Studies with a related phage make the additional point that *competition between strong and weak initiation sites* can be a factor when translation occurs from a polycistronic mRNA (Priano et al., 1997).

3.3. Novel regulatory mechanisms

Proteins that repress translation are more numerous and better studied than proteins that activate translation. The BipA protein in *E. coli* might be an example of the latter. Owens et al. (2004) postulate that BipA, which displays ribosome-dependent GTPase activity, activates the translation of an mRNA which has a stronger-than-normal SD interaction. Some but not all of the proffered data support this interesting hypothesis.

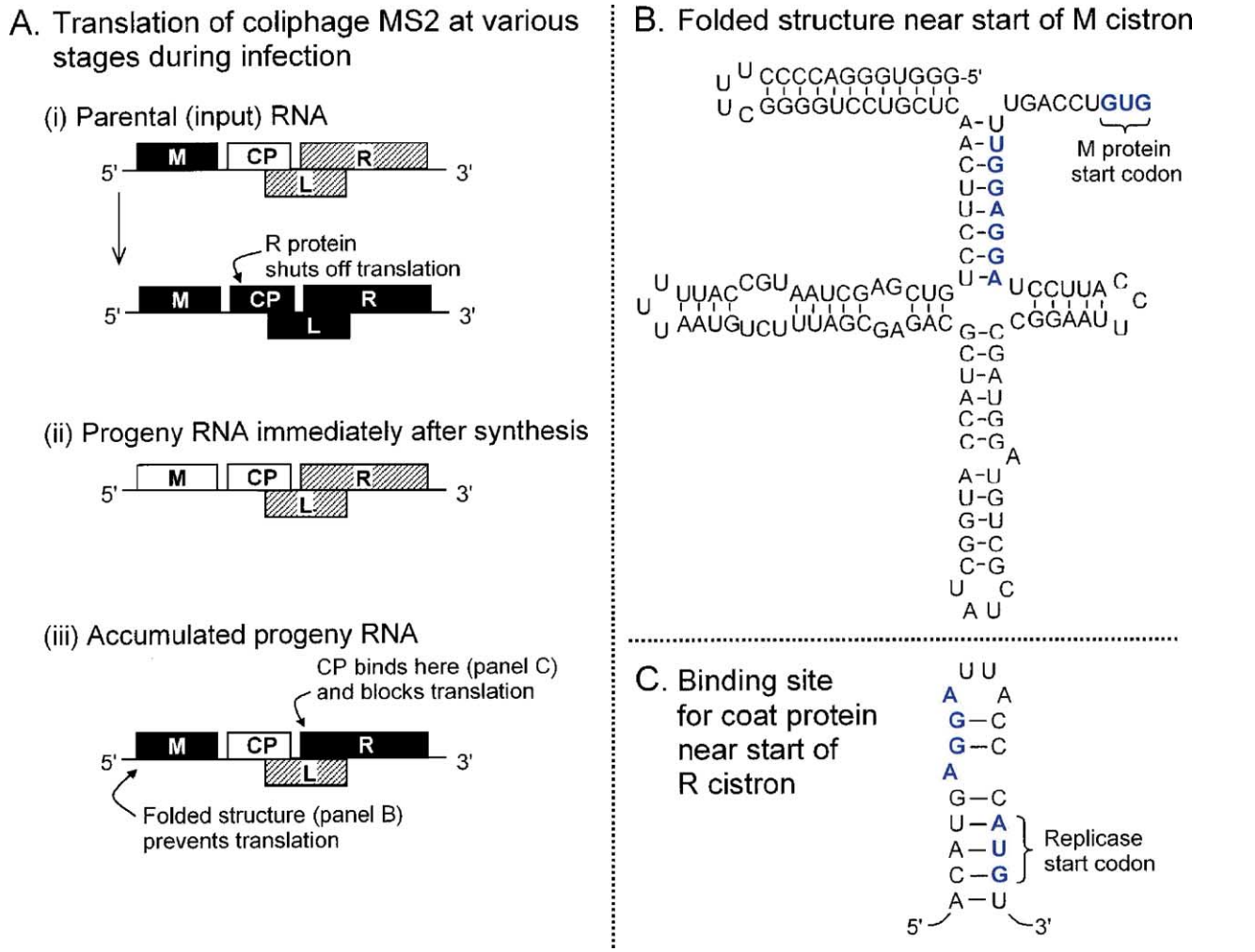

Fig. 2. Translation of coliphage MS2 mRNA is regulated by conformational constraints and RNA binding proteins. The single-stranded RNA genome encodes four proteins: coat protein (CP), the major structural component; a minor capsid protein (maturase, M); RNA polymerase (replicase, R); and a small protein (L) which promotes lysis of the host cell late in infection. Cistrons that are open for translation are shown as white blocks; those dependent on coupled translation are crosshatched; cistrons shown in black are silent. The coupled translation is the result of base pairing between the initiation site of L or R and the CP coding domain (Klovins et al., 1997; Licis et al., 2000). (A) (i) Infection begins with translation of mRNA released from parental virions. This brief phase ends when newly synthesized R protein binds near the start of the CP cistron, shutting off translation of CP and all downstream cistrons. [The evidence for repression by R comes from a related phage (Meyer et al., 1981); this point has not yet been verified for MS2.] The input mRNA next serves as template for RNA replication (not depicted), which generates a burst of new plus-strands able to serve as mRNA. Slow folding of the 5′ end of these new transcripts provides a brief interval (ii) during which M can be translated before base-pairing blocks access to the start codon (Poot et al., 1997). [The folded structure which eventually forms and shuts off translation of M is depicted in (B).] (iii) Progeny mRNA primarily directs translation of CP. L is also translated, but only via an inefficient coupled mechanism which keeps the yield low (Klovins et al., 1997). Translation of R is soon shut off by CP which, upon reaching a sufficiently high concentration, binds near the start of the R cistron in a way that blocks ribosome entry. The binding site for the coat repressor protein is shown in (C). The net effect of these translational controls is that each protein is produced in the required amount and at the appropriate time. Although viruses can replicate when some of these controls are lost, there is a significant reduction in efficiency (Licis et al., 2000).

The 5′ untranslated region (UTR) of certain mRNAs confers preferential translation during cold shock, when bulk protein synthesis is drastically diminished (Giuliodori et al., 2004; Yamanaka et al., 1999). The general decrease in translation appears to be mediated by a cold-shock induced “protein Y” which binds the 30S ribosomal subunit in a way that blocks the A and P sites and competes with initiation factors (Vila-Sanjurjo et al., 2004). Whether and how protein Y might account for the preferential translation of cold-shock mRNAs, however, awaits investigation.

It is not out of place here to mention chloroplasts, inasmuch as the mechanism of translation in that system is very similar to prokaryotes. Translational regulation is thought to underlie the interesting phenomenon wherein a block in production of one subunit causes disappearance of all the subunits in a given photosynthetic complex. Results described by Wostrikoff et al. (2004), for example, can be explained by a model wherein absence of one subunit (psaB) causes the other *unassembled* subunit (psaA) to block translation of its own mRNA. The inhibition is assumed to be at the initiation step, inasmuch as translation of a chimeric reporter gene that carries the psaA 5′ UTR was also blocked. Cytochrome *f*, which is part of a different photosynthetic complex, also inhibits translation of its own mRNA in the absence of its assembly partners (Choquet et al.,

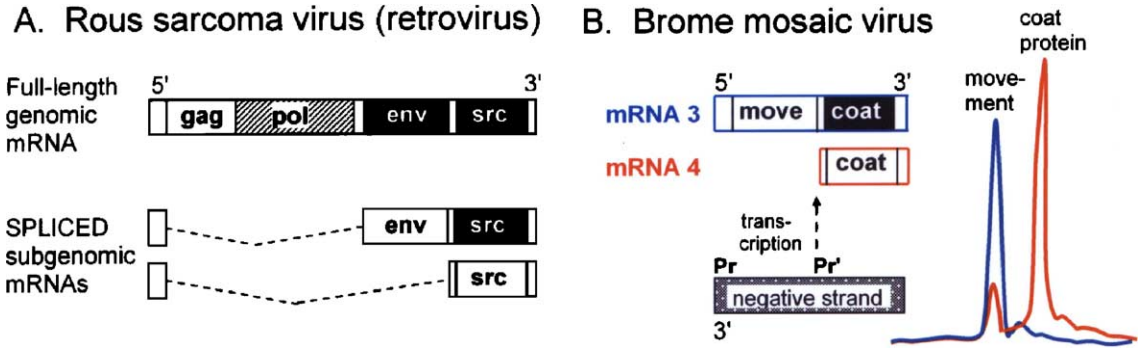

Fig. 3. Some plant and animal virus mRNAs are structurally polycistronic but functionally monocistronic, underscoring the rule that initiation of translation in eukaryotes is restricted to the 5′ end of the mRNA. Translationally silent cistrons are shown as black blocks. (A) Rous sarcoma virus genomic mRNA encodes four proteins (Gag, Gag-Pol, Env, and Src), but only the 5′-proximal *gag* initiation site is accessible to ribosomes. This full-length mRNA thus produces only Gag and (via frameshifting) Gag-Pol proteins; the downstream cistrons are silent. Translation of Env and Src occurs from spliced, subgenomic mRNAs wherein each cistron is moved closer to the 5′ end (Pawson et al., 1977; Purchio et al., 1977; Stacey et al., 1977). In similar fashion, many other viruses use splicing to restructure mRNAs and thus turn on translation of downstream cistrons (reviewed in Kozak, 2002a). (B) Other viruses generate the required subgenomic mRNAs via internal transcriptional promoters. Brome mosaic virus, shown here as an example, is historically important as the system in which silent downstream cistrons were first discovered. Genomic RNA3 is structurally dicistronic: the 5′ cistron encodes a protein required for cell to cell movement of the virus, and the 3′ cistron encodes the capsid (coat) protein. In vitro translation of RNA3 produces only the movement protein, as the gel electrophoresis profile (blue) shows. Coat protein can be translated only from subgenomic RNA4 (red profile). (These gel profiles are tracings of Fig. 6 in Shih and Kaesberg, 1976.) Initiation of transcription from an internal promoter (Pr′) in the negative RNA strand generates mRNA4 (Choi et al., 2004).

1998). Some evidence suggests the repression might be mediated by an unidentified ternary effector rather than by direct binding of cytochrome *f* to the 5′ UTR (Choquet et al., 2003).

4. Structural elements in eukaryotic mRNAs that control initiation of translation

4.1. Preamble

Whereas co-transcription of contiguous genes in prokaryotes produces polycistronic mRNAs, eukaryotic cellular genes are transcribed individually, producing monocistronic mRNAs. This fundamental difference in gene expression follows from a fundamental difference in the mechanism of translation: prokaryotic ribosomes can enter and initiate at multiple sites within an mRNA, but eukaryotic ribosomes routinely enter only at the 5′ end.

The *scanning mechanism of initiation* (Kozak, 2002a) postulates that the 40S ribosomal subunit enters at the 5′ end of the mRNA and migrates linearly until it encounters the first AUG codon, which is recognized by base-pairing with the anticodon in Met-tRNA$_i$ (Cigan et al., 1988a). eIF2, the factor that escorts Met-tRNA$_i$ onto the 40S subunit, is a latent GTPase; an associated factor (eIF5) activates GTP hydrolysis by eIF2 only when there is a sufficiently long pause in scanning. In other words, the eIF5-mediated step is a timing device that helps to distinguish authentic AUG start codons (long pause) from other contenders (e.g. short pause at UUG) (Das and Maitra, 2001; Huang et al., 1997).[5] In contrast with our growing understanding of the stop-scanning step, we know nothing about the mechanism that actually propels the 40S subunit/factor complex. Hints that scanning might be dependent on ATP hydrolysis (Kozak, 1980) suggest involvement of eIF4A, the only initiation factor that binds ATP; but this awaits verification. No real evidence underlies the often repeated claim that eIF4A unwinds the 5′ end of the mRNA prior to binding of the 40S subunit; secondary structure might be disrupted only as the 40S subunit/factor complex advances.

Initiation is not always restricted to the AUG codon nearest the 5′ end. The scanning model specifies certain conditions–described below under *context-dependent leaky scanning* (Section 4.3) and *reinitiation* (Section 5)–which allow limited escape from the first-AUG rule. In these cases, translation still initiates at the first AUG, but not exclusively.

Some investigators believe that, contrary to the restrictions imposed by the scanning mechanism, eukaryotic ribosomes can enter directly at internal positions in certain mRNAs. This idea is not discussed in detail herein because the so-called internal ribosome entry sites (IRES) have not been defined structurally (candidate IRES elements share no common sequence) or mechanistically. Evidence said to support the internal initiation hypothesis is described in other reviews (Hellen and Sarnow, 2001; Jackson and Kaminski, 1995), but serious questions have been raised about much of this evidence (Kozak, 2001a, 2003a).

The absence of natural dicistronic mRNAs (two full-length nonoverlapping cistrons) in eukaryotic cells is prima facie evidence against the internal initiation hypothesis. Occasional exceptions only underscore the rule: some viral transcripts are structurally dicistronic but only the 5′ proximal cistron gets translated; thus, even these mRNAs are *functionally* monocistronic (Fig. 3). In one case where the 3′ cistron appeared to be translated (Stacey et al., 2000), the interpretation was revised when a second, spliced transcript was found (Zheng et al., 2004). Other claims of dicistronic mRNAs were simply

[5] eIF5 interacts also with eIF1 and eIF3, and genetic evidence suggests these factors can influence the eIF5-mediated GTPase reaction (Valášek et al., 2004). One possibility is that eIF1 serves as a brake on eIF5. The stop-scanning step controlled by these factors is followed by joining of the 60S subunit, which requires yet another protein factor (Lee et al., 2002; Shin et al., 2002).

mistaken: the two proteins turned out to be generated by proteolysis following translation of a single large open reading frame (ORF) (Hänzelmann et al., 2002; Ritchie and Wang, 1997, corrected in Feng et al., 1998).[6]

In addition to eukaryotic mRNAs being basically monocistronic, four other structural features are important vis-à-vis initiation of translation. These are explained next. As in prokaryotes, the eukaryotic ribosome protects ~15 nt on each side of the AUG codon (Kozak, 1977), but it is not appropriate to use the term "ribosome binding site" for eukaryotes; because of the scanning mechanism of initiation, structural elements that affect initiation can be dispersed throughout the 5′ UTR. (The latter term also is not appropriate, inasmuch as small ORFs located within the 5′ "untranslated region" do get translated, as explained in Section 5; but we are stuck with the term).

The following discussion does not include structures at the 3′ end of the mRNA. Despite abundant evidence implicating 3′ UTR elements in translational control of developmentally regulated genes (Bashaw and Baker, 1997; Kuersten and Goodwin, 2003; Wickens et al., 2000), the mechanisms are not yet clear. A recent review explains why some proposed mechanisms require rethinking (Kozak, 2004).

4.2. m7G cap

The 5′ end of all cellular and most viral mRNAs is capped with 7-methylguanosine (Furuichi and Shatkin, 2000). Via interaction with eIF4E (Gingras et al., 1999; von der Haar et al., 2004), the m7G cap strongly promotes ribosome binding. This was demonstrated directly by varying the 5′ terminal structure on mRNAs used for in vitro translation (Both et al., 1975) and indirectly by the inhibitory effect of soluble cap analogues (Hickey et al., 1976). In vivo experiments confirmed a substantial reduction in translational efficiency (≥10-fold) when mRNAs lack the m7G cap (Horikami et al., 1984; Lo et al., 1998).

Initiation was shown to occur exclusively at the first AUG codon even in the absence of the cap (Kozak, 1998, Fig. 6) or in the absence of initiation factors required for cap function (Ali et al., 2001). These experiments underscore an important point: *it is not because of the m7G/eIF4E interaction that ribosomes enter at the 5′ end. Rather, it is because eukaryotic ribosomes enter at the 5′ end that the m7G/eIF4E interaction can augment initiation*. The inability of eukaryotic ribosomes to bind a circularized mRNA (Kozak, 1979) supports the hypothesis that entry occurs via the 5′ end.

In the aforementioned experiments, the cap might have been dispensable because the 5′ end of the mRNA was relatively free of secondary structure and thus accessible to ribosomes. Most natural mRNAs have considerable secondary structure near the 5′ end, however. For this or other reasons, the interaction between m7G and eIF4E strongly stimulates translation of most mRNAs.[7]

4.3. Context effects on recognition of AUG (or other) start codons

The optimal context for initiation of translation in mammals is GCCRCCaugG. In experimental tests, the biggest reduction in efficiency was seen when the purine (R) in position −3 or the G in position +4 was mutated (Kozak, 1986a, 1997). Thus, initiation sites are usually designated "strong" or "weak" based on those two positions. A start codon flanked by A −3 and G +4 can function >10-fold more efficiently than an AUG codon in the weakest context. The GCCRCC motif augments initiation only when it directly abuts the AUG codon (Fig. 4A, lane 4 vs. lane 5; Kozak, 1987a).

Ribosomes will initiate at the first AUG codon to a limited extent even when the context is weak, but the poor context allows some ribosomes to bypass the first AUG and thus reach a start codon farther downstream. This is called *leaky scanning*. Fig. 4A shows a test case wherein initiation was restricted to the first AUG when it resided in the optimal context (lane 3), while a weaker context allowed initiation from the first and second AUG codons (lanes 1, 2). The leaky scanning seen when the first AUG codon resides in a suboptimal context can be suppressed by downstream secondary structure, as demonstrated in Fig. 4B. Because this depends on precise positioning of the hairpin structure (compare lanes 2, 4 and 5), a reasonable interpretation is that the structured element slows scanning and that recognition of a weak start codon improves when the 40S subunit pauses with its AUG-recognition center right over the AUG codon.

When the first and second start codons are in the same reading frame, context-dependent leaky scanning generates long and short forms of the protein which can be targeted to different compartments in the cell (Leissring et al., 2004; Melén et al., 1996; Shang et al., 2001; Souciet et al., 1999). When the first and second start codons are in different reading frames, leaky scanning enables one mRNA to produce two completely different proteins. Many bifunctional mRNAs that use this mechanism have been identified in plant and animal cells and viruses,[8] and occasional examples have been found in yeast (Outten and Culotta, 2004). (Recognition of start codons in yeast is not sensitive to context in all cases; Cigan et al., 1988b).

[6] Even picornaviruses (e.g. poliovirus), which are deemed the prime example of internal initiation, do not employ dicistronic mRNAs. The full set of viral proteins is derived by proteolysis from a "polyprotein" which is translated from a single large cistron. The IRES (if such it is) is at the 5′ end of the mRNA.

[7] Stimulation means simply that more protein is produced because more of the mRNA is engaged by ribosomes. It merits repeating that selection of the correct start site (the first AUG codon) is not augmented by eIF4E, contrary to what some textbooks say (Alberts et al., 2002). Reports of initiation occurring at internal sites upon removal of the cap (Brown et al., 2000) are probably attributable to partial degradation of the mRNA by 5′ exonucleases.

[8] Table 3 (Kozak, 2002a) lists 33 examples with full references. In many of these examples, operation of the leaky scanning mechanism was verified by showing that mutations which improve the context around the first AUG codon reduce initiation from the second AUG. In rare cases, production of a viral protein does not respond to changes in start codon context, apparently because translation is limited at a step other than initiation (Fajardo and Shatkin, 1990). Note that leaky scanning can occur, irrespective of context, when the first AUG codon is positioned very close to the cap (Kaneda et al., 2000; Slusher et al., 1991).

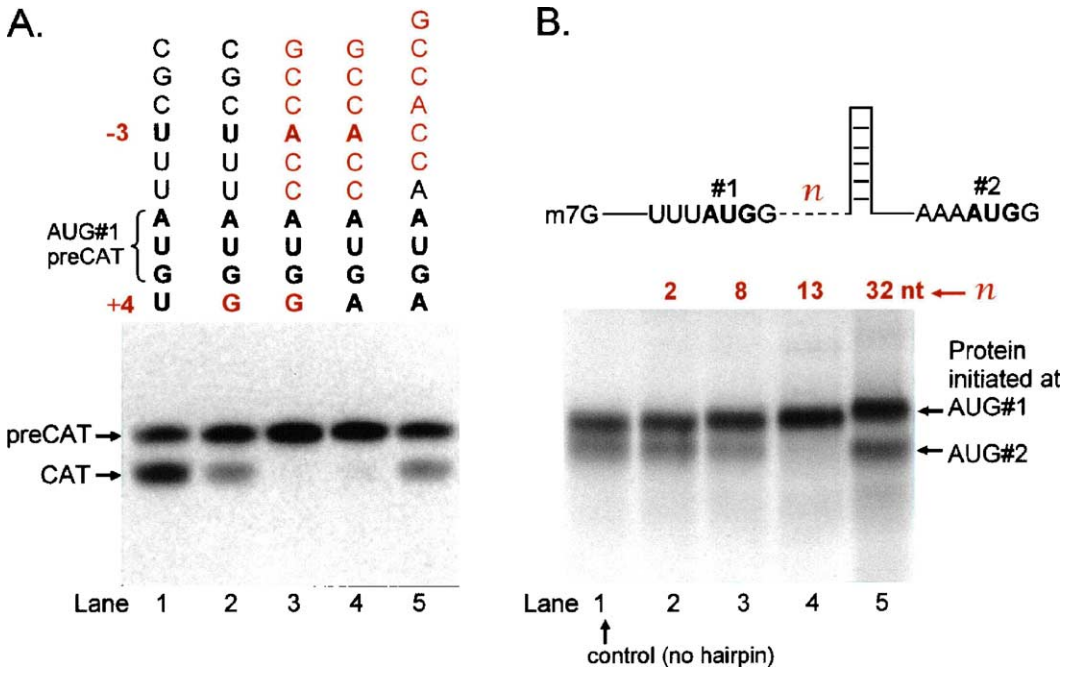

Fig. 4. Flanking sequences augment recognition of the AUG codon in eukaryotes. The autoradiograms show [^{35}S]Met-labeled proteins synthesized in vitro from capped mRNAs that encode chloramphenicol acetyltransferase. (A) Initiation at AUG#1 generates an N-terminally extended protein, labeled preCAT. A suboptimal context around AUG#1 allows some ribosomes to scan past that site and initiate instead at AUG#2. This leaky scanning produces a shorter protein, labeled CAT. (B) All mRNAs have a suboptimal context (U in position −3) around the first AUG codon and, except for the control in lane 1, a moderately stable base-paired structure between the first and second AUGs. The only variable is the distance (*n*) between AUG#1 and the base of the hairpin structure. When properly positioned, the downstream base-paired structure apparently suppresses leaky scanning (lane 4). A full description of these constructs and the adjustments required for the rabbit reticulocyte translation system to work properly are given in Kozak (1990a,b).

Whereas initiation at a codon other than AUG is common in prokaryotes, use of CUG, GUG or UUG as the primary initiation site is exceedingly rare in eukaryotes.[9] These nonstandard start codons are usually weak even when supported by the optimal context, as shown by an increase in protein production upon experimentally changing the codon to AUG. The weakness explains why *non-AUG codons are usually used only as supplementary initiation sites*; i.e. ribosomes initiate at an upstream non-AUG codon in addition to initiating at the first AUG (Carroll and Derse, 1993; Chang and Wang, 2004; Fütterer et al., 1997; Fuxe et al., 2000; Portis et al., 1994). The non-AUG initiated protein serves a useful function in those examples; but in some other cases, the N-terminally extended form of the protein has no biological relevance (Miles et al., 2003). Meaningless initiation events at upstream CUG codons might occur by accident when scanning is slowed by a GC-rich leader sequence.

4.4. Position determines which AUG functions as the start codon

The strongest evidence for the scanning mechanism is the position rule, which simply means that translation initiates at whichever AUG codon is closest to the 5′ end. (In the following examples, the first AUG was in a favorable context, thus allowing an uncomplicated test.) The earliest test of the position rule involved reiterating a block of nucleotides comprising the AUG start codon and nearby sequences in rat preproinsulin mRNA. Analysis of proteins produced by this mRNA in vivo revealed that translation initiated exclusively at the first of four tandemly repeated sequences (Kozak, 1983). An important followup test showed that the first AUG codon was used exclusively even when the second AUG was very close (2 or 5 nt downstream from the first AUG) and in the same favorable context (Kozak, 1995).

Selection-based-on-position was also verified in yeast via a clever experiment that involved changing the anticodon of Met-tRNA$_i$ to 3′-UUC-5′, whereupon initiation shifted to an AAG codon located upstream from the normal AUG start codon (Cigan et al., 1988a).

In the aforementioned tests, start codons were added or removed experimentally. When restructuring of mRNAs happens naturally via mutations, the pattern of translation again reveals the dominant role of position. Several examples are described in Fig. 5. In the first two cases, the mutation introduces an AUG codon upstream from the normal initiation site, whereupon the new AUG takes over. In the third case, a point mutation ablates the normal start codon, whereupon the next downstream AUG codon *which had been silent* becomes the new start site for translation. A few other naturally occurring mutations along these lines have been described (Cai et al., 1992; Liu et al., 1999; Mével-Ninio et al., 1996).

Elimination of the start codon via a mutation is a rare event, but the everyday production of alternative transcripts illustrates

[9] Examples and full documentation are provided in Kozak (2002a).

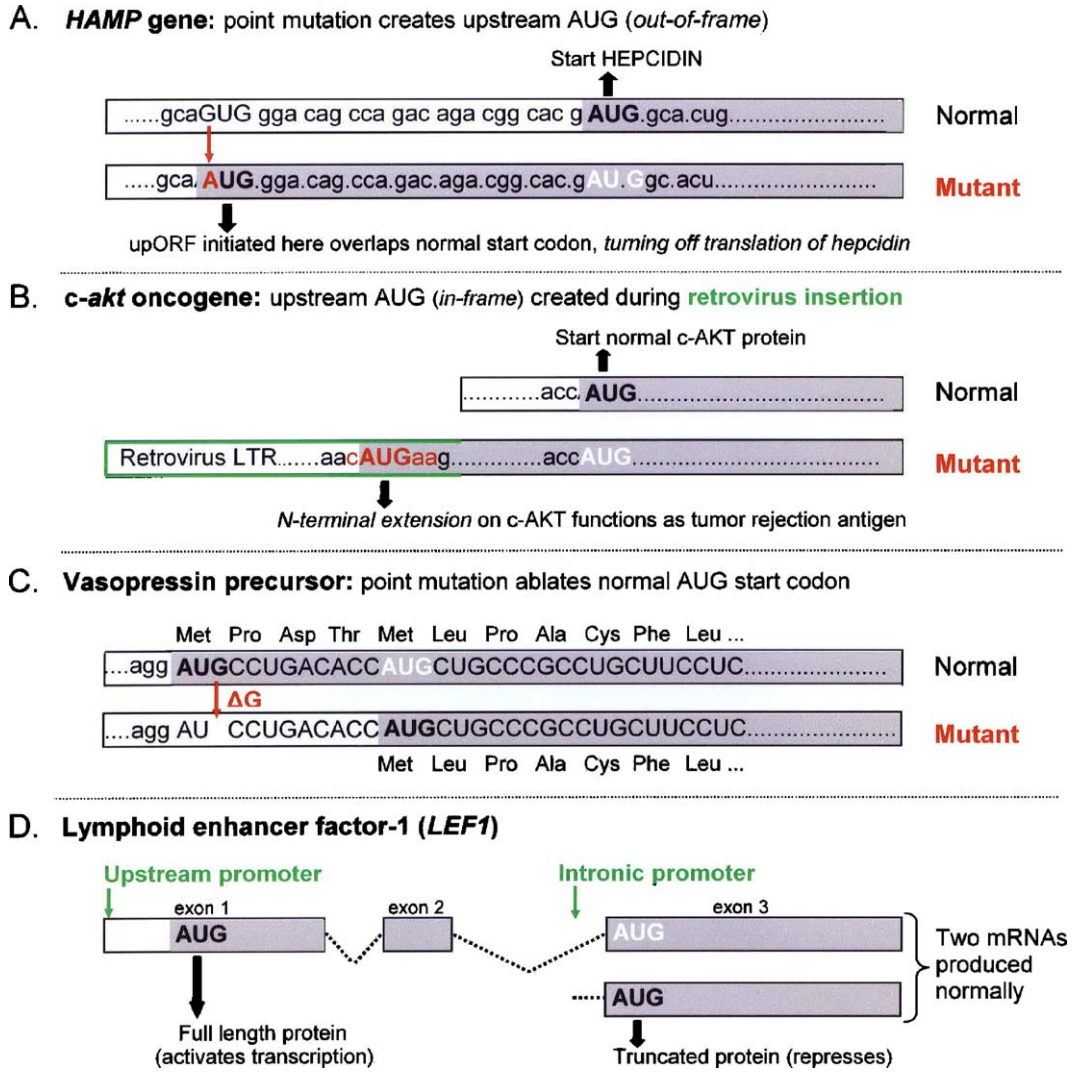

Fig. 5. Proximity to the 5′ end dictates which AUG functions as the start codon. White lettering indicates silent AUG codons. Only the portion of the mRNA relevant to understanding how an AUG codon is added (A, B) or removed (C, D) is shown. The resulting shifts in initiation have clinical consequences. (A) The G→A mutation that creates an upstream AUG codon, thereby shutting off translation of hepcidin, was found in patients with juvenile hereditary hemochromatosis (Matthes et al., 2004). Hepcidin is an important negative regulator of iron absorption. (B) Restructuring of the c-*akt* gene adds an upstream in-frame AUG codon which takes over as the initiation site, producing an N-terminally extended form of AKT. This was found in a retrovirus-induced mouse leukemia which undergoes regression due to recognition of the novel N-terminal peptide by cytotoxic T-lymphocytes (Wada et al., 1995). (C) This point mutation causes loss of only the first four amino acids, but the shortened polypeptide folds improperly and vasopressin is not produced (Beuret et al., 1999). (D) Activation of only the upstream promoter in colon cancer prevents modulation of LEF1 activity, which is accomplished normally by balanced production of long and short forms of the protein (Hovanes et al., 2001).

the same principle: when the normal AUG codon is eliminated via a change in splicing or a switch in the transcriptional start site, a downstream AUG which had been silent becomes the new initiation site. Fig. 5D depicts one example. There are many, many others.[9] This is important biologically because it enables a single gene to produce different forms of the protein which sometimes have complementary functions, as indicated in Fig. 5D for LEF1. From a theoretical perspective, *the need to produce a second form of mRNA to activate an internal start codon* is strong evidence for the scanning mechanism.

The position rule asks only "which AUG is first?" The actual distance from the 5′ end is irrelevant. The scanning mechanism was shown to operate with no measurable reduction in efficiency even when the first AUG codon was >1000 nt from the 5′ end of the mRNA (Berthelot et al., 2004). Thus, it is a mistake to think that a long leader sequence contravenes operation of the scanning mechanism.

4.5. Base-paired structures in 5′ UTR

Although leader length per se is not a problem, scanning can be difficult when a long leader sequence contains secondary structure. The GC-richness of mammalian 5′ UTR sequences predicts a considerable amount of secondary structure. Yeast mRNAs, in contrast, have remarkably AU-rich leader sequences (Shabalina et al., 2004).

Base-paired structures are most inhibitory when their proximity to the 5′ end blocks ribosome entry (Goossen and Hentze, 1992; Kozak, 1989; Wang and Wessler, 2001). Once

bound to mRNA, the scanning 40S subunit/factor complex has some ability to disrupt base-pairing, although this has limits (Kozak, 1986b). The bottom line is that a long 5′ UTR which contains substantial secondary structure can greatly reduce translational efficiency but does not completely preclude scanning (Short and Pfarr, 2002; van der Velden et al., 2002).

An often-repeated idea is that increased expression of initiation factor eIF4E might selectively elevate translation of mRNAs that encode critical growth-regulatory proteins (Graff and Zimmer, 2003). The selectivity is attributed to these mRNAs having leader sequences which are GC-rich, hence highly structured. One problem with the hypothesis is that the mRNAs produced by most housekeeping genes also have extremely GC-rich (>70%) leader sequences. Thus, there is no structural basis for the idea that increased expression of eIF4E specifically elevates translation of growth-regulatory genes.

When increased production of critical regulatory proteins is needed, a proven solution in some cases is to change the structure of the 5′ UTR via alternative splicing or activation of a downstream transcriptional promoter. In other words, the block to translation is relieved by truncating the long, GC-rich 5′ UTR (Charron et al., 1998; Han et al., 2003a,b; Sasahara et al., 1998).[9]

Occasionally, experimental manipulations of 5′ UTR sequences do not produce the expected effects on translation. In cases where a long, GC-rich leader inhibits translation in vitro but not in vivo (Hoover et al., 1997; Nikolcheva et al., 2002; van der Velden et al., 2002), the simplest explanation might be that the sequence harbors a cryptic promoter which generates a better-translated short-leader transcript in vivo.[10] The lesson is that, when pondering the function of 5′ UTR structures, it is a mistake to think only in terms of direct effects on initiation of translation.

5. Reinitiation as a device for regulating translation in eukaryotes

5.1. Mechanism of reinitiation

We know little about the molecular mechanisms underlying reinitiation. Ribosomes initiate in the normal way at the first AUG codon, producing the peptide encoded in the small upstream ORF (upORF). At the terminator codon (where the 60S subunit presumably dissociates), the 40S subunit apparently remains bound to the mRNA, resumes scanning, and initiates again at a downstream site.

Whereas reinitiation in prokaryotes is facilitated when the stop codon of the first cistron is closely apposed to the next start codon, that arrangement works poorly, if at all, in eukaryotes. *Reinitiation in eukaryotes improves as the distance increases between the stop codon and the re-start site* (Kozak, 1987b; Abastado et al., 1991). This reflects a requirement for the 40S subunit to reacquire Met-tRNA$_i$, without which the next AUG codon cannot be recognized. Reinitiation can occur only if there is enough time (distance) for the scanning 40S subunit to pick up Met-tRNA$_i$ before arriving at the next AUG. Reacquiring Met-tRNA$_i$ requires the help of eIF2 (i.e. a ternary complex must assemble consisting of Met-tRNA$_i$, eIF2, and GTP), and therefore, reinitiation can be regulated by manipulating eIF2·GTP levels. The significance of this is explained in Section 5.2.

Reinitiation is affected by certain changes in mRNA structure, enabling us to formulate working rules even though the biochemical mechanisms are uncertain.

One rule is that eukaryotic ribosomes can reinitiate following the translation of a small ORF but *not following the translation of a full-length protein-coding ORF*. Naturally occurring upORFs usually are only a few codons long. With experimental constructs, reinitiation was found to decrease as the upORF was lengthened, reaching a barely detectable level when the upORF was 35 codons long (Kozak, 2001b; Rajkowitsch et al., 2004). This might be explained if reinitiation depends on retention of certain initiation factors which gradually dissociate from 80S ribosomes during the course of elongation. This idea is somewhat supported by experiments wherein manipulations designed to slow elongation through the upORF (e.g. depleting tRNA levels or introducing a base-paired structure into the mRNA; Kozak, 2001b; Rajkowitsch et al., 2004) reduced the efficiency of reinitiation. The hypothesis cannot really be tested until we understand more about the cycling of initiation factors.[11]

Another rule is that eukaryotic ribosomes *cannot backup* to reinitiate at an AUG codon positioned far upstream from the termination site. It follows that translation of the major coding domain (the longest ORF) should be profoundly inhibited by an *overlapping* upORF. Many observations verify this prediction.[9]

The bottom line is that, when an upORF is relatively small and terminates before the start of the main coding domain, reinitiation can occur; but reinitiation is never efficient. The next section describes some consequences.

[10] Recent experiments confirm this prediction for the *pim-1* gene (Wang et al., 2005b).

[11] Cauliflower mosaic virus produces a transactivator protein called TAV which is postulated to interact with eIF3, retaining the initiation factor on ribosomes and purportedly allowing reinitiation even after translation of a full-length cistron (Park et al., 2001). Evidence for this idea is not convincing, however. Although TAV and eIF3b appear to co-sediment with small polysomes in a sucrose gradient, this interpretation was not verified by showing a shift in sedimentation of TAV/eIF3b upon disruption of polysomes. As for whether TAV indeed enables a dicistronic mRNA to be translated by reinitiation, the results from DNA transfection experiments are ambiguous. One study showed strong stimulation of expression of the downstream cistron by TAV (Park et al., 2001, Fig. 6). In another study, the efficiency of translation of the 3′ cistron was not revealed; the yield from a particular construct in the presence of TAV was simply called "100%" (Fütterer and Hohn, 1991, Fig. 2). In a third study–*the only one in which mRNA structure was checked*–the yield of protein from the downstream cistron in the presence of TAV was only about 5% of the yield from a monocistronic control mRNA; and the analysis of mRNA structure was not sensitive enough to rule out production of a monocistronic mRNA at 1/20th the level of the dicistronic mRNA (Bonneville et al., 1989). Inasmuch as TAV is present in both the nucleus and cytoplasm of infected cells (Haas et al., 2005), the possibility that TAV might augment splicing or transport of viral mRNAs, rather than directly promoting translation, needs to be considered.

5.2. Significance of reinitiation

The inefficient reinitiation mode of translation can regulate gene expression in eukaryotes in four ways.

(i) The simplest effect is a reduction in protein production from the major ORF. Countless experiments, wherein translation of an mRNA was found to improve when upstream AUG codons were mutated, confirm that small upORFs can down-regulate translation (Blaschke et al., 2003; Koš et al., 2002; Kriaucionis and Bird, 2004; Pecqueur et al., 2001; Son et al., 2003). Examples such as those in Fig. 6 support the hypothesis that small upORFs–which force translation to occur by reinitiation–are used to *limit expression of potent proteins* which are required in small amounts but would be harmful if over-produced (Kozak, 1991).

Many genes that produce barely translatable mRNAs, due to small upORFs, turn out to harbor another promoter downstream; and the short, AUG-free 5′ UTR thereby produced boosts translation when more of the protein is needed (Blaschke et al., 2003; Phelps et al., 1998; Smith et al., 1998).[9] Other genes accomplish this via alternative splicing (Son et al., 2003). Alternative promoters and/or splicing underlie some tumor-related changes in gene expression; e.g. elevated translation of a growth-promoting gene caused by switching to a shorter, simpler 5′ UTR (Arrick et al., 1994; Landers et al., 1997; Wang and Rothnagel, 2001), and reduced translation of a tumor suppressor gene caused by

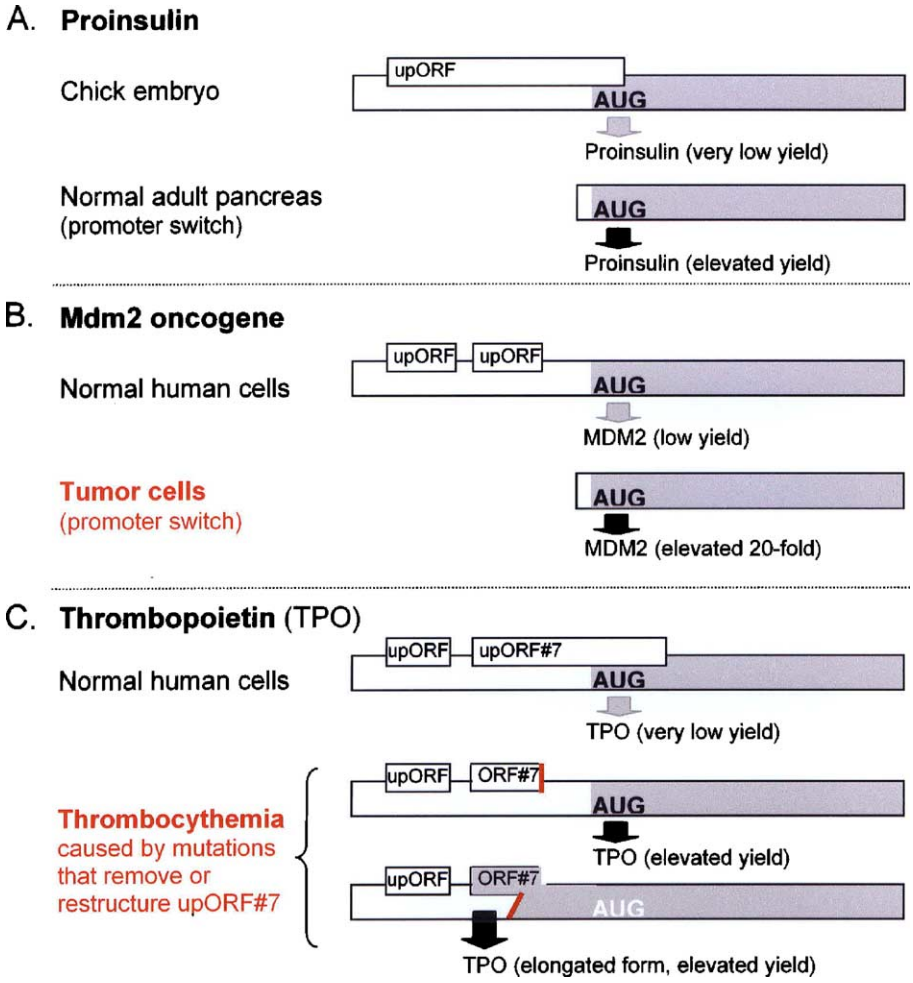

Fig. 6. Small upstream ORFs down-regulate translation by imposing an inefficient reinitiation mechanism. This constraint on translation ensures against harmful overproduction of potent proteins. (A) The presence of an overlapping upORF allows only low-level production of proinsulin in chick embryos (Hernández-Sánchez et al., 2003). In the adult pancreas where higher production of proinsulin is needed, a more efficiently translated form of mRNA is produced via a downstream promoter. Whereas the production of two forms of proinsulin mRNA is a normal event, the changes in mRNA structure in the next two examples are rare pathological events. (B) Overexpression of the *mdm2* oncogene in human tumor cells is caused by a switch in the transcriptional start site which eliminates two small upORFs, thereby elevating translation 20-fold (Landers et al., 1997). (C) Pathological overproduction of thrombopoietin (TPO) is caused by various mutations that restructure upORF#7. (It is so numbered because there are additional minor forms of mRNA with additional upORFs; but the story can be understood by focusing on the most abundant mRNA, depicted here.) In normal individuals, translation is strongly suppressed by this overlapping upORF, ensuring that production of TPO is kept very low. The depicted mutations elevate translation by truncating upORF#7 or (via deletion of 1 nt) fusing upORF#7 with the TPO coding domain. The resulting overproduction of TPO causes hereditary thrombocythemia (Cazzola and Skoda, 2000; Ghilardi and Skoda, 1999; Ghilardi et al., 1998).

imposing an AUG-burdened 5′ UTR (Anant et al., 2002; Frost et al., 2000).

The degree to which small upORFs inhibit translation varies. This can be rationalized to some extent based on whether the upstream AUG codons are in a strong or weak context (Wang and Rothnagel, 2004); i.e. leaky scanning can mitigate the inhibitory effects of upstream AUGs. With extremely GC-rich leader sequences, the old adage that "nothing bad can happen to a rotten eggplant" might apply: the inhibitory effects of secondary structure might be so profound that adding or removing an upstream AUG codon barely matters.

(ii) When eIF2·GTP levels are low, the slow reacquisition of Met-tRNA$_i$ might cause 40S subunits to bypass the closest downstream AUG codon and advance farther before reinitiating. Thus, *the site where translation reinitiates can be manipulated by the availability of eIF2·GTP*, and this can be manipulated by kinases which respond to growth conditions and other cues (Clemens, 2001).

The best studied example involves the yeast *GCN4* gene (Gaba et al., 2001; Hinnebusch, 1997). The GCN4 protein is a transcription factor which turns on expression of genes involved in amino acid biosynthesis. Translation of GCN4 itself is regulated by amino acid availability because the 5′ UTR has four small upORFs, forcing translation to occur via reinitiation; and reinitiation is controlled by a kinase activated by uncharged tRNAs. The kinase (GCN2) phosphorylates eIF2 in a way that impairs the exchange of GDP for GTP. When yeast are starved for amino acids, uncharged tRNAs accumulate and operation of the reinitiation mechanism changes, as explained in Fig. 7. (Although genetic manipulations point to involvement of eIF3 in some aspects of reinitiation (Nielsen et al., 2004), eIF2 is the only component directly altered in response to amino acid starvation).

Recent evidence suggests that translation of the ATF4 transcription factor in mammals is regulated via a GCN4-like mechanism (Vattem and Wek, 2004). In this case, upORF2 inhibits profoundly because it overlaps the start of the ATF4 ORF. Small upORF1 augments translation of ATF4 by causing upORF2 to be bypassed, to an extent that depends on the availability of eIF2·GTP.

(iii) In rare cases, the *peptide* produced from translation of a small upstream ORF has regulatory effects (Alderete et al., 2001; Fang et al., 2004; Law et al., 2001). This mechanism clearly is not involved in many other cases, inasmuch as mutations that change the amino acid sequence of the peptide do not change the inhibitory effects of the upORF.

(iv) Occasionally, the clinical effects of a premature nonsense codon in one or another gene are mitigated because

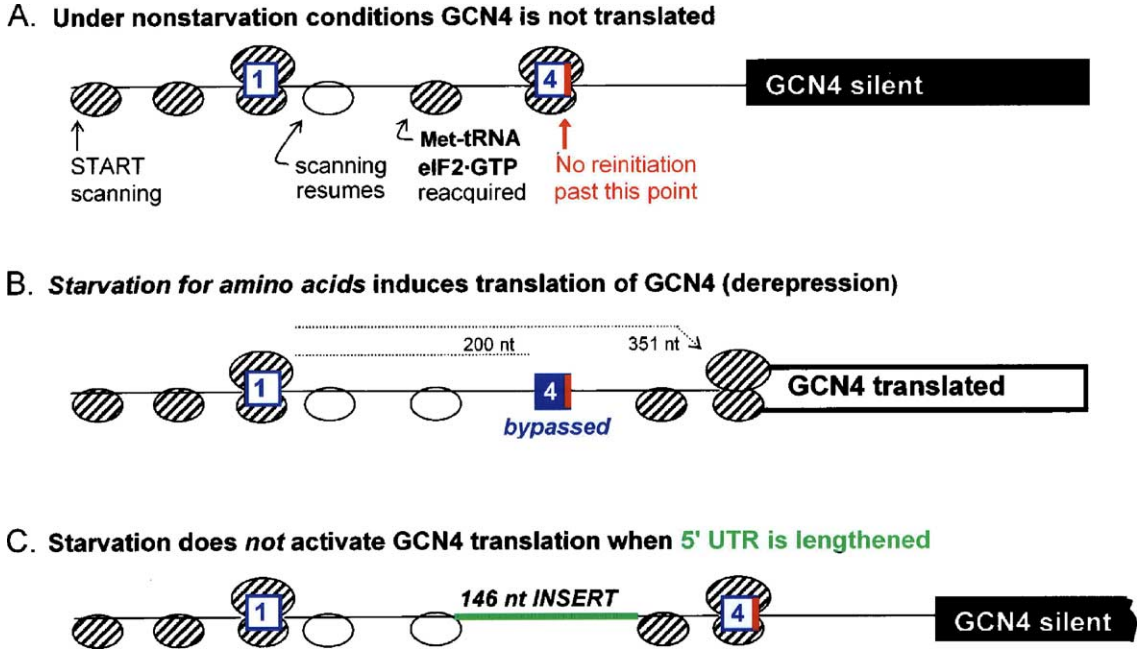

Fig. 7. Small upORFs set up a reinitiation mechanism which allows translation of GCN4 only under conditions of amino acid starvation. The natural leader sequence has four upORFs, but the two depicted here (boxes 1 and 4) are sufficient to reconstitute the regulation. Following translation of upORF1, 40S ribosomal subunits resume scanning and might reinitiate at upORF4 or the GCN4 start site, depending on how quickly Met-tRNA$_i$ rebinds. (A) Under nonstarvation conditions, 40S subunits quickly reacquire Met-tRNA$_i$ (indicated by cross-hatching), and reinitiation occurs at upORF4. upORF4 is peculiar in that, following its translation, further reinitiation is precluded. (A possible explanation is that a sequence flanking the termination site causes 40S subunits to dissociate from the mRNA, but this has not been shown directly.) The main point is that, if upORF4 gets translated, GCN4 cannot be translated. (B) Uncharged tRNAs, which accumulate in the absence of amino acids, activate a kinase which phosphorylates eIF2, thereby impairing the GDP→GTP exchange reaction. The reduction in eIF2·GTP levels, and resulting *slower reacquisition of Met-tRNA$_i$*, causes some 40S ribosomal subunits to bypass upORF4. The ribosomes become competent to reinitiate by the time they reach the GCN4 start site. (C) The foregoing explanation was tested by expanding the distance between upORF1 and upORF4. This resulted in failure to translate GCN4 even under conditions of starvation (Abastado et al., 1991).

a truncated, but still functional, form of the protein is produced by reinitiating at a fortuitously positioned downstream AUG codon (Chang and Gould, 1998; Ozisik et al., 2003). This is not a common escape mechanism because mRNAs that carry a premature terminator codon–the result of a point mutation or failure to remove an intron–usually get degraded via a translation-linked mechanism called nonsense-mediate decay (Baker and Parker, 2004; Holbrook et al., 2004).

6. Regulation via proteins targeted to the 5′ UTR of eukaryotic mRNAs

Ferritin is the major iron-binding protein in non-hematopoietic tissues. Its function is to sequester excess intracellular iron, thereby protecting against the generation of harmful oxygen and nitrogen radicals which can damage DNA. Translation of ferritin is turned on and off by an iron-response protein (IRP) which binds a defined sequence (iron-response element, IRE) near the 5′ end of the mRNA. The mechanism of inhibition is straightforward: binding of IRP blocks entry of the 40S ribosomal subunit (Muckenthaler et al., 1998). The biological importance of this mechanism is evident from the pathological consequences of mutations in the repressor protein (LaVaute et al., 2001) or the IRE (Allerson et al., 1999).[12] In addition to controlling translation of ferritin, the IRE–IRP mechanism regulates other genes involved in iron uptake and utilization. In the case of ferroportin, the IRE is near the 5′ end of the mRNA and regulation is at the level of translation (Mok et al., 2004). In the case of transferrin receptor, the IRE is in the 3′ UTR and regulation is at the level of mRNA stability (Casey et al., 1989).

Regulation of translation by IRPs is so simple and efficient that it seems surprising there are not more such examples. Eukaryotic cells contain a plethora of mRNA-binding proteins, but their primary roles are in processes other than translation (e.g. splicing, mRNA transport, mRNA turnover). The demonstrated effects on translation in vitro are slight (Fukuda et al., 2004; Timchenko et al., 2001) or require an unnaturally high concentration of the protein (Nekrasov et al., 2003). Although more substantial effects were observed when these proteins were tested in vivo, effects on mRNA stability or transport might have been misinterpreted as effects on translation. In short, claims of translational regulation by general RNA-binding proteins are not convincing.

A major emerging story concerns proteins that bind to and regulate translation of embryonic mRNAs. Many such proteins bind to the 3′ UTR of the targeted mRNA (Kuersten and Goodwin, 2003). Exactly how they function is unclear, probably because the 3′ UTR-binding protein is only one piece of the puzzle. Thus, an early attempt to explain how the Bicoid protein represses translation of *caudal* mRNA in *Drosophila* embryos (Niessing et al., 2002) proved to be mistaken because *the repression requires at least two proteins*: Bicoid, bound to the 3′ UTR, interacts with the recently discovered 4EHP protein, bound to the m7G cap (Cho et al., 2005). This protein–protein interaction effectively circularizes *caudal* mRNA; and we understand how circularization of an mRNA prevents ribosome binding (Kozak, 1979).

[12] Two genes encode proteins, designated IRP1 and IRP2, capable of binding the IRE. When the IRE was linked to reporter genes and their expression studied in cultured cells, IRP1 appeared to be the major player; but this is now recognized as an artifact caused by *degradation of IRP2* during cell lysis and *activation of IRP1* by the unnaturally high concentration of oxygen used for tissue culture (Meyron-Holtz et al., 2004a). Knockout experiments in animals confirm that iron homeostasis is mediated primarily by IRP2 (Meyron-Holtz et al., 2004b).

7. Common questions and misunderstandings about initiation of translation in eukaryotes

7.1. Does complementarity between mRNA and 18S rRNA augment translation?

No evidence implicates such a mechanism in eukaryotes. Investigators sometimes point to sequences in one or another mRNA that are partially complementary to short segments of 18S rRNA; but there is no consistency regarding which rRNA segment is invoked, and no attention is paid to the (in)-accessibility of the rRNA sequences. Students who remember the prokaryotic story (Section 2.4) will understand that claims of mRNA/rRNA complementarity are meaningless without mutagenesis experiments to test the significance.

7.2. Misunderstandings about context

The optimal context for AUG codon recognition in mammals was defined experimentally, as described in Section 4.3. cDNA surveys sometimes raise doubts about the context rules, but it is the surveys which are flawed: the calculations give equal weight to all positions (ignoring the primacy of positions −3 and +4), and the postulated start codons are only guessed (often guessed incorrectly because the cDNAs are incomplete). When these and other mistakes in interpretation are corrected, mammalian mRNA/cDNA sequences show strong adherence to the experimentally defined rules (Kozak, 1987c, 2000; Pesole et al., 2000).

There are no grounds for thinking the initiation mechanism in plants is substantially different from animals. Plant mRNA sequences adhere to the mammalian consensus motif in positions −3 and +4 (Pesole et al., 2000; Rogozin et al., 2001), and mutagenesis experiments confirm that A −3 and G +4 strongly stimulate translation in plants (Jones et al., 1988; Lukaszewicz et al., 2000). The consensus motif in plants differs from animals only in the less important positions (−1, −2, −4, −5), where A rather than C predominates.

Careless wording is used sometimes when describing the context rules. It is wrong to say that "*purines* in positions −3 and +4" define an efficient initiation site (Hershey and Merrick, 2000); in fact, G is the only effective base in position +4. It is also wrong to attribute the conservation of G +4 simply to a requirement for certain amino acids (Ala, Val, Gly) in the penultimate position of proteins. That facile explanation ignores

the fact that G +4 augments translation even when the assay is limited to the initiation step (Kozak, 1997).

The positive effect of G+4 can be negated by U in position +5 (Kozak, 1997). Other than that, no particular base in position +5 or +6 exerts an effect on initiation. Mutations introduced near the beginning of the coding domain can alter the stability of the encoded polypeptide, however, and that might explain the mistaken notion that the optimal context for initiation includes particular bases in positions +5 and +6 (Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994).

In some human and mouse genes, naturally occurring mutations or polymorphisms close to the AUG codon have pathological consequences (Kozak, 2002b). These mutations usually affect position −3 or +4, and the expected reduction in translational efficiency has been confirmed in vitro. It would be a mistake, however, to assume that every mutation in the "Kozak sequence" is deleterious because of effects on translation. Possible effects on mRNA stability or splicing must always be considered (Kozak, 2003b).

It is a mistake also to be distracted by distance when making predictions about leaky scanning. If the first AUG codon is in a strong context, it will be the unique initiation site even when the second AUG is very close (e.g. accAUGgcAUGg; Kozak, 1995). If the first AUG is in a poor context, the second AUG will also be usable no matter how close or far it resides from the first (e.g. 4 nt, Matsuda et al., 2004; 620 nt, Herzog et al., 1995).

7.3. Upstream AUG codons do not constitute "evidence against scanning"

Some people mistakenly think the scanning hypothesis is undermined by the presence of small upstream ORFs in many mRNAs. This does not contradict the first-AUG rule because *the small upORFs are translated* (Hackett et al., 1986; Hernández-Sánchez et al., 2003; Oyama et al., 2004; Wang and Wessler, 2001). Whereas the encoded peptides were detected directly in those examples, sometimes this is difficult because small peptides are degraded rapidly. When the mRNA is reconfigured in a way that fuses the upORF with a longer coding domain, however, the upstream AUG codon can be shown to function (Chen et al., 2005; Tanaka et al., 2001). This is true even for picornaviruses (Borman and Jackson, 1992; Pöyry et al., 2001).

It is hard to know how many cellular mRNAs actually have upstream AUG codons. Tallies of mammalian cDNA sequences sometimes indicate a high frequency, but these statistical calculations present a distorted picture. In some cases, only a small fraction of transcripts from the gene in question carry the AUG-burdened 5′ UTR (Kriaucionis and Bird, 2004; Laurin et al., 2000; Perälä et al., 1994). Sometimes, sequencing errors give the false appearance of upstream AUG codons (Kozak, 1996; Sanz et al., 1995, corrected in Rekdal et al., 2000). In other cases, the cDNAs with upstream AUG codons turn out to derive from incompletely processed transcripts; i.e. the upstream AUGs are in an intron which gets removed from the functional mRNA (Kozak, 2000). Reliance on a curated database, such as RefSeq, reduces but does not eliminate these problems.[9]

Not all upstream AUG codons are artifacts, of course. In some–perhaps many–cellular mRNAs, small upORFs are used deliberately to reduce translational efficiency, as shown for TPO (Fig. 6C). This regulatory device works *because the mRNA is translated via scanning.*

7.4. Cap-independent translation does not necessarily indicate an IRES

Internal initiation is sometimes postulated upon finding that translation of an mRNA is not strongly cap-dependent, but studies with TMV show this reasoning to be faulty. This viral mRNA is naturally capped, but its translation is relatively resistant to inhibition by cap analogues or depletion of eIF4E (Altmann et al., 1990; Hickey et al., 1976), and synthetic uncapped transcripts bearing the TMV 5′ UTR are translated efficiently in vitro (Sawasaki et al., 2002). Despite this unusual lack of dependence on the m7G cap, three experiments rule out the possibility that the sequence functions as an IRES. (i) The TMV leader sequence promotes efficient translation only when positioned directly at the 5′ end of a transcript (Sleat et al., 1988). (ii) Ribosomes cannot bind to a *circularized* form of the TMV leader sequence (Konarska et al., 1981). (iii) Insertion of the TMV sequence at the midpoint of a *dicistronic* mRNA does not allow translation of the 3′ cistron (Akbergenov et al., 2004). The simplest explanation for the lack of dependence on the m7G cap is that the long TMV leader sequence, A-rich and G-poor, is nearly devoid of secondary structure.

With some other mRNAs, "cap-independent translation" is claimed wrongly because of misunderstandings about initiation factors. Textbooks are mistaken when they identify eIF4E as the limiting component in translation. The concentration of this factor is normally quite high (Rau et al., 1996; von der Haar and McCarthy, 2002), and therefore, eIF4E levels can be reduced without necessarily causing cap-dependent translation to fail.[13]

In the case of poliovirus, there is no doubt that translation is cap-independent–the viral mRNA is not capped–but the accompanying rationalization is wrong. Because the cap-binding factor eIF4E normally associates with eIF4G, a popular idea is that cleavage of eIF4G impairs cap-dependent translation. Thus, textbooks tell us that cleavage of eIF4G during poliovirus infection shuts down host translation while allowing viral translation to continue via an IRES (Alberts et al., 2002). This rationalization is contradicted, however, by the timing of events in poliovirus-infected HeLa cells: the major form of eIF4G is cleaved rapidly, but host protein synthesis persists for some time thereafter (Malnou et al., 2004). The discrepancy in timing is even greater in a neuronal cell line: eIF4G is cleaved extensively by 3.5 h post-infection and cleavage is complete by 5 h, but host protein synthesis

[13] An additional complication, when eIF4E levels are manipulated in vivo, is that the m7G/eIF4E interaction also affects mRNA stability and transport. In the case of cyclin D1, for example, the step most affected is mRNA transport rather than translation (Rousseau et al., 1996).

continues unabated for at least 9 h (Yanagiya et al., 2005).[14] Reconstruction experiments confirm that the truncated (100 kDa) form of eIF4G generated by the poliovirus-encoded protease can still support translation of capped mRNAs (Ali et al., 2001). Thus, *translation under conditions where eIF4G gets cleaved is not grounds for postulating a cap-independent (IRES-mediated) mechanism of initiation*.

What does constitute grounds for postulating internal initiation? The most common test involves transposing a suspected IRES from its normal 5′ position to the midpoint of a synthetic dicistronic transcript and asking whether this allows translation of the 3′ cistron. The test is undermined in many cases, however, because the candidate IRES turns out to harbor a cryptic promoter or splice site, causing the dicistronic DNA vector to produce an unintended monocistronic mRNA (Dumas et al., 2003; Han and Zhang, 2002; Han et al., 2003a,b; Hecht et al., 2002; Liu et al., 2005; Sherrill et al., 2004; Van Eden et al., 2004; Vergé et al., 2004; Wang et al., 2005b). In many other cases, the RNA analyses required to rule out a cryptic promoter or splice site simply were not done. An unexpected (and disconcerting) finding is that the ability of putative IRESs to support translation from a dicistronic vector depends on the choice and arrangement of reporter genes (Hennecke et al., 2001). In short, although much has been written in defense of the internal initiation hypothesis (Hellen and Sarnow, 2001), there are grounds for doubting much of the evidence (Kozak, 2001a, 2003a). Students might want to look carefully at the experiments before accepting what textbooks say about this.

8. Experimental deficiencies underlie other misunderstandings about translation

A widespread problem is failure to recognize artifacts caused by cleavage of the mRNA during incubation in cell-free translation systems. Because proximity to the 5′ end is the main determinant of start-codon selection in eukaryotes, *silent internal AUG codons can be converted to functional start sites simply by fragmenting the mRNA*.[15] mRNA cleavage might explain why upORFs which strongly inhibit translation in vivo are sometimes less inhibitory in vitro (Ghilardi et al., 1998; Meijer et al., 2000; Pecqueur et al., 2001; Tanaka et al., 2001) and why translation sometimes initiates in vitro at far-downstream AUG codons which are not used in vivo (Byrd et al., 2002, Fig. 3C; Hassin et al., 1986; Meulewaeter et al., 1992; Peeters et al., 2004). In vitro translation reactions that generate an array of low molecular weight polypeptides are a sure sign of mRNA degradation (e.g. Van Eden et al., 2004, Fig. 2B, lane 2). Failure to recognize this problem underlies many faulty interpretations, including claims of IRES activity (Venkatesan and Dasgupta, 2001) and exaggerated estimates of the size of the human proteome (Kettman et al., 2002). The latter study showed that single transcripts, each derived from a cloned cDNA, gave rise to multiple polypeptides when translated in vitro; but the possibility of artifacts caused by mRNA cleavage was not considered.

When translation is studied in vivo, a major deficiency is failure to search for all possible forms of mRNA. It is wrong to claim tissue-specific inhibition by upstream AUG codons (Muller and Danner, 2004; Zimmer et al., 1994)–i.e. wrong to claim regulation at the level of translation–without having looked for possible tissue-specific changes in structure of the 5′ UTR. A popular idea is that, because eIF4E is elevated in tumor cells (as are many other components of the translational machinery), eIF4E might augment translation of critical regulatory genes (Buechler and Peffley, 2004; Nikolcheva et al., 2002; Sunavala-Dossabhoy et al., 2004). But it is unwise to look only at initiation factors and to ignore possible changes in structure of the mRNA. Restructuring of the 5′ UTR, via alternative splicing or promoter usage, is a common phenomenon in tumor cells (Dabrowska and Sirotnak, 2004; Perrais et al., 2001a,b; other examples are cited in Section 5.2).

Some investigators invoke a shunting mechanism (i.e. discontinuous scanning) upon finding that upstream AUG codons or base-paired structures do not cause the expected block to translation (Sen et al., 2004, and references therein). But the structures that failed to block translation might have been eliminated via splicing. Claims of shunting published without verification of the mRNA structure are meaningless (Rogers et al., 2004). The shunting hypothesis ignores big questions (e.g. how do ribosomes decide where to resume scanning?) and is supported by no credible evidence.

There are occasional reports of other bizarre translation mechanisms, such as initiation without Met-tRNA$_i$ (Cevallos and Sarnow, 2005). This study employs unproven assays (e.g. use of agarose gels rather than sucrose gradients to demonstrate "initiation complexes") and points to a smear of uncharacterized peptide (?) products as evidence that an initiation-factor-independent IRES supports translation from a dicistronic mRNA. Because the mRNA in question encodes the viral capsid protein, which is required in large amounts, a credible story requires showing that the unusual initiation mechanism works efficiently (e.g. by comparison to a normal, Met-tRNA-dependent, monocistronic mRNA). The question of efficiency was ignored. Moreover, elaborate experiments with *synthetic* dicistronic constructs were undertaken without first showing

[14] Some investigators try to rescue the hypothesis by suggesting that cleavage of a second form of the factor (eIF4GII) is what shuts off host translation, but this explanation requires testing. It merits mentioning that the yield of poliovirus obtained from neuronal cells was nearly identical to that from HeLa cells, although only HeLa cells showed substantial inhibition of host protein synthesis (Yanagiya et al., 2005). This undermines the popular belief that, for viral translation to succeed, competition from host mRNAs must be eliminated.

[15] This artifact was recognized long ago (Bendena et al., 1985; Lawrence, 1980; Pelham, 1979) but has gradually been forgotten, even as conditions for studying translation in vitro have evolved in ways that exacerbate the problem. Some commercial reticulocyte translation systems are not strongly cap-dependent (Kozak, 1998, Fig. 6) and this invites translation of broken mRNAs. mRNAs are rapidly degraded in HeLa cell-free extracts, especially when an elevated temperature (37 °C) is used. It is unwise to incubate reactions for ≥90 min and then interpret what one sees as an effect on the initiation step of translation. Formation of initiation complexes takes only about 5 min. The longer the incubation, the more one has to worry about artifacts caused by cleavage of the mRNA. Artifactual initiation from a downstream AUG codon can be caused also by failure to adjust the Mg^{2+} concentration in reticulocyte lysates (Kozak, 1990b). For this or other reasons, coupled transcription/translation systems often show inappropriate selection of start codons.

that the *natural* dicistronic mRNA actually supports translation of the capsid protein. A subgenomic, monocistronic mRNA for the capsid protein might be found in virus infected cells, if someone were to look.

Similar claims about a putative IRES from cricket paralysis virus (Hellen and Sarnow, 2001) are called into question by a recent study in which IRES activity, assayed via synthetic dicistronic constructs, and actual virus replication were tested in a range of insect cell lines. The notable finding is that the *test for IRES activity failed completely* in the two cell lines that produced the *highest yield of virus* (Masoumi et al., 2003). This is a small warning against the growing practice of "reconstructing" translational regulatory mechanisms, using reporter genes in vitro, without first having gathered the basic facts about the natural process. Molecular biologists are supposed to explain biology, not invent it.

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