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Determinants of translational fidelity and efficiency in vertebrate mRNAs

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Summary — This article reviews current knowledge on the mechanisms affecting the fidelity of initiation codon selection, and discusses the effects of structural features in the 5'-non-coding region on the efficiency of translation of messenger RNA molecules.

initiation of translation / scanning model / mRNA structure

Introduction

Two questions about the initiation of protein synthesis in higher eukaryotes are considered here: i) how do ribosomes find the correct AUG codon for initiation of translation?; and ii) how is the efficiency of translation modulated by aspects of mRNA structure, especially near the 5' end? The suggested answers to both questions are most easily understood by invoking the scanning model for initiation [1], which postulates that the 40S ribosomal subunit binds initially at the capped 5' end of the mRNA and migrates linearly until it encounters the first AUG codon, at which point the 60S subunit joins and the resulting 80S ribosome is poised to form the first peptide bond. Evidence in support of the scanning mechanism has been summarized previously [1, 2].

Determinants of translational fidelity

The fidelity of initiation (*ie* selection of the correct start site) is determined primarily by the position of the AUG codon relative to the 5' end of the mRNA, with contributions from the surrounding primary sequence and in some cases from downstream secondary structure. The importance of the fidelity of initiation can be grasped intuitively, but the point is made concrete by reports in which truncated proteins, initiated inappropriately from internal AUG codons, have been shown to be unstable, or sorted improperly, or capable of interfering with the function of the full-length protein [3–5].

The dominant role of position in determining the site of initiation has been shown experimentally by introducing AUG codons upstream from the normal start site: insertion of a strong, upstream, out-of-frame AUG codon dramatically inhibits translation, while a strong, upstream, in-frame AUG codon supplants the original site of initiation (reviewed in [1]). A rigorous test of the latter point was carried out by constructing an mRNA in which the translational start site – contained within a block of 66 nucleotides derived from the rat preproinsulin gene – was reiterated four times in tandem [6]. Although each of the four repeats contained an in-frame AUG codon in an identical context, ribosomes initiated exclusively from the first AUG codon in the tandem array [6]. This experiment gave a clear demonstration of the 'first-AUG rule' because the initiator codon in preproinsulin mRNA occurs in (what was later recognized to be) a good context. Initiation may not be limited to the first AUG codon when the surrounding context is less favorable, as discussed below.

Systematic mutagenesis of nucleotides in the vicinity of the AUG codon revealed that GCCACCaugG is the optimal context for initiation of translation in vertebrates [7, 8]. The experimentally determined optimal context matches the consensus sequence derived from inspection of published vertebrate mRNA sequences [9]. In experimental tests of context effects, the strongest contributors were a purine (preferably A) in position –3 and a G in position +4. (In the numbering scheme used here, the A of the AUG codon is designated +1, with positive and negative integers proceeding 3' and 5', respectively.) The com-

bination of A^{-3} and G^{+4} can increase translation ≥ 10-fold *in vivo* [7] and *in vitro* [10]; and those are the two most highly conserved positions in the leader sequences of vertebrate mRNAs [9]. The small number of vertebrate mRNAs in which the AUG initiator codon occurs in an extremely poor context (*eg* pyrimidines in positions –3 and +4) includes several growth factor and cytokine genes. In these cases, the poor context might be a deliberate ploy to throttle the expression of potent proteins, the overproduction of which might be deleterious. Although context effects have been studied most thoroughly in vertebrate systems, there is some evidence that A^{-3} and G^{+4} augment AUG-codon recognition in plant [10–12] and insect [13] translation systems. *S cerevisiae* is the only organism thus far studied in which context effects seem to contribute only slightly to AUG-codon recognition [14–16, 88]. In vertebrates, the deleterious effects of a suboptimal context can be mitigated by downstream secondary structure [17], which has been postulated to slow scanning and thus to provide more time for the 40S ribosomal subunit to recognize the AUG codon. By extension, the absence of strong context effects in *S cerevisiae* might be rationalized by postulating that the rate of scanning by 40S ribosomal subunits is inherently slow in lower eukaryotes. But that idea awaits experimental study.

In vertebrate mRNAs in which the first AUG codon is in the optimal context, the usual outcome is that all 40S ribosomal subunits stop scanning at the first AUG and translation initiates uniquely from that site. When the first AUG codon is in a suboptimal context vis-à-vis positions –3 and/or +4, some 40S ribosomes bypass that site and initiate instead at the second (or, rarely, even the third) AUG. This 'leaky scanning' mechanism thus enables two independently-initiated proteins to be produced from one mRNA. Nearly 30 examples of bifunctional mRNAs that fit this description have been identified [18] and, in ten cases, the postulated connection between leaky scanning and a suboptimal context has been confirmed by mutational analysis [19–28]. Thus, the importance of context for recognition of the AUG initiator codon has been confirmed in many laboratories. While most instances of leaky scanning are attributable to a poor context around the first AUG codon, in rare cases the second AUG codon may be accessible because the first AUG lies too close to the cap to be recognized efficiently [16, 29–31].

Whereas an unfavorable context around the first AUG codon enables some 40S ribosomes to scan past that site and initiate farther downstream, the presence of a highly favorable context (possibly augmented by downstream secondary structure [17]) around certain non-AUG codons, such as CUG or ACG or GUG, may encourage 40S ribosomal subunits to pause and initiate at these adventitious, upstream sites in addition to initiating at the first AUG codon. Initiation of translation from an upstream non-AUG codon is growth-regulated or developmentally-regulated in some cases [32, 33]. In a few cases, the N-terminally extended protein initiated from an upstream non-AUG codon has been found to function differently from the shorter protein initiated from the first AUG codon ([34–36]; see [89], this issue). But it would be wrong to expect *a priori* that every polypeptide initiated from an alternative upstream site serves a special function. In some cases, initiation from spurious upstream sites may be an inadvertent consequence of the passage of 40S ribosomal subunits across the entire 5' non-coding region *en route* to the AUG start codon. (Because initiation at codons other than AUG occurs rarely and usually inefficiently in eukaryotes, some genetic diseases are attributable to point mutations in the AUG initiator codon [37]. On the other hand, the fact that some non-AUG codons can support at least a low level of initiation explains the moderate phenotype of some genetic diseases [38].) Initiation at non-AUG codons is best evaluated *in vivo*, since it can be artificially enhanced by choosing inappropriate reaction conditions *in vitro* [10].

The leaky scanning that occurs when the first AUG codon is in an unfavorable context provides one means of escape from the rule that eukaryotic ribosomes are limited to initiating at the first AUG codon. The ability of eukaryotic ribosomes to reinitiate (see below) provides a second escape from the first-AUG rule. These escape mechanisms mean that the scanning model does not have to be abandoned as the list of cDNA sequences with AUG-burdened leader sequences grows longer [18]. There are rules (leaky scanning, reinitiation) that allow the first-AUG rule to be broken. But breaking the rule usually is paid for by a reduction in translational efficiency, and therefore a cDNA sequence in which the presumptive 5' non-coding sequence has many upstream AUG codons should be treated sceptically. Indeed, follow-up studies have revealed that many cDNA sequences with problematical leader sequences do not correspond to functional mRNAs. Some AUG-burdened 5' non-coding sequences have been traced to artifacts during cDNA construction and cloning [1, 18]. More interestingly, some cDNA sequences have been shown to derive from mRNA precursors that still retain a 5' intron: the upstream AUG codons reside within the intron and therefore do not compromise translation of the mature mRNA [18]. Another common solution to the upstream-AUG conundrum is promoter switching; *ie* in certain tissues or under certain growth conditions, activation of a downstream promoter produces a second form of mRNA that lacks the long, AUG-burdened leader sequence and supports translation more efficiently [18, 39–41]. An important ancillary lesson

from these studies is that the rather insensitive Northern blotting assay does not always detect all the functionally important transcripts from complex genes. Even with the exhaustively studied SV40 system, for example, new mRNA species have been found recently by devising more sensitive assays [42]. The importance of not accepting Northern blots as the definitive measure of transcription is further discussed elsewhere [2] in connection with the problematical 'internal initiation' hypothesis.

Determinants of translational efficiency

The efficiency of initiation of translation (*ie* the yield of protein per unit of mRNA) is affected by five structural elements near the 5' end of the mRNA: i) the m7G cap; ii) the primary sequence or context surrounding the AUG codon; iii) the position of the AUG codon (*ie* whether or not it is 'first'); iv) secondary structure; and v) leader length. Because the interplay of these five features in controlling the translation of synthetic mRNAs was reviewed previously [43], I will summarize succinctly what we have learned.

The ability of the m7G cap to increase translational efficiency was first shown by Dr Aaron Shatkin and has been confirmed many times since. As far as we know, all cellular mRNAs and nearly all viral mRNAs are capped, and therefore this nearly universal requirement probably does not underlie common differences in translational efficiency. The second structural feature that affects the initiation of translation is the GCCACC...G sequence flanking the AUG codon; this was discussed in the preceding section.

The third feature, the position of the AUG codon, has important effects on both the fidelity and efficiency of initiation. When the first AUG codon is in a favorable context, virtually all 40S ribosomes will initiate at that site, usually to the exclusion of downstream sites. When the first AUG codon is followed shortly by an in-frame terminator codon, however, some initiation from downstream site(s) may occur ([44] and references cited therein). The usual interpretation is that, after an 80S ribosome has translated the first small ORF, the 60S ribosomal subunit dissociates while the 40S subunit remains bound to the mRNA, resumes scanning, and reinitiates at the next AUG downstream. While this mechanism gets around the limitation of the first-AUG rule, there is a cost in terms of efficiency because, in higher eukaryotes, reinitiation is nearly always inefficient. (In yeast the efficiency of reinitiation can be regulated [45], but in multicellular eukaryotes only low-level constitutive reinitiation has been documented. In all eukaryotes the ability to reinitiate appears to be limited to the 5' end of the mRNA, *ie* reinitiation is possible following the translation of a short ORF but not following the translation of a full-length cistron.) The reduction in translational efficiency imposed by an upstream ORF, and hence by the need to reinitiate, appears to be important in controlling the developmental [46] or tissue-specific [47] expression of some genes, modulating the synthesis of some proteins that might be harmful if overproduced [48], and regulating the replication [49] or pathogenicity [50, 51] of some viruses. The presence of spurious upstream ORFs might also be a means to prevent the translation of unrearranged immunoglobulin genes [52].

The fourth structural feature that we have explored using synthetic mRNAs is secondary structure. A base-paired structure near the 5' end of the mRNA can affect translation in ways that depend on the stability and position of the structure. A very stable stem-and-loop structure (ΔG –61 kcal/mol, calculated according to Tinoco *et al*'s rules [53]) inhibits translation profoundly by blocking an early step in initiation. We showed that when a hairpin of this sort was positioned 72 nucleotides from the m7G cap, a 40S ribosomal subunit was able to bind to the mRNA and apparently migrate up to, but not through, the base-paired structure [54]. In contrast with the strong inhibition imposed *in vitro* [54] and *in vivo* [55] by structures in the range of –50 to –61 kcal/mol, a –30 kcal hairpin structure inhibited translation only when it was close to (*eg* 12 nucleotides from) the 5' end of the mRNA. In that position, the base-paired structure prevented 40S ribosomal subunits from engaging the mRNA [54]. When the same –30 kcal structure was repositioned 52 nucleotides from the 5' end, however, it no longer inhibited translation [54]. A reasonable interpretation is that, as long as there is room for a 40S ribosomal subunit to bind at the 5' end of the mRNA, the subsequent migration of the 40S ribosome/factor complex (the set of initiation factors associated with 40S ribosomal subunits at this stage has not been defined exactly. As discussed elsewhere [2], the putative (limited) helicase activity of eIF-4A has not been directly implicated in scanning) can disrupt base-paired structures that occur downstream. There is a limit to this ability, however, as evidenced by the aforementioned inhibitory –61 kcal hairpin. It is striking that a –30 kcal hairpin, positioned some distance from the cap, did not impair translation even when the base-paired domain included the AUG initiator codon [54, 55]. This is profoundly different from the situation in prokaryotes where translation is virtually abolished by marginally stable (ΔG –10 kcal/mol) base-paired structures that impinge on the AUG codon. The explanation, of course, is that prokaryotic ribosomes engage the mRNA near the AUG codon while eukaryotic ribosomes enter upstream, near the m7G cap.

In contrast with the foregoing predictable inhibitory effects of upstream secondary structure, a modest amount of secondary structure (ΔG –19 kcal/mol) positioned downstream from the AUG codon was found to augment translation, apparently by suppressing the leaky scanning that would otherwise have occurred when the AUG codon was in a suboptimal context [17]. This unexpected positive effect of downstream secondary structure was strongest when the base-paired structure was positioned 14 nucleotides from the AUG codon [17]. Since RNase-protection experiments have shown that a ribosome bound at the AUG codon protects 12 to 15 nucleotides 3' of the AUG codon [56], we postulated that a hairpin positioned 12 to 15 nucleotides downstream from the AUG codon causes the scanning 40S ribosome to pause with its AUG-recognition center right over the AUG codon, thereby favoring initiation. (If this explanation for the enhancing effect of downstream secondary structure is correct, it would seem that the –30 kcal hairpin positioned shortly upstream from the AUG codon must also have slowed scanning, even though the –30 kcal hairpin did not reduce translational efficiency [54, 55]. In other words, pausing during scanning is not necessarily inhibitory, and may even be helpful, as long as the ribosome, after pausing, can eventually move on.) Although this phenomenon has been demonstrated so far only with synthetic transcripts, it is interesting that some natural mRNAs in which the AUG codon occurs in a weak primary sequence context have the potential to form a base-paired structure in an appropriate position downstream [57–61]. The ability of secondary structure to slow scanning, and thus to favor initiation, might also encourage adventitious initiation from upstream non-AUG sites, as discussed elsewhere [17, 43].

The fifth structural feature that has been shown to modulate the translation of test transcripts is leader length. Lengthening the 5' non-coding sequence beyond the 20 or so nucleotides (exactly how long the 5' non-coding sequence has to be to ensure recognition of the first AUG codon depends on whether the sequence 3' of the AUG codon is structured or unstructured [29]) required for the fidelity of initiation can dramatically increase the efficiency of translation *in vitro* [62] and, under certain conditions, *in vivo* [63]. The increased efficiency was clearly attributable to leader length, rather than to any particular sequence, inasmuch as insertion of three different synthetic oligonucleotides, each 60 nucleotides long, stimulated translation identically [62]. The only feature common to all three sequences was a paucity of G residues, which ensured against the formation of secondary structure. The ability of long leader sequences to enhance translation irrespective of the particular nucleotide sequence might be explained by the apparent binding of extra 40S ribosomal subunits to such mRNAs [62]. It seems likely, although not proven, that this 'early recruiting' of 40S subunits gives mRNAs an advantage under conditions of competition. The facilitating effect of leader length has not yet been verified widely in other laboratories, perhaps because a long 5' non-coding sequence helps only if it lacks secondary structure; most random RNAs squences do not meet that requirement.

Evaluation of natural mRNA leader sequences

The five structural features discussed above were delineated primarily by studying synthetic leader sequences, an approach that engenders clear results because it enables each feature to be studied in isolation. With natural mRNAs, in contrast, the effects of flanking nucleotides on AUG codon recognition might be underestimated if there happen to be downstream secondary structure(s) that slow scanning and thus compensate for a poor context. And the potential advantage of lengthening the 5' non-coding sequence might be missed if the long leader sequence contains inhibitory secondary structures. Notwithstanding these and other complications encountered with natural mRNA sequences, some progress has been made in understanding why certain mRNAs are translated more efficiently than others.

Because probing of various 'good' mRNA leader sequences has failed to identify any particular motif capable of enhancing translation [64–67] (in a few cases where a particular sequence has been claimed to facilitate translation [68–71], the possibility that the so-called enhancer sequence works simply by reducing secondary structure and/or increasing leader length was not rigorously evaluated), a reasonable view is that a moderately long, moderately unstructured 5' non-coding sequence may be sufficient to support efficient translation. In the case of β-globin mRNA, for example, we found that translational efficiency was not reduced by substitutions introduced in nearly every position of the 53-nucleotide leader sequence [72], suggesting that the 5' non-coding domain of this unusually good mRNA contains no special effector motifs. Consistent with the view that efficient translation requires only a moderately long leader sequence that is not extensively base-paired, probing of the α- and β-globin mRNA leader sequences and derivatives thereof revealed a perfect inverse correlation between 5' secondary-structure content and translational efficiency [72]. The long-noted [73] two-fold difference in translatability between α- and β-globin mRNAs may thus be explained. Significantly, the secondary structure that apparently restricts translation of wild type α-globin mRNA is far less stable than the –30 kcal hairpin

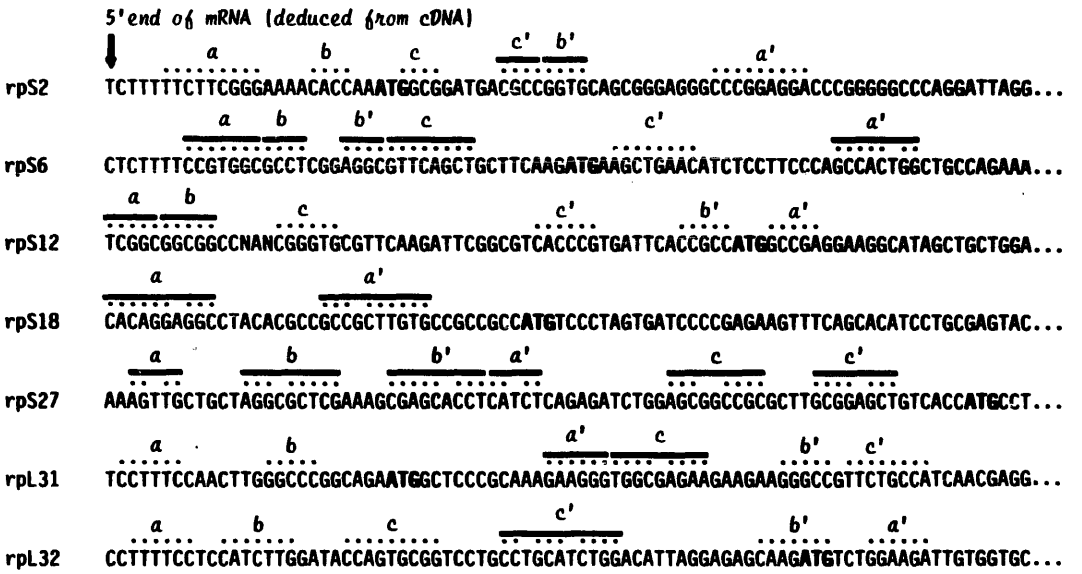

Fig 1. Potential for base-pairing near the 5' end of some ribosomal protein mRNAs. Complementary sequences that might participate in base-paired structures are labeled a and a', b and b', c and c'. Alternative structures are possible in some cases. The sequences are from [74–80]. The ATG (AUG) codon shown in boldface is the start of the protein coding domain. Participation of the coding domain in some of the proposed structures does not necessarily contradict evidence from leader-shuffling experiments that translational regulation is a function of the 5' non-coding domain [81], since secondary structure could be preserved if a GC-rich sequence in the reporter gene were to substitute for a GC-rich segment of the original ribosomal protein coding sequence.

structure discussed above in connection with synthetic mRNAs. The work with α-globin mRNA suggests that even a −10 kcal/mol base-paired structure can limit translation, under some reaction conditions, when the structure occurs very near the 5' end of the mRNA.

The poor translation of many mRNAs that encode oncoproteins, growth factors, transcription factors and other critical regulatory proteins can probably be attributed to the highly structured leader sequences on these mRNAs [18]. In some cases, for example, the G+C content of these leader sequences is 80% or greater, which implies an extraordinary potential for base pairing. But secondary structure might also limit the translation of mRNAs in which the G+C bias is less extreme. As illustrated in figure 1, for example, the 5' ends of ribosomal protein mRNAs might form secondary structures that are comparable in strength to the structure that restricts α-globin translation. With secondary structures in this modest range, inhibition of translation would not be absolute; inhibition should and does depend on the proximity of the structure to the 5' end of the mRNA [81] and on the imposition of conditions that force mRNAs to compete [82].

Notwithstanding the failure of mutational analyses to identify *cis*-acting effector motifs [64–67, 72], some investigators continue to assert vague claims that a particular mRNA leader sequence contains a specific motif that facilitates translation [68–71], and some continue to postulate special *trans*-acting factors to explain the preferential translation [71, 83, 84]. But the fact is that no mRNA-specific translational initiation factor has yet been demonstrated. It is easy enough to find proteins that bind to the 5' non-coding sequence of certain mRNAs, but binding is not indicative of function, and attempts to show stimulation of translation by such proteins have not been very convincing [85]. (Even negative regulation requires more than mere binding of a protein to the mRNA leader sequence. To inhibit translation, a potential repressor protein has to bind with very high affinity to a site near the 5' end [86, 87].) In contrast with the lack of compelling evidence for positive-acting, mRNA-specific *cis*- and *trans*-acting elements involving the 5' non-coding sequence, there is growing evidence for positive-acting elements near the 3' end of eukaryotic mRNAs. What is not clear, however, is whether these 3' non-coding elements act directly at the level of translation. While these and other special instances of translational regulation remain to be unraveled, the five recognized structural elements in 5' non-coding sequences – cap, context, position, secondary structure, and leader length – seem capable of explaining many aspects of translational regulation in higher eukaryotes.

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