Minireview

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Structural Features in Eukaryotic mRNAs That Modulate the Initiation of Translation*

Marilyn Kozak

From the Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

In higher eukaryotes, translation is modulated at the level of initiation by five aspects of mRNA structure: (i) the m7G cap; (ii) the primary sequence or context surrounding the AUG codon; (iii) the position of the AUG codon, *i.e.* whether or not it is "first"; (iv) secondary structure both upstream and downstream from the AUG codon; and (v) leader length. Here I briefly discuss how experimental manipulation of these features affects the fidelity and/or efficiency of initiation. Elsewhere (1) I discuss the extent to which natural mRNA leader sequences conform to these experimentally determined requirements for initiation.

Although my primary concern is to document the occurrence and consequences of the five structural elements in mRNAs, I will allude now and then to why each feature has the effect it does. The explanations will invariably hark back to the scanning process by which ribosomes are thought to initiate translation. In its simplest form, the scanning model (2) postulates that a 40 S ribosomal subunit, carrying Met-tRNA$_i^{Met}$ and an imperfectly defined set of initiation factors (3), enters at the 5′-end of the mRNA and migrates linearly until it reaches the first AUG codon, whereupon a 60 S ribosomal subunit joins and the first peptide bond is formed (Fig. 1). Evidence in support of the scanning model has been adduced previously (2, 4, 5). The most recent evidence includes the apparent queuing of 40 S ribosomal subunits on a long, unstructured leader sequence (6) and the demonstration that 40 S subunits stall on the 5′-side of a stable hairpin structure introduced between the cap and the AUG codon (7).

m7G Cap

That the ubiquitous m7G cap increases the efficiency of translation *in vitro* was first shown by Shatkin (8) and has been confirmed many times since. While the dependence of *in vitro* translation on the m7G cap may vary with the choice of reaction conditions, *in vivo* translation of most mRNAs is stringently cap-dependent, as shown in studies with vesicular stomatitis virus mutants that are defective in methylation (9) and with various other test systems (10, 11). Parenthetically, the experiments with vesicular stomatitis virus distinguish nicely between the ability of the cap to stabilize transcripts and its ability to stimulate translation; a guanylylated, unmethylated cap is sufficient to protect transcripts from 5′-exonucleases, but N-7 methylation is essential for efficient translation. A methylated cap and the associated cap-binding protein may be less important for the translation of mRNAs that have a rather long, unstructured leader sequence (6, 12), but such mRNAs are rare. Among animal cells and viruses, the only mRNAs that clearly are translated without benefit of a cap derive from picornaviruses.

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Context

Systematic mutagenesis of nucleotides in the vicinity of the AUG codon revealed that GCC$\begin{matrix}A\\G\end{matrix}$CCAUGG is the optimal context for initiation of translation in cultured monkey cells (13, 14). (The A of the AUG codon is designated +1, with positive and negative integers proceeding 3′ and 5′, respectively.) A purine, preferably A, in position −3 and a G in position +4 have the strongest effects, modulating translation at least 10-fold; the smaller effects of other nucleotides near the AUG codon are seen most easily in the absence of A^{-3} and G^{+4}. To be effective, the GCCACC motif must abut the AUG codon. Shifting the motif by just one nucleotide to the left or right abolishes its facilitating effect (13, 14). The strong contributions of A or G in position −3 and G in position +4, deduced initially in transfection assays with COS cells, have been confirmed in experiments with transformed plants (15, 16) and with standard *in vitro* translation systems from plants (17) and animals (17–21). One set of constructs used for the *in vitro* translation experiments was designed with two in-frame AUG codons, positioned to produce "long" and "short" versions of chloramphenicol acetyltransferase (17). The experiments carried out with those constructs revealed that a suboptimal context around the first AUG codon causes some 40 S ribosomal subunits to bypass the first AUG and initiate instead at the second AUG codon. Thus, context affects the fidelity as well as the efficiency of initiation. When the first AUG codon lies in a weak context, it is recognized inefficiently irrespective of the mRNA concentration and irrespective of the presence or absence of competing mRNAs (17), consistent with the scanning model which postulates that recognition of the AUG codon occurs *after* the competition-sensitive binding of the 40 S ribosome to the 5′-end of the mRNA. The leaky scanning that results from a suboptimal context around the first AUG codon enables some viral mRNAs to produce two proteins by initiating at the first and second AUG codons, as described elsewhere (1, 22).

The experimentally determined optimal context for initiation (13, 14) matches the consensus sequence derived from inspection of 699 vertebrate mRNA sequences (23). Except for yeasts (24), other eukaryotic organisms that have been examined show context effects similar to those described for vertebrates. Thus, ACC in positions −3 to −1 promotes translation in *Drosophila* (25), although the actual consensus sequence in flies differs slightly from mammals. Plant mRNAs have the expected purine in position −3 (in 93% of 252 mRNA sequences examined)[1] and the expected G in position +4 (in 74% of the mRNAs examined); and those conserved nucleotides augment translation in plants (15–17). A recent compilation of translational start sites in protozoa also shows a strong preference for A in position −3 (26).

Position

The scanning model predicts that ribosomes should initiate at the *first* AUG codon in a good context, a prediction that is upheld by most (perhaps 90% of) vertebrate mRNAs (23). (The number cannot be fixed more precisely because reports of cDNA sequences with upstream AUG codons often turn out to reflect errors or misinterpretations, as documented elsewhere (1, 2, 23).) The importance of position in determin-

[1] M. Kozak, unpublished compilation.

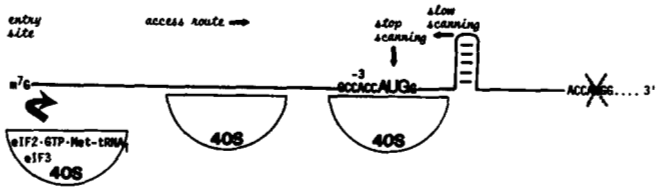

FIG. 1. **The scanning model for initiation of translation in eukaryotes.** The 40 S ribosomal subunit, carrying Met-tRNA$_i^{Met}$ and initiation factors, binds initially near the capped 5′-end of the mRNA and then migrates linearly to the first AUG codon. The contributions of context and downstream secondary structure are discussed in the text.

ing 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 (2) while a strong, upstream, in-frame AUG codon supplants the original site of initiation (2, 27). A common mistake in trying to deduce translational start sites is to screen an entire cDNA sequence for the AUG codon that best matches the consensus sequence. That approach misses the point that the scanning 40 S ribosome evaluates AUG codons sequentially; the position of an AUG codon, relative to the 5′-end of the mRNA, is as important as its context. Indeed, position is more important since *some* ribosomes may initiate at the first AUG codon even when it occurs in the weakest context; and an A in position −3, irrespective of the rest of the context,[2] is usually sufficient for *most* ribosomes to select the first AUG codon.

While efficient translation thus requires that spurious upstream AUG codons be avoided, under some circumstances an upstream AUG codon in a favorable context will reduce but not abolish initiation from downstream. This happens when the first AUG codon is followed shortly by a terminator codon, creating a small open reading frame (ORF)[3] at the 5′-end of the mRNA. The simplest explanation is that, after an 80 S ribosome translates the 5′-"mini-cistron," the 40 S ribosomal subunit remains bound to the mRNA, resumes scanning, and may *reinitiate* at another AUG codon downstream. Some rudimentary rules for reinitiation have been deduced for mammals (28) as well as yeasts (29). In eukaryotic systems, expanding the distance between the 5′- and 3′-cistrons increases the efficiency of reinitiation, in contrast with bacterial systems where overlapping of the affected cistrons often enhances reinitiation. An important caveat is that reinitiation has been shown to occur fairly efficiently in eukaryotes only when the 5′-ORF is short (28–31). Thus, there are no naturally occurring bicistronic mRNAs from yeasts or mammals that express two *full-length* proteins from nonoverlapping cistrons,[4] and artificially constructed bicistronic transcripts of that sort allow only very inefficient translation of the second cistron (32–34). There are many plant and animal virus mRNAs that are structurally bicistronic, encoding two full-length proteins in nonoverlapping ORFs, but they are functionally monocistronic, *i.e.* only the 5′-proximal ORF is translated (35). The reason why eukaryotic ribosomes can reinitiate efficiently only near the 5′-end of the mRNA, *i.e.* after translating only a short 5′-ORF, might have to do with the kinetics of release of initiation factors (28).

Secondary Structure: Positive Effects

While there have long been hints that secondary structure[5] in mRNAs can reduce the efficiency of translation, it was surprising to find that a small amount of secondary structure near the start of the coding sequence can actually enhance recognition of the preceding AUG codon. Downstream secondary structure apparently contributes to the fidelity of initiation by preventing the 40 S ribosome from scanning too fast or too far. The effect is striking in that the introduction of downstream secondary structure (ΔG, −19 kcal/mol) can completely suppress the leaky scanning that otherwise occurs when the first AUG codon lies in an unfavorable context (36). The simplest rationalization is that downstream secondary structure slows scanning, thereby providing more time for recognition of the preceding AUG codon. The maximal effect was seen when 14 nucleotides intervened between the base of the hairpin and the preceding AUG codon, and that number fits nicely with RNase protection experiments which mapped the leading edge of an initiating ribosome 12–15 nucleotides 3′ of the AUG codon. Thus, our working hypothesis is that a hairpin located 12–15 nucleotides downstream from the AUG codon causes the 40 S ribosomal subunit to stall momentarily with its AUG-recognition center right over the AUG codon, thereby facilitating initiation.

There are several situations in which this positive effect of secondary structure might be important. Although 97% of vertebrate mRNAs have the required purine in position −3, very few possess the full consensus sequence. Thus, some feature in addition to primary structure would seem to be required to explain the usual absence of leakiness, and the possibility that secondary structure near the start of the coding sequence compensates for the less-than-perfect context around the AUG codon seems an attractive solution. The contribution of downstream secondary structure might be especially important for the handful of vertebrate mRNAs that initiate translation at an AUG codon in a very weak context (*i.e.* lacking both a purine in position −3 and G in position +4 (23)) or at a non-AUG codon. The experimental imposition of appropriately positioned secondary structure indeed increases initiation from cryptic non-AUG codons in test cases (36), and nearly all of the mRNAs that naturally support initiation from upstream non-AUG codons have extraordinarily GC-rich (hence highly structured) leader sequences (Fig. 2).

Secondary Structure: Negative Effects

In contrast with the positive effects of secondary structure introduced downstream from the AUG codon, stem-and-loop structures introduced between the cap and the AUG codon never facilitate initiation. Whether or not secondary structure upstream from the AUG codon impairs translation depends on the strength and position of the hairpin. A summary of the rules follows. (i) A modest amount of secondary structure near the cap (*i.e.* within the first 12 nucleotides) can drastically inhibit translation (7). Secondary structure in this position has been shown to prevent mRNA from binding to 40 S ribosomes (7), as expected if the 5′-end of the mRNA is the entry site for ribosomes (Fig. 1). The much discussed inhibition of ferretin mRNA translation by the IRE-binding protein (48, 49) might be attributable to stabilization by the repressor protein of 5′ secondary structure and consequent inhibition of ribosome entry. (ii) When secondary structure occurs sufficiently far from the cap that the initial binding of 40 S ribosomal subunits is not impaired, the stability of the hairpin determines whether or not it inhibits scanning. Stem-and-loop structures with a free energy of −30 kcal/mol positioned 50 or 60 nucleotides from the cap did not impair translation in COS cells (50) or in cell-free extracts (7). This seems remarkable inasmuch as base-paired structures as slight as −12 kcal/mol can drastically impair initiation in prokaryotic systems (51). It is clear that 40 S ribosomal subunits get past a −30 kcal/mol hairpin by migrating through it, rather than jumping over it, inasmuch as AUG codons that were buried

[2] This lack of dependence on the complete consensus sequence may be explained by the compensatory effect of downstream secondary structure, as discussed in the next section.
[3] The abbreviation used is: ORF, open reading frame.
[4] A few reported exceptions to this rule, discussed in Ref. 1, are not compelling.
[5] The discussion of secondary structure here and in the next section is limited to cases in which stem-and-loop structures were proven to exist by biochemical or genetic means.

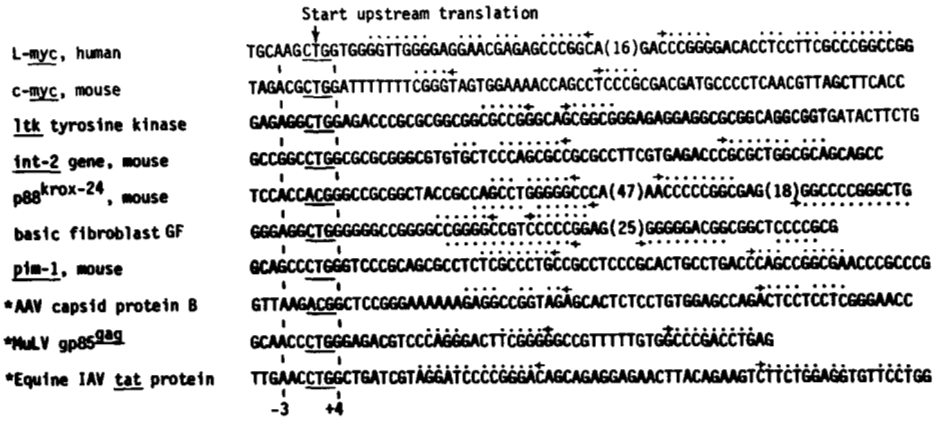

FIG. 2. **Sequences of eukaryotic mRNAs that initiate translation at upstream non-AUG codons, usually in addition to initiating at the first AUG codon.** The resulting N-terminally extended polypeptides produced by the three viral mRNAs (marked with *asterisks*) serve unique functions. In contrast, most cellular proteins initiated from upstream non-AUG codons have not been shown to mediate unique, essential functions; and their synthesis might be the inadvertent result of the way eukaryotic ribosomes reach the AUG codon (see text). *Dots* placed above each sequence (and below, when there is an alternative base-pairing scheme) indicate nucleotides that might anneal to form stem-and-loop structures; the overall GC-richness of most of these leader sequences makes many alternative pairings possible, however. In addition to the postulated contribution of downstream secondary structure, initiation at non-AUG codons requires a favorable primary sequence, especially in positions −3 and +4, as noted in the figure. Abbreviations: *GF,* growth factor; *AAV,* adeno-associated virus; *MuLV,* murine leukemia virus; *IAV,* infectious anemia (lenti)virus. The sequences are from Refs. 37–46. Not shown is the transcription enhancer factor TEF-1, which appears to initiate translation exclusively at an AUU codon by a mechanism that may be unique (47). For *ltk*, the upstream CUG initiator codon resides in an intron; AUG is the unique initiator codon in the mature mRNA (71).

in some of the hairpin structures became accessible to ribosomes (50). This raises the question of how such structures get melted. The possibility that certain initiation factor(s) may have helicase activity has been raised (52) and is evaluated elsewhere (4). The short answer is that it isn't known whether individual initiation factors or the 40 S ribosome-plus-factor complex actively unwinds secondary structures or whether the 40 S complex simply waits for such structures to breathe and then advances. Regardless of the mechanism, the ability of 40 S initiation complexes to penetrate base-paired structures has limits, as discussed next. (iii) Translation is profoundly inhibited *in vivo* (50) and *in vitro* (7) upon inserting into the 5′-noncoding domain a stem-and-loop structure with a free energy of −50 or −60 kcal/mol. Translation is inhibited even when the hairpin impinges on neither the cap nor the AUG codon. A very stable base-paired structure apparently inhibits translation by blocking the migration of 40 S ribosomes, as evidenced by RNase protection experiments which showed a 40 S ribosome trapped on the 5′-side of the hairpin (7). In contrast with the inability of 40 S ribosomal subunits to unwind a stem-and-loop structure of −60 kcal/mol, 80 S elongating ribosomes can, to some extent, penetrate such structures (7).

(iv) Some effects of secondary structure on the initiation of translation might be regulable, but this idea is much more speculative than the preceding points. It may be pertinent that a −30 kcal/mol hairpin (which normally does not inhibit translation, as explained above) becomes inhibitory when cells in culture are subjected to hypertonic stress (53). A somewhat related issue is whether the inhibitory effects of secondary structure are more pronounced in some cell types than in others. That question was raised by Muller and Witte (54) but not really answered, inasmuch as each time they switched cell types they also switched vectors. (If the vector-derived portion of the 5′-noncoding sequence is unstructured to begin with, as in baculovirus and riboprobe vectors, the introduction of a structure-prone leader sequence might be expected to inhibit translation more profoundly than if a bit more secondary structure is added to an already structured leader!) The recent report (55) that structure-prone mRNAs are translated more readily after fertilization of *Xenopus* eggs could indicate some novel, developmentally regulated helicase activity, although it is possible that covalent modification of hairpin-containing mRNAs by a previously recognized unwinding activity (56) is responsible for the enhanced translation.

Leader Length

Recognition of the first AUG codon may be impaired when it is positioned too close to the cap (57–59). When this issue was explored systematically, using synthetic transcripts in which the first AUG codon was in a favorable context, about half of the ribosomes bypassed the first AUG codon and initiated instead at a downstream site when the first AUG occurred within 12 nucleotides of the cap (60). The leakiness was suppressed when the leader sequence was lengthened to 20 nucleotides or when a modest amount of secondary structure was introduced downstream from the first AUG codon (60). In natural mRNAs the amount of secondary structure near the beginning of the coding region varies, making it hard to say *a priori* whether a particular short leader sequence will pose a problem.

Further lengthening of the 5′-noncoding sequence beyond the 20 or so nucleotides required for the fidelity of initiation can dramatically increase the efficiency of translation *in vitro* (6). 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 (6). The only feature common to all three sequences was a paucity of G residues, which ensured against the formation of secondary structure. The efficiency of translation *in vitro* was proportional to leader length in the range of 17 to about 80 nucleotides (6). Augmentation of translation by long, synthetic leader sequences mimics the effects of certain natural leader sequences (61–65). The fact that the precise sequence of these translational "enhancers" is not critical (6, 61–64) makes it unlikely that their facilitating effect on translation is mediated by proteins that recognize particular sequence motifs. Rather, the observed loading of extra 40 S ribosomal subunits on long leader sequences (6) seems likely to underlie the improvement in translation. In keeping with that interpretation, a long unstructured leader sequence augments translation only when it is at the exact 5′-end of the transcript (6, 53, 63). The introduction of a moderately long, unstructured, synthetic leader sequence turns out to be an easy way to increase the efficiency of *in vitro* expression vectors (6).

Closing Notes

The trick to identifying elements within 5′-noncoding sequences that modulate translation is to isolate each feature. There are many ways that the effects of context, upstream AUG codons, etc., might not be seen. Mutations that change the primary sequence around the initiator codon may have little effect if the sequence 3′ of the AUG codon is structured, inasmuch as downstream secondary structure compensates for absence of the preferred context (36). Since features in addition to context modulate initiation, as described herein, it follows that virtually nothing can be learned from comparing two completely different leader sequences in which context is only one of many variables (66). When matched mRNAs are compared,[6] converting a good context to a poorer one usually reduces the translational yield, but converting a poor context to a better one may not increase the yield of protein if some other step (such as elongation (67) or protein processing (68)) is limiting. Biological assays, albeit very sensitive, are so many steps removed from translation that failure to see the expected effects of context are difficult to interpret (69). In cases where less mRNA accumulates in cells under conditions (such as the imposition of a poor context) that impair translation, the gesture of "correcting" protein yields for differences in mRNA levels may ablate the perceived effects of context on translation (25).[7] Thus, the best systems for testing effects of context are those in which differences in translatability do not affect mRNA stability. Context effects and inhibition by upstream AUG codons may also be missed if inappropriate reaction conditions are used for *in vitro* translation. While the conditions recommended by some commercial suppliers of reticulocyte lysates give excellent overall incorporation of amino acids, in some cases they do not support the proper selection of initiator codons (70). With mRNAs that are translated poorly due to extensive secondary structure near the 5′-end, manipulating upstream AUG codons may be expected to have little impact because the effects of secondary structure dominate. Finally, the possibility of reinitiation complicates predictions about the effects of removing upstream AUG codons, since certain ORFs (those that favor reinitiation) can actually facilitate translation by blocking access to other upstream ORFs that are less conducive to reinitiation (28, 29).

The extent to which natural mRNA leader sequences conform to these experimentally determined requirements for initiation is examined elsewhere (1). A surprising realization is that, although nearly all vertebrate mRNAs have features that ensure the fidelity of initiation, many mRNAs that encode critical regulatory proteins do not appear to be designed for efficient translation. Thus, throttling at the level of translation may be an important component of gene regulation in vertebrates.

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[6] Indeed, interpretation can be complicated even when context is the only variable. Because the consensus sequence for initiation is GC-rich, mutations that improve the primary structure may inadvertently increase secondary structure to a point that becomes inhibitory. Some evidence of this was seen in Ref. 17, but the facilitating effect of context could nevertheless be discerned in those experiments by monitoring initiation from the first *versus* the second AUG codon.

[7] The problem is that a defect in translation sometimes accelerates mRNA degradation, in which case expressing the yield of protein as a function of the (lowered) steady-state level of mRNA makes the efficiency of translation appear greater than it really is.