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A Consideration of Alternative Models for the Initiation of Translation in Eukaryotes

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KEY WORDS: Although recent biochemical and genetic investigations have produced some insights into the mechanism of initiation of translation in eukaryotic cells, two aspects of the initiation process remain controversial. One unsettled issue concerns a variety of functions that have been proposed for mRNA binding proteins, including some initiation factors. The need to distinguish between specific and nonspecific binding of proteins to mRNA is discussed herein. The possibility that certain initiation factors might act as RNA helicases is evaluated along with other ideas about the functions of mRNA- and ATP-binding factors. A second controversial issue concerns the universality of the scanning mechanism for initiation of translation. According to the conventional scanning model, the initial contact between eukaryotic ribosomes and mRNA occurs exclusively at the 5′ terminus of the message, which is usually capped. The existence of uncapped mRNAs among a few plant and animal viruses has prompted a vigorous search for other modes of initiation. An "internal initiation" mechanism, first proposed for picornaviruses, has received considerable attention. Although a large body of evidence has been adduced in support of such a mechanism, many of the experiments appear flawed or inconclusive. Some suggestions are given for improving experiments designed to test the internal initiation hypothesis.

KEY WORDS: protein synthesis, scanning model, internal initiation (of translation), picornaviruses, mRNA binding proteins.

I. INTRODUCTION

In recent years much has been learned about mRNA sequences that govern basal[16,73,78] and regulated[1,48,60] translation in eukaryotic cells. Some understanding of the functions of transacting protein factors is also beginning to emerge.[17,25a,28,42,60,121,123] Rather than recapitulating yet again what most workers in the field of translation have already agreed on,[42,83,94] this review focuses on two aspects of the initiation process that remain controversial. One issue concerns the possible functions of proteins, including some initiation factors, that bind to mRNA. The other issue concerns possible alternatives to the conventional scanning model for selection of the AUG initiator codon. These issues remain problematical as a result of inadequate data, notwithstanding the burgeoning literature on both subjects. Exposition of some repeated flaws in the design and interpretation of past experiments might improve future investigations on these difficult topics.

By way of orientation, the discussion begins with the scanning model, for which there is considerable evidence, although molecular details of the process remain obscure.

II. MODELS FOR THE TRANSLATION OF CAPPED mRNAs

A. Evidence for a Scanning Mechanism

The basic scanning model[71] postulates that a 40S ribosomal subunit, carrying Met-tRNA$_i^{met}$ and an imperfectly defined set of initiation factors,[42] enters at the capped 5′ end of the mRNA and

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migrates linearly until it reaches the first AUG codon, whereupon a 60S subunit joins and the first peptide bond is formed. The model is supported by the following evidence.

1. 40S ribosomal subunits can migrate, as shown by the formation of polysome-like complexes when mRNAs are incubated with ribosomes in the presence of the antibiotic edeine.[19,44,80,91] The complexes were shown to consist of multiple 40S ribosomes bound throughout the length of each (monocistronic) mRNA under circumstances which suggested that 40S subunits could enter only at the 5′ end of the mRNA.[80] (Here and in the remainder of this section, references to "migrating 40S subunits" are not meant to exclude the possible involvement of initiation factors. The sucrose gradient assays that were employed were adequate to reveal the presence of 40S ribosomal subunits in complexes with mRNA but were not adequate to reveal which initiation factors might also have been present.)
2. 40S ribosomal subunits are trapped upstream from the AUG codon when ATP is depleted[63] or when a stable hairpin structure is introduced between the cap and the AUG codon.[72] The presence of 40S subunits upstream from the initiator codon is clearly predicted by the scanning model but not by alternative models that would have ribosomes enter directly at the AUG start site.
3. Some long, unstructured 5′ leader sequences appear to load up with "extra" 40S ribosomal subunits.[75] Again, this observation supports a scanning mechanism and contradicts alternative "direct entry" models.
4. Circularization of a RNA template prevents its engagement by eukaryotic ribosomes.[61,62] The AUG start site appeared to be equally accessible in linear and circular forms of those transcripts, as evidenced by the ability of prokaryotic ribosomes to bind both forms. (Indeed, some of the circular RNA molecules were big enough to accommodate two 70S ribosomes from *E. coli*.[62]) Thus, the failure of these circular templates to form initiation complexes with wheat germ or rabbit reticulocyte ribosomes implies that an AUG codon and flanking sequences are not sufficient for eukaryotic ribosomes to bind. Eukaryotic ribosomes apparently require a free 5′ end, even when the end is not capped, and the simplest explanation is that the 5′ end of the mRNA constitutes the ribosome entry site.
5. Proximity to the 5′ end generally determines which AUG functions as the initiator codon in eukaryotic mRNAs. For example, when the initiation site from preproinsulin mRNA was reiterated four times near the 5′ end of a chimeric transcript which was then translated *in vivo*, ribosomes initiated exclusively at the first site in the tandem array.[64] Inasmuch as the local context was identical around all four potential start sites, the first AUG codon was unique only by virtue of its position; and sensitivity to the position of an AUG codon is a key prediction of a scanning mechanism. (This experiment was later repeated, under more sensitive conditions, with identical results.[74] One might argue that ribosomes actually can initiate translation from all four potential start sites in the transcript but that 80S (elongating) ribosomes advancing from the first AUG codon impede access to the downstream initiation sites. That possibility seems remote in view of the *complete* absence of initiation from the downstream AUG codons, but it has not been rigorously excluded by assaying initiation complexes under conditions that preclude elongation.) Among natural mRNAs from vertebrate sources, initiation is usually limited to the first AUG codon,[68] except when the leader sequence is extremely short[76] or when the context around the first AUG codon is unfavorable for initiation.[65] In such cases, "leaky scanning" allows ribosomes to initiate from the first and second AUG codons.[77] The phenomenon of reinitiation (see Section IV.A), which also gives ribosomes access to downstream AUG codons, appears to involve a resumption of scanning and not direct internal initiation.[1,34,69] Ad-

ditional evidence relating to position effects on the selection of AUG codons is described elsewhere.[71]

6. When the anticodon in one of the $tRNA_i^{met}$ genes from yeast was changed from 3′-UAC-5′ to 3′-UCC-5′, the mutated form of $tRNA_i^{met}$ directed ribosomes to initiate at the first AGG instead of the usual AUG codon.[15] That clever experiment indicates that the basic scanning process is conserved between unicellular and multicellular eukaryotes, although some details of the initiation mechanism may differ.[16]

B. Possible Variations on the Scanning Mechanism

In one alternative to the above-described scanning model, the role of migrating across the 5′ end of the mRNA is attributed to eukaryotic initiation factors (eIFs) -4A and -4F rather than to 40S ribosomal subunits. This new model postulates that ribosomes enter "at a site downstream of the cap that could include the AUG initiator" codon — a site exposed by eIF-4A/F-mediated unwinding of the 5′ end of the mRNA.[113] The extreme versions of the new model seem untenable. Thus, the suggestion that the AUG codon itself might be the entry site ignores the fact that 40S ribosomal subunits can be detected under a variety of circumstances upstream from the AUG codon.[63,72,75] Because eukaryotic ribosomes can initiate with some efficiency at an AUG codon as close as three nucleotides to the cap,[76] and because there are many mRNAs (reviewed in Reference 76) in which an AUG codon positioned 7 to 13 nucleotides from the cap serves uniquely as the initiation site, there is little room for an *obligatory* ribosome entry site between the cap and the AUG codon. That is, at least with some mRNAs, 40S ribosomes must enter directly at the 5′ end. The model thus acquires an arbitrary cast: with mRNAs in which the AUG codon is some distance down, ribosomes may enter at an exposed region somewhere downstream from the cap but before the AUG codon. The challenge is to explain how eIF-4A/F know where to start and stop unwinding. Because the AUG codon closest to the 5′ end of the mRNA almost always initiates translation, unwinding must start at the 5′ end of the mRNA. Unwinding would have to stop when eIF-4A/F reached the first AUG codon because, again, the first AUG is usually the unique site of initiation. There is no evidence, however, that eIF-4A/F (with or without eIF-4B) can recognize an AUG codon — and much evidence that the initiator codon is recognized by the 40S ribosome/Met-tRNA complex.[15] Thus, one is forced to postulate that *a 40S ribosomal subunit scans in such close proximity to the putative helicase that they are effectively a unit.* And in that form, which seems to be the only tenable form, the new model becomes indistinguishable from the original scanning model. The underlying idea that an unstructured stretch of mRNA might allow ribosomes to enter internally, rather than scanning from the 5′ end, has actually been tested by inserting into the interior of a mRNA a sequence predicted to be free of secondary structure; transcripts thus modified showed no evidence of internal initiation.[27,75]

If the original scanning model[71] proves to be correct, a key question still to be answered is whether the 40S ribosomal subunit engages mRNA by chemical contact or by encirclement. The latter "ring-on-a-string" version of the model makes several testable predictions: (1) Scanning should be strictly linear. The observed inability of 40S ribosomes to jump across the base of a hairpin structure[66] indeed conforms to that prediction. Although occasional claims of discontinuous scanning can be found in the literature,[20,33] the rather limited data in those reports can be explained in other ways.[71] (2) The encirclement model requires that the entry of 40S ribosomal subunits, like the threading of a needle, should be strictly limited to the 5′ end of the mRNA — a prediction that would be contradicted if claims of internal initiation proved correct. Those claims and the supporting evidence are discussed in Section IV. (3) The encirclement model predicts that 40S ribosomal subunits should be released only at the 3′ end of the mRNA, rather than when the 80S elongating ribosome reaches a terminator codon. Experiments addressing that point have not yet been carried out with eukaryotic translation systems. In a more flexible version of the ring-on-a-string model, the 40S ribosome would only partly encircle the

mRNA chain; binding of initiation factors to the 40S ribosomal subunit would close the ring. This second version of the model would not flatly preclude internal initiation. Moreover, the model predicts that 40S ribosomal subunits could be released from the mRNA when 80S elongating ribosomes reached a terminator codon, provided that the initiation factors that close the ring had previously dissociated. That idea has significance vis-à-vis the phenomenon of reinitiation.[1,69]

By analogy with what is known about the processivity of some DNA polymerases,[114] a ring-on-a-string mechanism should allow ribosomes to slide bidirectionally and (probably) without a direct requirement for ATP hydrolysis. The limited evidence on those points is inconclusive. Although the overall direction of migration, dictated by the obligatory 5′ entry site and the AUG "trap" downstream, is evidently 5′ to 3′,[63] locally the 40S ribosome might flutter back and forth before advancing. The only hint of fluttering is the unexpected ability of eukaryotic ribosomes to initiate at two AUG codons when the first AUG codon (in a favorable context) lies very close to the second.[120] As for whether the migration of 40S ribosomal subunits requires ATP hydrolysis, as previously suggested,[63] upon reflection those experiments seem compatible with at least three explanations: (1) ATP hydrolysis might be needed for 40S ribosome migration per se; (2) ATP hydrolysis might be used to unwind downstream regions of the mRNA, which in turn would allow 40S ribosomes to slip forward (the helicase activity implied here is discussed again in Section III.A); or (3) ATP hydrolysis might alter the initiation complex — e.g., by promoting the addition, release, or rearrangement of factors — in a way that would augment the processivity of 40S ribosomal subunits. These ideas are elaborated in Figure 1. Some insightful studies of how ATP hydrolysis enhances the processivity of DNA polymerases raise additional ideas,[54,114] although the behavior of polymerases on a smooth DNA track differs in important ways from the behavior of ribosomes on mRNA. Given so little hard data about the initiation of translation, the point here is not to argue for any particular version of the scanning model but to caution against premature acceptance of any one version.

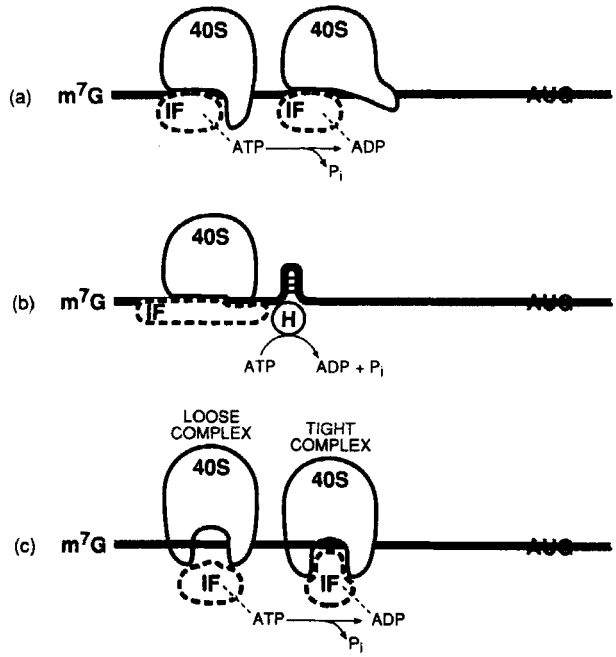

FIGURE 1. Hypothetical mechanisms whereby the advance of 40S ribosomal subunits, as postulated by the scanning model, might depend on ATP-hydrolysis. The 40S subunit is shown associated with initiation factors (IF). The protein factors are not specified by name because, apart from eIF-2 and Met-tRNA$_i^{met}$, it is not known with certainty when each factor enters and leaves the initiation complex. In scheme (a), the hydrolysis of ATP is postulated to be directly required for migration of the 40S ribosome/factor complex in the 5′-to-3′ direction. The complex might advance in inchworm fashion by switching between two conformations. By virtue of binding both to the 40S ribosomal subunit and (nonspecifically) to mRNA, initiation factors might stabilize the complex and thus enhance its processivity. In contrast with the ATP-driven, unidirectional migration depicted in a, schemes b and c show the initiation complex advancing passively. Scheme b uses ATP hydrolysis only to unwind the mRNA in advance of the 40S ribosome. The responsible helicase (H) could be a soluble protein, as shown, or it could be associated with and lead the 40S ribosomal subunit. If helicase activity is essential for the 40S subunit to reach the AUG codon, and if the unwinding activity resides in one of the recognized initiation factors, a likely component of the helicase is eIF-4A, the only initiation factor known to bind ATP (see text). The need for a conventional, ATP-driven helicase to unwind the 5′ end of the mRNA is plausible but not proven. An alternative possibility is that the processivity of the advancing 40S ribosome/factor complex is sufficient to disrupt base-paired structures in the 5′-noncoding domain, much as 80S elongating ribosomes are able to penetrate secondary structures in the coding domain. Scheme c differs from the foregoing models by postulating that the 40S ribosome/factor complex engages the mRNA by encirclement rather than by chemical contact. The hypothesis is that cycles of binding and hydrolysis of ATP serve to enlarge and then shrink the size of the hole in the "donut". Whereas enlarging the hole permits the ribosome/factor complex to slip forward, reducing the size of the hole is necessary for the initiation complex to sample the mRNA sequence, and thus to identify the AUG codon.

III. FUNCTIONS OF eIF-4A, CAP-BINDING PROTEIN, AND OTHER mRNA BINDING PROTEINS

Some settled aspects of how eukaryotic initiation factors function have been described in detail elsewhere.[42] eIF-2, the factor that escorts Met-$tRNA_i^{met}$ onto the ribosome, is probably the best understood initiation factor and arguably the most important vis-à-vis regulation of translation.[100] eIF-3 is probably the least well understood, in part because its complex structure[89] makes it difficult to purify. This section focuses on the eIF-4 group of factors, which somehow mediate the binding of 40S ribosomes to mRNA. Despite countless studies carried out with these factors, their mechanisms of action remain obscure. The discussion begins with eIF-4A, for which the most specific claims have been made.

A. eIF-4A and the RNA Helicase Hypothesis

Although arguments outlined in the preceding section seem to rule out a model[113] in which initiation factor-mediated unwinding of the mRNA, independently of ribosomes, actually specifies the AUG start site for translation, the possibility that initiation factors have some ability to unwind mRNA remains tenable. The problem is that this attractive idea, which is supported by a little evidence, has been too quickly promulgated as fact. In the most direct test of the hypothesis, the bidirectional helicase activity observed with eIF-4A (in the presence of eIF-4B) required a 200-fold molar excess of protein to mRNA.[102] This contrasts with a nucleolar p68 RNA helicase for which activity was demonstrated with a 1:1 ratio of protein to substrate.[45] (The absolute concentration of mRNA in the latter case was very similar to that used in the eIF-4A experiments.) A possible explanation for the discrepancy is that the unwinding activity attributed to eIF-4A, which has not yet been independently verified, actually resides in some contaminating protein. A more interesting possibility is that eIF-4A does have unwinding activity but the activity is normally ribosome dependent, much as is the ability of G proteins to hydrolyze GTP[90,107] or the ability of SRP to bind nascent signal peptides.[8] That could explain the need for a large amount of eIF-4A when the assay is conducted without ribosomes, and it would explain how the unwinding activity is targeted to where it is needed rather than to random sites in the mRNA. But it would contradict the view — already incorporated into several textbooks — that unwinding by eIF-4A prepares the mRNA for subsequent binding of 40S ribosomes.

It has been clearly established that eIF-4A is essential for translation[11,104] and that eIF-4A is an ATP-binding protein,[103,105] but what the bound ATP does is not yet known. It has been claimed that eIF-4F (of which eIF-4A is a subunit) in combination with eIF-4B forms a complex with mRNA which dissociates in the presence of ATP concomitantly with unwinding of the RNA.[53] But that experiment — i.e., forming a complex without ATP and *showing that it dissociates upon ATP addition* — does not seem to have actually been done.[53] The significance of the observed binding of eIF-4F/4B to duplex mRNA[53] might be questioned inasmuch as no such complexes were seen when eIF-4A was used in place of eIF-4F. Because the RNA substrate in those experiments was not capped, eIF-4F should have had no advantage over eIF-4A. A previous report from the same laboratory asserted that eIF-4A/4B and eIF-4F/4B are equally effective in unwinding base-paired structures in uncapped transcripts.[102] The obvious inconsistencies between the unwinding assay and the demonstration of protein-RNA complexes by gel-shift analysis suggest that one or both assays might have been misinterpreted.

If eIF-4A and conjoined proteins indeed turn out to have helicase activity, experiments will have to be devised to determine when and where the helicase acts. Sonenberg[113] has proposed that eIF-4A mediates unwinding of the entire sequence between the cap and the AUG codon, but no evidence favors that interpretation over one in which ATP-driven unwinding is needed only to expose the cap-proximal sequence, thus enabling a 40S ribosomal subunit to engage the mRNA. The additional ATP that appears to be required for the 40S ribosomal subunit to move beyond the 5′ entry site[63,115] might have nothing to do with helicases. Indeed, the fact that deple-

tion of the ATP pools in wheat germ or reticulocyte extracts prevented the migration of 40S subunits even with transcripts in which guanosine was replaced by inosine[63,115] appears to contradict the idea that ATP is used solely to power a helicase. (The possibility that inosine-substituted mRNA still has enough secondary structure to require a helicase cannot be dismissed outright, but it seems unlikely.) Consistent with these hints that the ATP requirement for initiation involves something more than unwinding, there is evidence from a yeast system that the requirement for eIF-4A is not abolished by eliminating most of the secondary structure from the 5′ end of the mRNA.[4]

B. Cap-Binding Protein(s)

By definition, the 24-kDa cap-binding protein (CBP, p24, eIF-4E) binds the 5′ m7G cap.[108,112] But exactly when that interaction takes place and how it facilitates initiation are debatable. A popular model reproduced in several textbooks shows CBP binding in solution to the 5′ end of the mRNA, followed by other initiation factors and eventually by the 40S ribosomal subunit.[112] However, the indisputable fact that free CBP *can* bind to mRNA in the absence of 40S subunits does not prove that the initial contact with mRNA is made by free CBP when 40S subunits are also present. No evidence rules out an alternative mechanism in which the initial contact with mRNA is made by a 40S ribosomal subunit, after which CBP *carried by the 40S ribosome* grabs the cap and thus prevents the mRNA from slipping off. The fact that translation is less dependent on the m7G cap when the 5′-noncoding sequence is moderately long and unstructured[4,27,35,75,111] could then be explained by postulating that interaction with CBP is especially important for stabilizing mRNA-ribosome complexes when constraints near the 5′ end of the transcript prevent the 40S subunit from advancing as soon as it touches. The question of whether CBP indeed binds to 40S ribosomal subunits in advance of mRNA has been answered both ways.[43,44] The association of CBP with ribosomes might be stabilized when mRNA binds, much as the binding of SRP to ribosomes is stabilized by contact with the signal peptide,[8] and thus a gentler assay than sucrose gradient sedimentation[43,44] might be required to detect unambiguously an association of CBP that precedes mRNA binding. The point is simply that credible alternatives to the standard model for CBP function can be envisioned.

There have long been hints that p24-CBP may function not as an isolated subunit (eIF-4E) but as part of a complex (eIF-4F) that consists of eIF-4E, eIF-4A, and a 220-kDa polypeptide (p220).[112] For example, in a recent study with translation-deficient extracts derived from HeLa cells that expressed antisense RNA against eIF-4E mRNA, activity was restored only upon addition of eIF-4F.[23] The novel 220-kDa component of eIF-4F is postulated to be a major player in some schemes for translational regulation,[112] but there is really little hard data about p220. The provenance of a monoclonal antibody that is often used to quantitate p220 is questionable.[31] The belief that cleavage of p220 is the primary mechanism whereby host protein synthesis is shut off in poliovirus-infected cells[112] has been questioned on a variety of grounds.[67,79] Thus, the functions of the putative 220-kDa subunit of CBP complexes remain enigmatic.

An extraordinary variety of proteins appears to bind near the cap, as evidenced by the transfer of radioactivity (''cross-linking'') during UV irradiation. Besides the paradigmatic 24-kDa eIF-4E discussed above, the list includes eIF-4B,[88] eIF-2,[118] a 68-kDa subunit of eIF-3,[118] and at least seven proteins recovered from messenger ribonucleoprotein particles (mRNPs).[38] eIF-4A can also be cross-linked to the cap when the reaction is accomplished chemically rather than by UV irradiation.[29] Inasmuch as the cap is a small structure and there is no evidence for sequential interactions, the list of cap-reactive proteins seems much too long! Fluorescence studies have confirmed that the 24-kDa protein, alone or as a subunit of eIF-4F, interacts directly with the cap, but those studies produced no evidence that eIF-4A or -4B binds the cap directly.[37] The ability of eIF-2 to stimulate cap recognition by eIF-4B and -4F does not justify the claim that eIF-2 itself is a specific cap-binding protein.[118] Rather, the

documented ability of eIF-2 to bind nonspecifically to internal sites in mRNA[118] might reduce spurious binding by eIF-4B and -4F and thus increase the effective concentrations of the latter proteins for interaction with the cap. The cross-linking of numerous mRNP proteins to the cap might also be dismissed as nonspecific. If one focuses on the four major cap-reactive proteins (p65, p52, p15 and p24), one notices that only the first three are detectable as structural components of RNPs.[38,39] The 24-kDa band has never been detected as even a minor component of mRNPs, meaning that it is a *very* minor component. The implication might be that p24 is the only genuine CBP in RNPs: thus, p24 gives a strong band in the cross-linking assay despite its presence in mRNPs in minuscule amounts, while p65, p52, and p15 must react very weakly with the cap because, despite their great abundance in RNPs, their signal in the cross-linking assay is only equal to that of p24.

C. Other mRNA-Binding Proteins

It is important to remember that the mere binding of proteins to mRNAs reveals little if anything about function. Apart from p24-CBP, no initiation factor has been shown to recognize specific sites in 5′-noncoding sequences in a way that appears functionally significant for translation. The strongest case might be construed for eIF-2, as some mutations in that factor allow yeast ribosomes to initiate translation at a UUG codon which cannot otherwise be used.[17,28] But, as the authors recognize, that intriguing result does not necessarily imply specific recognition of UUG by eIF-2. If mutations in eIF-2 increase its nonspecific affinity for mRNA, that could compensate for the weakened codon/anticodon interaction and thus allow initiation at the nonstandard UUG codon. (The effects of the eIF-2 mutations on initiation would thus resemble the effects of streptomycin on polypeptide elongation in bacteria, in the sense of allowing nonspecific binding to dominate over specific codon-anticodon recognition, thereby lowering accuracy.) A recent claim that eIF-2 can influence selection of the first vs. the second AUG codon in reticulocyte lysates is suspect inasmuch as the maximum effect, demonstrable only at a high concentration (144 μg/ml) of the factor, was only twofold.[22] No effect of eIF-2 on AUG selection in reticulocyte lysates was seen by other investigators.[116] The (proven) nonspecific binding of translation factors to mRNA should not be viewed as less interesting than (hypothetical) binding to specific sites, inasmuch as the general affinity of initiation factors for RNA could play a crucial role in stabilizing the ribosome/mRNA complex. In turn, the function of the ubiquitous mRNP proteins[39] might be simply to saturate nonspecific binding sites on mRNA so that translation factors would not be diverted.

If caution is required in interpreting the functional significance of initiation factors that bind to mRNA, then caution-plus is required in ascribing significance to other RNA-binding proteins. The mere binding of various host proteins to picornavirus leader sequences[24,52,87] does not implicate those proteins in translation, inasmuch as the 5′ end of picornavirus mRNA also mediates RNA replication and possibly packaging. In the case of a protein designated p52,[87] deleting the site in poliovirus mRNA to which the protein binds slowed growth but did not reduce the ultimate yield of virus,[47] which argues against an essential involvement of p52 in viral translation. These ideas are taken up again in the following sections.

IV. MODELS FOR THE TRANSLATION OF UNCAPPED VIRAL mRNAs

A. Internal Initiation vs. Reinitiation with Picornaviruses

The fact that picornavirus leader sequences are unusually long and uncapped and contain numerous upstream AUG codons has prompted novel ideas about how they might be translated. For poliovirus (PV) and encephalomyocarditis virus (EMCV), the general hypothesis that has emerged is that a large (incompletely defined) segment of the 5′-noncoding sequence directs ribosomes to bind to an internal AUG codon without having traversed the entire 5′ end of the mRNA. A thoughtful summary of the evidence for internal initiation in picornaviruses has been

compiled by Jackson and colleagues.[49] However, their summary overlooks some alternative interpretations and underemphasizes some uncertainties in the data.

For example, recent experiments showed clearly that ribosomes initiate at the 11th and not the 10th AUG codon in the EMCV leader sequence.[55] That result has been said to rule out any possibility that ribosomes reach AUG-11 by scanning the entire 5′-noncoding sequence.[49,55,113] But *if* one were to postulate that ribosomes scan the EMCV leader sequence, it would have to be a scanning/reinitiation mechanism, in view of the ten upstream open reading frames (ORFs). And one thing we know about reinitiation in eukaryotes is that ribosomes do not reinitiate efficiently at every internal AUG codon.[69] The well-studied yeast *GCN4* message, which has four upstream ORFs and is almost certainly translated by a linear scanning-and-reinitiation mechanism,[1] illustrates the point: compelling genetic evidence suggests that *AUG-4* is skipped by ribosomes that (re)initiate translation of the *GCN4* protein from AUG-5.[1] Thus, by analogy with *GCN4* in yeast and the earlier studies with synthetic constructs in mammals,[69] a scanning/reinitiation mechanism for picornaviruses is not ruled out by the mere failure to detect initiation at AUG-10 in EMCV[55] or by the fact that individually mutating each of the upstream AUG codons in PV mRNA did not prevent virus replication.[96] With so many upstream AUG codons, one could argue that their functions are redundant.

In the case of *GCN4*, partial phosphorylation of eIF-2 has the effect of increasing access to the internal initiator codon,[25a] apparently because the scanning 40S ribosome becomes competent to reinitiate translation only after it has (re)acquired eIF-2·GTP·Met-tRNA. The acquisition of competence understandably takes longer when the eIF-2 pool is depleted (by partial phosphorylation), and AUG codons encountered before the scanning 40S ribosome becomes competent are bypassed. The net effect is that phosphorylation of eIF-2 makes it easier for ribosomes to bypass upstream AUG codons and initiate instead from a downstream site. Because there is some evidence of hyperphosphorylation of eIF-2 in picornavirus-infected cells,[10,25] the possibility that infection by those viruses alters the translational machinery in a way that favors reinitiation seems worth exploring.

Absent some such change in the translational machinery, a reinitiation mechanism operating on a leader sequence that has eight or ten upstream ORFs is not likely to support efficient translation, but translation dependent on picornavirus leader sequences is demonstrably inefficient in (uninfected) COS cells. Thus, appending the PV leader sequence at the 5′ end of a transcript caused a sixfold reduction in the yield of protein in one study (Figures 2A and 2B in Reference 117); in another study, appending the EMCV leader sequence caused a tenfold reduction in protein yield.[59] That the EMCV leader sequence supports translation inefficiently *in vivo* is also suggested by experiments with T3-phage/vaccinia virus vectors: although protein yields from those cleverly designed vectors are very high, mRNA yields are even higher, and thus the specific activity of transcripts bearing the EMCV leader sequence is quite low.[124]

Because small peptides such as might be produced from the 5′-''untranslated'' domain of picornaviruses are notoriously unstable,[40] the anecdotal failure to detect such peptides during picornavirus translation[99a] is not compelling evidence against a reinitiation mechanism. Recent experiments suggest that some upstream AUG codons in PV mRNA actually facilitate translation, as evidenced by the tendency of large-plaque revertant viruses to have gained an upstream AUG codon.[99a] That remarkable finding seems more compatible with reinitiation than with a direct internal initiation mechanism.

B. Dicistronic Transcripts as a Test of the Internal Initiation Model

Dicistronic mRNAs have been designed in which the picornavirus leader sequence resides at the midpoint, followed by the chloramphenicol acetyl transferase (CAT) coding sequence and preceded by some other reporter gene, such as thymidine kinase (TK). The ability to translate CAT when its coding sequence is at the 3′ end of a dicistronic transcript is taken as evidence for internal initiation.[49] Confidence in that conclu-

sion depends, however, on how certain one is that the dicistronic mRNA is the only transcript produced. That premise has been previously questioned for experiments that utilized the PV leader sequence.[71] One of the criticisms concerned the faintness of the Northern blot in which the dicistronic transcript appeared to be the only form of mRNA on polysomes.[95] It seemed that a much longer exposure of the autoradiogram was needed to rule out the presence of monocistronic transcripts. That criticism has been criticized on the grounds that the presence of *any* dicistronic RNA in polysomes under conditions where the first cistron is silent should mean that ribosomes have directly engaged the second cistron.[42] Such an argument would seem compelling if we knew where in the gradient the silent transcripts sediment, but that key control — a polysome profile from cells transfected with the control TK-CAT construct which lacks the PV insert — was not presented. (It is not unheard of for a transcript to cosediment with polysomes even when it does not appear to be directing protein synthesis. See, for example, Reference 57.) Thus, the poliovirus experiments lacked at least one crucial control.

For EMCV, a paper describing the translation of dicistronic mRNAs *in vitro* included no documentation that the transcripts were intact.[50] The putative ability of the EMCV leader sequence to support internal initiation has also been studied *in vivo*, using plasmids that produce mono-, di-, and tricistronic transcripts. In the first such study, Northern blots of total cellular mRNA revealed no evidence of aberrant lower-molecular-weight transcripts.[51] Because the functional mRNA pool might be a small fraction of the total transcript pool, however (recall from the previous section that these mRNAs are translated inefficiently), a more telling assay would have been to analyze mRNAs from immunoprecipitated polysomes that were actually synthesizing CAT. Apart from the insensitivity of Northern analyses of total cellular mRNA, the *in vivo* studies with the EMCV leader sequence were compromised by unexplained variability in the yields of CAT protein. For example, when CAT synthesis was monitored by ^{35}S-methionine incorporation, CAT translation appeared to be greatly reduced when EMCV-CAT (i.e., the CAT coding sequence preceded by the EMCV leader) was the third rather than the first cistron in the transcript (Figure 2B of Reference 51), which is not predicted by the internal initiation model; but when CAT enzymatic activity rather than ^{35}S-methionine incorporation was monitored, a construct in which EMCV-CAT was positioned at the 5′ end of the transcript inexplicably produced far less CAT protein than that produced when EMCV-CAT was the second or third cistron (Figure 3 in Reference 51). Such variability could be explained if the functional mRNA had a structure different from that intended by the design of the vectors. Indeed, in another study, some dicistronic transcription units that contained the EMCV leader sequence at the midpoint clearly did produce an unintended monocistronic transcript (Figure 3A in Reference 59). (The monocistronic transcript is not seen in all lanes of that figure but, in many of the lanes that fail to show it, even the dicistronic transcript is faint; the authors admit that a longer exposure of the blot might have changed their interpretation.) Another follow-up study with the EMCV leader sequence, introduced this time into a retrovirus vector, posed other problems in interpretation. Adam et al.[2] assert that a dicistronic transcript, in which the EMCV leader sequence preceded the 3′ proximal neomycin phosphotransferase (NPT) coding sequence, expressed NPT at a level approaching that of a monocistronic transcript initiated from an internal SV40 promoter, but that claim ignores the fact that the SV40-initiated transcript was far less abundant than — and hence must have been translated much better than — the dicistronic transcript. The structure of the retrovirus vectors was odd in that the EMCV initiator codon was retained shortly upstream from, and out of frame with, the NPT coding sequence; thus, an internal initiation mechanism designed to deliver ribosomes to the EMCV initiator codon should *not* have allowed efficient translation of NPT. (The arrangement of AUG codons in those constructs does not conform to the recognized requirements for reinitiation by eukaryotic ribosomes.) Expression of NPT under such odd circumstances again raises the suspicion that the functional mRNA might have had a structure different from that intended by the design of the vectors. The experiments point up the danger of monitoring translation by a very sensitive enzymatic assay while monitoring tran-

scription by a rather insensitive Northern blotting assay of total cellular mRNA. As it is not unheard of for a minor transcript to be the major functional mRNA,[93,106] the question of whether a dicistronic vector inadvertently produces a small amount of monocistronic mRNA is more than academic. At least three claims of initiation by unusual mechanisms[7,18,58] have undergone drastic reinterpretation[56,59,81] upon discovering alternative transcripts that were missed in the initial analyses. Thus, it is not captious to ask whether the detected transcript from a given gene functions uniquely, or functions at all, in synthesis of the encoded protein.

To minimize the possibility that a vector designed to generate a di- or tricistronic transcript might also produce some monocistronic transcripts, vaccinia virus vectors, which replicate in the cytoplasm, could be helpful. The commonly used SV40- and retrovirus-based vectors, which replicate in the nucleus, can and do produce unintended transcripts by using cryptic splice sites;[26,46,85,122] and picornavirus leader sequences, which do not normally pass through a nucleus, have not evolved to be free of potential splice sites. Thus, it might be informative to use the cytoplasmic vaccinia virus-based expression system to compare the yield of CAT protein from a monocistronic EMCV-CAT transcript vs. a dicistronic transcript in which EMCV-CAT lies at the 3′ end: according to the internal initiation model, the yield of CAT protein should be identical. The expectation of *position-independent expression* of proteins translated by internal initiation clearly is not met by the polymerase gene of hepatitis B virus: Chang et al.[14] reported an unexplained tenfold increase in translation when a *pol-lacZ* fusion gene was moved from the 3′ to the 5′ end of the transcript. In short, although some results obtained with dicistronic test transcripts seem to support the internal initiation hypothesis, there are enough anomalies to keep the jury out. Even if better designed experiments were to reveal unequivocally that picornavirus leader sequences can mediate internal initiation, it seems important to recognize that the experiments described above have not proven the point.

In an interesting twist, Molla et al.[90a] have devised an infectivity assay to evaluate the ability of the EMCV leader sequence to support translation from an internal position. They constructed a dicistronic transcript of the form 5′-PV-P1-EMC-P2P3-3′, where PV and EMC represent the 5′-noncoding domains of poliovirus and EMCV, and P1, P2, and P3 represent the (usually contiguous) coding domains of poliovirus. Remarkably, when that transcript was introduced by transfection into HeLa cells, infectious progeny viruses emerged. The simplest interpretation is, as the authors contend, that the EMC insert supports translation of P2P3 from an internal position, but the experiments were not conclusive because they included no RNA analyses. RNAs from both infected cells and plaque-purified virions must be analyzed to rule out the possibility that polypeptide P2P3 might have been translated from a subgenomic mRNA. The authors claim that their dilution analysis, in which plaque counts were found to be proportional to virus concentration, rules out the possibility that infection requires two hits, as might be the case if the full-length genome and a subgenomic transcript were separately packaged. But an aggregated virus preparation, or a preparation in which the subgenomic "helper" particles are in excess, could give a linear response even if two hits were required. It's worrisome that the plaques were small and heterogeneous in size and that the recovered virus was not plaque purified. Although the incompleteness of the study contradicts the assertion that it constitutes proof for internal initiation, the general approach and some of the initial results are nonetheless promising.

V. DOES AN INTERNAL INITIATION MECHANISM OPERATE WITH CELLULAR mRNAs?

No convincing evidence suggests the occurrence of internal initiation with any nonviral mRNA. It has been postulated that internal initiation might operate with the mRNAs of proto-oncogenes,[42,87] inasmuch as their AUG-burdened leader sequences should preclude efficient operation of the scanning mechanism. But inefficient scanning, resulting in low translational yields, might be sufficient (indeed, might be necessary) for the expression of oncoproteins and other potent regulatory proteins.[77] Because many

cDNAs with upstream AUG codons have been recognized belatedly as intron-containing precursors rather than functional mRNAs,[71,77] the mere existence of cDNAs with AUG-burdened leader sequences argues neither against scanning nor for internal initiation.

The suggestion that cap-independent translation might be indicative of mRNAs that use internal initiation[42,49,113] is also too facile. Some evidence suggests that a moderately long, unstructured leader sequence may be all that is needed to reduce dependence on the m7G cap.[4,27,75,111] But long, unstructured leader sequences have been shown to promote efficient translation only when they are positioned at the 5′ end of the transcript.[27,70,84,111] Thus, even when translation is no longer highly cap dependent, it remains end dependent. Whereas mRNAs that function without a methylated cap are rare among animal viruses (and, as far as we know, nonexistent among cellular mRNAs), cap-independent translation is somewhat more common among plant viruses. However, when the "dicistronic mRNA test" was carried out with 5′ leader sequences from cowpea mosaic virus M RNA[6] and plum pox potyvirus,[101] the conclusion was that those naturally uncapped leader sequences do not support internal initiation. (With cowpea mosaic virus M RNA, the test for internal initiation has been carried out *in vitro* and *in vivo* with different results. Although the *in vitro* results, using reticulocyte lysates, were taken as evidence for internal initiation,[116,119] the same mRNA leader sequence showed no evidence of internal initiation *in vivo*.[6] As noted elsewhere, it is not uncommon for *in vitro* translation systems artifactually to give the appearance of internal initiation[71] due, for example, to cleavage of a small amount of the input mRNA.) In short, the demonstration that two naturally uncapped viral mRNAs initiate translation by a 5′ end-dependent mechanism[6,101] negates the simple idea that cellular mRNAs that show reduced dependence on the m7G cap are likely candidates for internal initiation.[42,49,113]

A recent report by Macejak and Sarnow[82] makes the strongest claim for an internal initiation mechanism with a nonviral RNA. They assert that the 5′ leader sequence of the cellular BiP mRNA mediates internal initiation, as evidenced by the ability to translate luciferase from a vector that produces dicistronic transcripts of the form CAT-BiP-LUC (where *BiP* designates the 5′-noncoding sequence of the immunoglobulin heavy-chain binding protein, and *LUC* designates the luciferase coding sequence). As explained in the preceding discussion of picornavirus translation, the critical question is whether the intended dicistronic mRNA really is the functional template for translating the downstream LUC cistron. Although the data indeed show that dicistronic transcripts cosediment with polysomes, the faint, splotchy Northern blots in Figure 3b of Reference 82 are not sensitive enough to rule out the presence of some monocistronic transcripts. In Figure 3c of Reference 82, where the blots are cleaner, the authors probed for 5′ CAT sequences (detecting dicistronic as well as unexplained smaller transcripts), but inexplicably they did not probe for the critical 3′ LUC sequence to see if it was present exclusively in dicistronic transcripts, as their interpretation requires. As to why, if ribosomes cannot initiate internally, there would be *any* dicistronic mRNA in polysome-like complexes under conditions that preclude translation of the 5′ cistron, the answer may be that the hairpin structure which was introduced to restrict translation of the 5′ CAT cistron was positioned sufficiently far from the 5′ end that several 40S ribosomal subunits might have been able to bind upstream from the hairpin. Thus, a serious omission is that the *control transcript,* in which sequences other than BiP separate the CAT and LUC cistrons, *was not shown to be disengaged from polysomes* under conditions where the CAT-BiP-LUC transcript appeared to be engaged. The same critical control was omitted from the original poliovirus experiments, as noted above.

VI. OTHER CAUTIONARY NOTES ABOUT INTERNAL INITIATION

Apart from picornaviruses, most other claims of internal initiation[5,21,41,116] arguably are attributable to cleavage of the mRNA. (Some of these claims were discussed point by point in an earlier review.[71]) Indeed, RNA breakage would account for the otherwise unexplained synthesis of prominent low-molecular-weight polypeptides, in ad-

dition to the expected full-length proteins, in some *in vitro* experiments.[97,116]

The so-called IRES motif (internal ribosome entry site) has not been defined for any mRNA. Because the 5′ end of picornavirus mRNAs mediates RNA replication as well as translation, the fact that certain structural motifs are conserved, as nicely summarized by Jackson et al.,[49] is not *a priori* evidence that those features are important for translation. To delimit sequences that specifically affect translation, investigators have used deletion mutagenesis, inhibition by complementary oligonucleotides, and occasional point mutations (reviewed in Reference 49); but the available data are insufficient to define the essential features of the ~450-nucleotide IRES. Moreover, the mapping results from independent laboratories do not always agree. Thus, for EMCV, a key result reported by Jang and Wimmer[52] is contradicted elsewhere;[28a,109] and for poliovirus (PV) there are major contradictions among References 9, 97, and 110. Even from one laboratory there are troubling inconsistencies between the sequences said to be needed for the PV IRES to function at the 5′ end vs. the midpoint of a dicistronic transcript. (Thus, in one study, deletion to position 134 reduced translation fourfold and deletion to position 265 virtually abolished translation,[92] whereas, in another study, deletion to position 140 had little if any effect and deletion to position 320 reduced translation only twofold.[97]) Another seeming anomaly is that a polypyrimidine stretch said to be essential for translation in HeLa cell extracts appeared to be completely dispensable in Krebs-2 extracts;[98] wild-type PV mRNA is translated equally well in both extracts, and it seems unlikely that two different mechanisms are employed. The often repeated speculation that the "essential" polypyrimidine motif might base-pair with a sequence near the 3′ end of 18S rRNA[99a] is pure speculation. A recent claim that a functional IRES could be reconstituted by annealing unlinked fragments of the EMCV leader sequence[13] is not credible, inasmuch as the employed conditions (85 to 90°C for 3 to 4 min followed by cooling to 0°C) should preclude rather than promote annealing of unlinked RNA fragments.[102a]

A major limitation of all these experiments is that the effects of disruptions in the IRES have been assessed simply by measuring protein yields. It would be much more informative to monitor the formation of initiation complexes and thus to determine whether a deleterious mutation actually prevents ribosomes from binding. The internal initiation and the reinitiation models make very different predictions in that regard. Also needed are footprinting and/or RNase protection assays to establish where, within the long IRES, ribosomes actually contact the mRNA.

The internal initiation model would seem far more compelling if at least one IRES were rigorously defined and if the essential features of that element were detectable in just one other unrelated mRNA. It seems reasonable to expect mRNAs that use a common mechanism to share common structural features. (One might defend the apparent absence of a common structure among PV, BiP mRNA, etc., by postulating that a different initiation factor mediates internal initiation in each case; but *not one of the putative mRNA-specific factors has been purified and shown directly to affect translation.* As explained above, the mere binding of proteins to mRNA leader sequences does not justify calling them initiation factors.) If the sequence requirements for internal initiation could be fully defined for one mRNA, we would have criteria for extrapolating the model. In the absence of such criteria, it is too easy to invoke internal initiation as an explanation — or, more correctly, an excuse — for every anomalous observation that comes along.[14,21,41,116]

VII. CONCLUSIONS AND PROSPECTS

This review considers some difficult, unsettled issues regarding the mechanism of initiation of translation in eukaryotes, with particular emphasis on questions around which "consensus answers" seem to have emerged in advance of compelling experimental data.

The use of reconstituted translation systems — i.e., washed ribosomes and purified initiation factors — would seem an obvious way to resolve some of the uncertainties about what eIF-4A and other initiation factors do, and when they do it. Unfortunately, the low activity of reconstituted systems and the fact that few of the protein factors

are completely pure limit what can be learned from that approach. The clever exploitations of yeast genetics that are underway in several laboratories[17,25a,28,86,123] may offer the best hope of finding out how translation initiation factors really work. The ability of some mammalian factors to substitute for their yeast counterparts[3] may justify the extrapolation of some results from "lower" to "higher" eukaryotes. But certain differences between the yeast and mammalian initiation mechanisms[16] mean that some questions will be answerable only by working with mammalian systems. Recent advances in cloning and expressing the human gene for initiator $tRNA^{met}$ provide one valuable tool for manipulating mammalian translation systems.[32] One may hope that, with the emerging tools, our understanding of how initiation factors participate in cap recognition, scanning, etc. will soon improve.

The other major question addressed herein is whether some mRNAs, most notably from picornaviruses, might be translated in a radically different manner from cap-dependent mRNAs. The unusual structure of picornavirus mRNA leader sequences could have explanations other than the use of an odd mechanism of initiation. For example, the presence of upstream AUG codons might reflect an actual need for translation to be inefficient, inasmuch as the genomes of plus-stranded viruses at some point must be cleared of ribosomes in order to serve as templates for RNA replication. (A similar argument has been advanced for retroviruses.[99] For both retroviruses and picornaviruses, the deliberately inefficient translation might be compensated by an efficient transcription mechanism that floods the cell with mRNA.[12]) Viruses that replicate in the cytoplasm must encode their own capping and methylating enzymes, a requirement that may be incompatible with the small size of picornavirus genomes; thus, the absence of a methylated cap may result from default rather than preference. Absence of a cap might not be a liability if the translation of picornavirus mRNAs has to be throttled anyhow. In any event, the absence of a cap and the presence of an AUG-burdened leader sequence do not imply that picornaviruses *must* use an internal initiation mechanism, inasmuch as other naturally uncapped mRNAs[6,101] and other mRNAs with upstream AUG codons[1,40] nevertheless initiate translation in a 5′ end-dependent manner.

The questions raised herein about the proposed internal initiation mechanism for picornaviruses do not negate the utility of incorporating the EMCV leader sequence into vectors that promote the co-expression of genes[36,59] or that allow the translation of uncapped transcripts,[24a,30,124] but the mechanisms by which such vectors work require more thorough investigation. Because the cap normally contributes to mRNA stability as well as translation, picornavirus 5′-noncoding sequences must have compensatory stabilizing features; it might be those stabilizing elements that make the EMCV leader sequence useful in the construction of vectors. (The poor vector performance of the PV leader sequence, in contrast with that of EMCV, remains to be explained.) The results of some experiments reviewed here and elsewhere[49] are most easily explained by invoking internal initiation. But until additional experiments and numerous missing controls are done, internal initiation of translation should be considered a possibility rather than a fact.

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