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New Ways of Initiating Translation in Eukaryotes?

This letter to the editor is a response by a large number of investigators in the field of protein synthesis to the minireview published by Dr. Kozak in Molecular and Cellular Biology (9). This minireview attempts to create significant doubts regarding the published literature that we believe are unwarranted and to bolster Dr. Kozak's own point of view regarding translation initiation. We therefore take serious issue with the scholarliness of the Kozak minireview. As will be shown, the Kozak minireview contains numerous distortions of fact and of published data and selectively utilizes the published literature. In every field of research there are legitimate concerns regarding the interpretation of results and the reproducibility of certain published data. Several of the issues raised by Dr. Kozak are legitimate in this regard, but they are not new and have hardly gone unnoticed, having been raised in scholarly and critical reviews elsewhere. At issue here is not the right to critically question results and interpretations but rather whether the Kozak minireview is scholarly and its tone is professional.

We point out that much of the work challenged in the Kozak minireview was published in *Molecular and Cellular Biology*, as well as other leading peer-reviewed journals, and forms a mainstream of research on protein synthesis which is taking place in scores of laboratories around the world. In this minireview, Dr. Kozak dismisses three novel mechanisms for translation initiation which have now been well studied and extensively documented. One mechanism is internal ribosome entry, which she rejects in favor of ribosome scanning, a mechanism for translation initiation which she proposed over 20 years ago. In ribosome scanning, it is proposed that the 40S small ribosome subunit enters the mRNA from the 5′ cap and undergoes a linear 5′-to-3′ search for the initiation codon, which is typically an AUG. Internal ribosome entry involves the internal association of ribosome subunits at or near the initiation codon without the need for entry from the 5′ end of the mRNA. A second mechanism opposed by Dr. Kozak is the initiation of protein synthesis without Met-tRNA, a universal and key component, as shown for several insect virus mRNAs. The ability to carry out translation without this initiator tRNA, and from the A site of the ribosome, has enormous implications for our understanding of protein synthesis and its evolution. A third mechanism of translation initiation which Dr. Kozak takes issue with is known as ribosome shunting or discontinuous scanning, which combines features of 5′ entry of ribosomes by scanning and the internal translocation of ribosome subunits without further scanning to the initiation codon. It is clear to a great many researchers, as represented by the signatory list below, that the initiation of protein synthesis in eukaryotes is dynamic and flexible, involving a variety of mechanisms that have evolved to meet the complex demands of eukaryotic cells and viruses.

Dr. Kozak has spent more than 10 years in strenuous opposition to the evidence for viral internal ribosome entry and the recognition of specific viral *cis*-acting internal ribosome entry site (IRES) elements. The minireview now attempts to use almost entirely the same kinds of arguments against cellular IRESs and other means of nonscanning translation initiation that Dr. Kozak used previously in her unsuccessful efforts to disprove viral IRESs. Whether Dr. Kozak explicitly acknowledges internal ribosome entry as an established fact, at least for viruses, is not at all clear in the minireview, although she does compare translation functions to the encephalomyocarditis virus (EMCV) IRES, but without comment or acceptance. It would be fairer to the reader and more intellectually honest to explicitly acknowledge internal ribosome entry as an established fact, at least for viruses, or—if she still wishes to oppose the idea—to do so openly. Needless to say, it is now difficult to mount a convincing case for blanket repudiation of IRESs in the face of overwhelming data, including elegant and compelling evidence from viral IRES-dependent translation of a circular RNA (3), which was not cited in the Kozak review.

It is not practical to document here all of the examples in which published results were inappropriately presented in the minireview by Dr. Kozak. We refer readers to a recent comprehensive review which summarizes the current evidence in support of viral and cellular IRES elements and alternate mechanisms of translation initiation in eukaryotes and briefly overviews some of the key techniques which were questioned by Dr. Kozak (7). Consequently, we list below just several specific examples which are emblematic of the serious issues which are of concern to us.

Several reasons are described by Dr. Kozak for dismissing reports of cellular IRESs. Dr. Kozak argues that because cellular IRESs often represent modest translation increases over background levels, they result from fortuitous positioning of RNA sequences in experimental constructs. This argument ignores the evidence that IRESs have been shown to represent a range of activities from weak to strong and to function by a variety of mechanisms. Indeed, the expression of many cellular genes encoding regulatory proteins is often tightly controlled at multiple steps to guarantee that correct protein levels are achieved, which is not generally equivalent to high protein levels. An IRES may therefore be relatively weak, but in combination with other levels of gene control, it achieves significant or correct protein expression levels under different physiological conditions. One example is the IRES of the proto-oncogene c-*sis*, which encodes platelet-derived growth factor 2 (PDGF2). This IRES is activated severalfold during megakaryocyte differentiation, in conjunction with induction of PDGF2/c-sis gene expression during differentiation (1, 16). The modest translation stimulation directed by the cellular PDGF2 IRES fine tunes PDGF2/c-sis gene expression during differentiation. Similar mechanisms are likely employed by other critical regulatory genes and may have widespread implications for cellular growth and development. Thus, it is arbitrary to dismiss cellular IRES elements as physiologically irrelevant artifacts merely because their effects on translation are moderate. A more considered view is that regulatory elements act at all levels of gene control, including transcription, mRNA transport, and mRNA stability and translation, and permit exquisite control precisely because they involve multiple and modest additive effects which can be independently combined and regulated.

There are several functional ways to study IRESs. Construction of a dicistronic mRNA containing an internal downstream second open reading frame that is ordinarily not translated is typically used to detect IRES activity. Other approaches include insertion of very stable, translation-blocking hairpin structures upstream of the IRES and biochemical detection of IRES interaction with initiation factors and ribosome subunits. Important control studies must be performed to validate the integrity of the dicistronic mRNA and to exclude the presence of cryptic promoters or aberrant splicing that could lead to

production of subgenomic mRNAs or removal of intervening RNA sequences that would normally prevent internal translation initiation by ribosome scanning. With this in mind, apart from one "potential" cellular IRES, Dr. Kozak dismisses all other published reports as artifacts arising from improper experimental methodologies, a lack of proper control studies, or poor experimental design. However, most but not all cellular IRES studies included the use of other RNA segments that did not contain IRES elements or IRES sequences with defined mutations, which failed to mediate internal ribosome initiation. Thus, selective translation by internal ribosome entry was in fact shown to be specific for a small number of RNA elements. These controls were largely ignored in the Kozak minireview, inappropriately casting doubt on the integrity of the conclusions from these reports. Dr. Kozak is particularly critical of cellular IRES reports because the background control level of translation in the absence of the IRES varies between different constructs and because it is not zero. This argument can be misapplied to most molecular systems. For instance, deletion of all transcription elements seldom completely abolishes activity, and the basal activities of different control constructs typically vary. As proof for this view, Dr. Kozak points out that an antisense version of a putative cellular IRES directed translation at 40% of the level of the sense form (13). However, in other examples the antisense verification did not function as an IRES. In other cases, Dr. Kozak asserts (in the absence of any evidence to support her view) that a control RNA sequence has depressed translation, making it only appear that the cellular IRES element directs translation initiation. As one example, the Kozak minireview inappropriately compares experiments described in two papers (11, 21). In the *Nature* paper (11), the BiP IRES mediated translation of the second cistron 15-fold over the Antp control sequences. Importantly, introduction of a hairpin at the 5′ end of the dicistronic mRNA completely abolished translation of the second cistron (Fig. 2 in reference 11), demonstrating that the BiP sequence has IRES activity. In the *Nucleic Acids Research* paper (21), the BiP IRES was stimulated 10-fold over the Antp sequence; as Dr. Kozak pointed out, translation was lower (2.5-fold) compared to the "empty" vector control. This was interpreted by the authors as readthrough mediated by the 30-nucleotide sequence located between the two cistrons. This does not constitute a serious "discrepancy of results," in contrast to its presentation by Dr. Kozak. In addition, while studies have shown that varying the length of the intercistronic region influences translation initiation frequency (5), a potential confounding problem, the effect acts predominantly on scanning-dependent rather than internal initiation of translation.

Dr. Kozak asserts that cryptic promoters or cryptic splicing of RNAs cannot be excluded as a source of smaller mRNAs that could be translated from truncated positions, providing the false impression of internal ribosome initiation or initiation by ribosome shunting. Dr. Kozak is not alone in expressing concern regarding some claims for internal ribosome entry, particularly when there is no accompanying data verifying the integrity or size of the mRNA species. Indeed, a few studies noted unanticipated smaller transcripts and noted that they likely arose from splicing, generally at low levels, from a few of the dicistronic constructs (e.g., see reference 6). However, this study demonstrated that the translation of the second cistron could not have occurred from the low-abundance monocistronic mRNA. Dr. Kozak cites the fact that unanticipated splicing was sometimes detected but does not present the data fully and accurately. Additionally, many studies involved in vitro-synthesized mRNAs that were monitored in cell-free systems or examined after expression in cultured cells, and the RNAs were found to be intact. While Dr. Kozak highlighted instances in which important RNA structural data were absent, she often failed to reference studies in which it was included and the RNAs were found to be intact. In some other cases she inappropriately dismisses the data as of poor quality or not sufficiently sensitive. For example, Dr. Kozak criticized published work on the vascular endothelial growth factor (VEGF) IRES as not convincing because of the presence of an internal promoter but failed to cite another paper which showed that translation initiation from an internal promoter cannot account for VEGF translation results (8). Again, it is not appropriate to assume that moderate translation effects, which can be quite important biologically, are artifacts because they do not conform to an arbitrary value. Internal initiation of c-Myc2 protein synthesis was similarly dismissed by Dr. Kozak despite evidence for only a single mRNA because transfection of the mRNA itself into cells, compared to its expression from a DNA vector, failed to lead to translation (17, 18). It was suggested by the authors of these two papers that the c-*myc* IRES might require nuclear binding proteins to function, which is reasonable given the importance of noncanonical factors for the activity of certain viral IRESs (7). This was rejected by Dr. Kozak, and other well-controlled studies which demonstrated c-Myc IRES function were not cited (e.g., reference 12). Thus, the minireview provides the false impression that only limited and poorly controlled research has been performed on cellular IRESs.

Dr. Kozak also asserts that biochemical studies have never been conducted to show that initiation factor 4G (eIF4G), a key factor that promotes ribosome binding, can associate with sufficient affinity to a natural IRES so as to mediate internal ribosome entry. This conclusion is meant to cast doubt on the validity of translation by internal ribosome entry in eukaryotic cells. In fact, Lomakin, Hellen, and Pestova (10) directly measured the affinity of the central domain of eIF4G alone and as a complex with eIF4A for the EMCV IRES and for β-globin mRNA. They found that the eIF4G/4A complex binds the EMCV IRES with an affinity sufficient for the IRES to be able to compete with cellular capped mRNAs for eIF4F, a complex of factors which contains eIF4G and helps to direct ribosome binding to capped mRNA. While these data do not prove a mechanistic function, they account for a critical first step. This reference was not cited by Dr. Kozak, nor in fact was any of the literature that analyzed the functional, specific interactions of eIF4G/4A/4F with EMCV-like IRESs and of eIF3, another essential initiation factor that binds to the 40S small ribosome subunit, with hepatitis C virus-like IRESs. True, these are viral IRESs. However, this is a well-known literature published in leading journals, and it provides a quantitative and partial mechanistic understanding of IRES function that may be applied to cellular IRESs.

Dr. Kozak questioned the quality and integrity of work which demonstrated the possibility of initiator-independent translation from the ribosome A site, as shown to occur in the cricket paralysis virus (CrPV) mRNA (19). This is a recent seminal finding in the field of protein synthesis. Importantly, a landmark paper (15), which demonstrated that a related insect virus IRES is also translated without $tRNA_i^{Met}$ and is therefore highly supportive, was not cited in the minireview. While this paper is included in a review cited by Dr. Kozak, that review was referenced in a manner so as to cast doubt on these findings. Figure 3 in the PNAS paper (15) provides unambiguous data demonstrating that the CrPV IRES does not use initiator tRNA to initiate translation, strongly arguing that

general rules of scanning-dependent initiation do not apply in this case. The *Cell* CrPV paper (19) challenged by Dr. Kozak confirmed these earlier findings from a related insect virus genome and disclosed an important and unexpected alternate molecular mechanism for protein synthesis. Dr. Kozak claims that the IRES-ribosome complexes that were reported in reference 19 are merely aggregates that are not translationally active complexes and ignores the fact that the authors examined the oligomeric state of the RNA molecules in these studies. Results also showed that 80S ribosome/CrPV IRES formation does not need GTP hydrolysis and is quite insensitive to the addition of L-methioninol (an approach also used by Dr. Kozak). Notwithstanding this evidence for unconventional initiation, Dr. Kozak questions the validity of the formation of initiator $tRNA^{Met}$-independent 80S ribosome/CrPV complexes on the basis of the concentrations of edeine used in the experiments. It is true that edeine at 1 to 10 μM inhibits translation initiation at the 40S ribosome-AUG recognition step. These concentrations of edeine will inhibit the pausing of 40S subunits at the AUG initiation codon on all mRNAs examined so far, except the CrPV IRES. A 40S ribosome can be detected at the CrPV-IRES initiation codon by toeprinting analysis. While CrPV IRES-mediated translation is unaffected in the presence of 0.5 μM edeine, translation is inhibited by 80% at 1 μM. This finding could be explained if edeine has an affect on a step in translation that is subsequent to the 40S subunit-start codon recognition step. Indeed, it has been shown (2) that the enzymatic association (aided by eEF1 and GTP) of Phe-tRNA to the ribosomal A site is abolished by 80% in the presence of 1 μM edeine. The sucrose gradient-toeprinting data in reference 19 support the hypothesis that edeine inhibits a postinitiation step in CrPV IRES-mediated translation. As this concentration of edeine inhibits the AUG recognition by 40S ribosome subunits in all examined mRNAs, a subsequent affect of edeine on elongation would not be trivial to detect. It is therefore difficult to understand Dr. Kozak's claim to have disproven that the CrPV-like IRESs have an unusual mechanism of initiator tRNA-independent translation initiation, which does not use the ribosomal P site. Dr. Kozak also states that CrPV may synthesize subgenomic mRNAs that are translated, providing initiation from 5′-truncated transcripts that only appear to constitute internal translation initiation. This claim ignores compelling and rigorous literature (none of which was cited) demonstrating that CrPV does not produce subgenomic mRNAs in infected cells and that full-length genomic RNA extracted from virions is directly translatable to yield the protein in question from the downstream open reading frame (4, 14, 20).

A number of studies have identified yet another alternate mechanism for translation initiation known as ribosome shunting. As it occurs in adenovirus mRNAs expressed during the late stage of infection, ribosome shunting was shown to involve sequences in the viral 5′ noncoding region that are complementary to 18S rRNA (22). Dr. Kozak's minireview misrepresents the central conclusion of this paper, falsely stating that this study claims to have demonstrated direct interaction between mRNA and 18S rRNA for initiation of translation by ribosome shunting. In fact, this study concluded that ribosome shunting on adenovirus late mRNAs might occur by any of several mechanisms that involve sequences complementary to 18S rRNA, including but not limited to structural RNA mimicry or direct interaction with 18S rRNA. Dr. Kozak also asserts that only rudimentary mapping, large deletions, and a failure to conduct mRNA integrity analysis underlie the conclusion that 5′ noncoding sequences in adenovirus late mRNAs facilitate ribosome initiation by utilizing sequences complementary to 18S rRNA. This assertion ignores control Northern mRNA analyses presented in this paper and elsewhere, and it improperly represents the size of deletions introduced in the 5′ noncoding region in such a way as to leave the impression that they are nonspecific.

The examples cited above represent only a sampling of numerous significant errors in the minireview published by Dr. Kozak. Careful inspection of this minireview reveals a lack of scholarly accuracy that will only serve to confuse and mislead readers. While Dr. Kozak is entitled to her opinions, we believe very strongly that only manuscripts of acceptable scholarly standards should be published in Molecular and Cellular Biology.

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Author's Reply

The letter to the editor ignores or responds with lame defenses to major points in my minireview. The letter diverts attention to side issues, which I shall address, and ad hominem issues, which I shall overlook. The letter to the editor was substantially rewritten after the authors were shown a draft of this response. Because of that unusual maneuver, their arguments appear to anticipate some of the points below.

The first section of my minireview (39) questioned whether appropriate criteria were used in identifying putative IRES elements in cellular mRNAs. My main concerns in this regard, along with issues raised in the letter, are discussed in the first section ("Does internal initiation via IRES elements occur with cellular mRNAs?") below. The second part of the minireview pertained to the claim that some insect virus mRNAs initiate translation via a novel route that does not require Met-tRNA$_i$. This is discussed in the second main section ("Initiation without Met-tRNA$_i$?").

The third part of the minireview simply pointed out that it is too easy to find, in various mRNAs, a short segment that is complementary to rRNA. Invoking "elusive SD-like sequences" (25) enables one to imply base pairing between IRES elements and 18S rRNA without even pointing to anything, but in some cases a particular mRNA sequence has been singled out. I briefly discussed three examples: Gtx, hsp70, and adenovirus late mRNAs. The main question is not how the complementary sequence functions, which the letter to the editor focuses on, but whether the complementarity is due merely to chance. In none of these cases was the significance of the complementarity tested by introducing point mutations. I think nothing more needs to be said.

Does internal initiation via IRES elements occur with cellular mRNAs? (i) Vectors. Given the absence of natural dicistronic mRNAs that support independent translation of the downstream cistron, testing for internal initiation requires constructing synthetic dicistronic mRNAs by transplanting a 5′ untranslated region (UTR) to an internal position. My minireview raised two concerns about these artificial constructs. One big worry was whether a low-end positive result is really a positive result. The letter responds to this concern in a way that sidesteps the main issue. The letter argues that weak IRES activity can be physiologically important because low-level translation might be required to produce just the right amount of critical regulatory proteins, such as c-sis. I completely agree that some cellular mRNAs are designed to be translated poorly (21, 38), but if one wants to investigate the mechanism of that low-level translation, one still needs an appropriate assay. The scanning mechanism can be demonstrated even when it operates inefficiently (21, 41, 55, 58, 60). When testing for internal initiation, however, a low-end positive result using synthetic dicistronic vectors is not credible for reasons explained in the next paragraph. A better assay is needed. One cannot simply ignore the limits of reliability of the assay on the grounds that it doesn't have to work well. We still need to know what "it" is.

The question when using dicistronic vectors is this: if translation of the 3′ cistron preceded by a candidate IRES sequence is close to background level, is it anything more than background variation? The problem is that the "negative control" (empty vector) is never negative. There is always some expression of the 3′ gene. Without understanding how that translation is achieved (via mRNA breakage? via a cryptic promoter?), everyone simply sets it at 1.0 and looks for expression greater than 1.0 when the candidate IRES is inserted. Extraneous sequences that merely lengthen the intercistronic region have been shown to elevate background translation of the 3′ cistron as much as 10-fold (Fig. 9 in reference 18), perhaps by providing room for RNases to cleave and thus release a translatable 3′ RNA fragment. The variability in and uncertain cause of the background expression set limits on the reliability of the dicistronic assay.

The analogy to transcriptional promoters is an inappropriate defense for IRES elements that score barely above background. With a promoter, there might be uncertainty about which sequences or which factors mediate transcription but the basic mechanism is not in question. With candidate IRES elements that function close to background level, however, the uncertainty is fundamental: are we seeing internal initiation of translation or just a slight increase in the undefined mechanisms that generate background? It is not natural dicistronic mRNAs that are being studied. These are artificial constructs in which a 5′ UTR has been inserted between two convenient reporter genes. It seems injudicious to argue that a certain level of translation of the 3′ cistron is just background—re-

quiring no explanation—while a 2.5-fold increase is real. The increase was only 2.5-fold above background when sequences from c-sis (3) or ornithine decarboxylase mRNA (52) were tested. With six other candidate IRES elements the stimulation was ≤5-fold (Table 1 in reference 39).

The letter misrepresents my position on a related issue. I did not make a broad assertion "in the absence of any evidence to support [my] view" that IRES elements give positive results only because the IRES replaces an inhibitory control sequence. I raised that possibility specifically in connection with BiP (45), where the negative control was not the usual empty dicistronic vector but one into which a 400-bp inverted segment of the *Drosophila Antp* gene was inserted between the 5′ and 3′ cistrons. It is an unusual starting point. Upon replacing the *Antp* sequence with the BiP IRES, translation of the 3′ cistron was stimulated 15- to 30-fold (45); but the stimulation was ≤4-fold when the BiP sequence was inserted into, and judged against, an empty-vector control. The letter (paragraph six) inappropriately accuses me of inappropriately comparing one BiP study (45) with another (66): I did not cite or discuss the second of those studies at any time in any way. The straightforward studies I did cite showed 2.6-fold (37) or 4-fold stimulation (26, 36) when the BiP IRES was evaluated in vivo against an empty-vector control.

Instead of faulting me for pointing out the quantitative discrepancies in tests of the BiP IRES, I wish the letter had explained the reason for starting with a vector that contains a 400-bp segment of the *Drosophila Antp* gene. That vector is still in use (16). Even more problematic is a dicistronic vector that contains at the midpoint a mutated version of the EMCV IRES to which candidate cellular IRES sequences were appended (5, 34). I expressed concern that although the mutation in the EMCV insert prevents it from functioning independently as an IRES, it might still bind protein factors without which the test sequences would not have scored. Neither the letter nor the new review on internal initiation (25) offers any justification for using this vector which seems to invite misinterpretation.

Control sequences inserted into the intercistronic region of a dicistronic vector are not the only potential problem. The choice of reporter genes and their arrangement (i.e., which is 5′ and which 3′) can profoundly affect whether a putative IRES supports downstream translation (27). It will be important to understand the mechanisms behind the unexpected findings in that study of viral (poliovirus and EMCV) and cellular IRES elements. RNA analyses, as discussed next, might help.

(ii) RNA analyses. The second big concern I raised vis-a-vis synthetic dicistronic vectors was whether extraneous mechanisms (splicing, use of a cryptic promoter, or mRNA breakage) might subvert simple interpretation of the results even when activity is substantially (e.g., 10-fold) above background. The simplest interpretation is that if the 3′ cistron gets translated, the intercistronic sequence is an IRES; but that assumes that the aforementioned extraneous mechanisms did not generate a monocistronic mRNA from which the downstream cistron is actually translated. Very careful RNA analyses are needed to rule out this possibility. Several points raised in the letter revolve around this issue.

The RNA analyses I am criticized for not citing were not determinative; the assays in those papers simply were not sensitive enough to prove the point. Routine RNA assays used to document presence of the intended dicistronic mRNA are not adequate to prove absence of an unintended monocistronic mRNA. According to one calculation explained in the minireview, even when an IRES supports 10-fold better translation of the 3′ cistron than does the empty vector, that is only ~5% as efficient as translation from a capped monocistronic mRNA. Thus the RNA assay must be able to detect—to rule out—a monocistronic transcript produced at 1/20 the level of the dicistronic form. Northern blots and other routine RNA assays do not have that level of sensitivity. Even greater sensitivity is required to rule out an internal promoter in the c-sis IRES, which supports translation only 2.5-fold better than the empty vector.

The letter calls attention to my failure to cite controls in which mutations in putative IRES sequences abolished translation of the 3′ cistron, but such mutations have little meaning without careful RNA analyses. The question is not whether putative IRES activity is sequence-specific but how the sequence functions. Consider some examples.

- A putative IRES in eIF4G mRNA was shown by mutagenesis to require a polypyrimidine tract (20) that was later identified as a 3′ splice junction sequence (22).
- The activity of the XIAP IRES was abolished upon deleting a polypyrimidine tract ($Y_{10}AG$) that strongly resembles, but has not yet been shown to function as, a splice acceptor site (28).
- Recent analysis of alternative transcripts produced by the AML1 gene (43) revealed that the putative IRES from that gene includes a 3′ splice junction sequence.
- The GC-rich VEGF IRES includes a proven transcriptional promoter (1). The authors of that study continue to call the VEGF sequence an IRES because there was residual low-level translation after the recognized promoter elements were deleted. As the letter (paragraph six) points out, however, "deletion of all transcription elements seldom completely abolishes [promoter] activity." The VEGF promoter was not recognized in another study (30), but the quality of RNA analyses therein certainly did not rule it out.
- The 1-kb long 5′ UTR from human c-sis mRNA which is said to function as an IRES contains binding sites (GGG CGG) for transcription factor Sp1. The corresponding rat gene has been shown to produce a second transcript with a short 5′ UTR initiated 15 nt upstream from AUG^{START} (53), which suggests the presence of an internal promoter.
- The putative IRES in Gtx mRNA might also function as a promoter, although this has not been proved. The active component of the "IRES" (CCGGCGGGT) imperfectly resembles an Sp1 binding site. RNA analyses that could have tested the promoter hypothesis were not carried out with the construct in which expression was elevated by reiterating this GC-rich element (6).

In short, the fact that a certain sequence allows downstream translation, while various controls do not, does not prove that the sequence that works is an IRES.

There are other reasons to worry about inadvertent production of spliced mRNAs from dicistronic vectors. The RP vector designed by A. E. Willis has been used to identify many candidate IRES elements (6, 7, 8, 9, 35, 51, 61, 62). An intron built into this vector upstream from the first cistron might make it easy to generate a surreptitious monocistronic mRNA: the candidate IRES need contribute only a cryptic 3′ splice site. Inadvertent splicing indeed occurred with the Willis vector in at least one case (51), although internal initiation was said to persist after removal of the intron. The vector used to test for IRES activity in NRF mRNA also has an upstream intron (49). The importance of careful RNA analyses is illustrated by a viral system in which a dicistronic vector unexpectedly pro-

duced both dicistronic and monocistronic mRNAs (23). Production of the spliced, monocistronic transcript occurred much more readily with LUC as the 3′ cistron than with the natural viral gene, which is noteworthy given the frequent use of *LUC* as the reporter gene. The authors speculated that translation of the natural viral gene occurs primarily from the dicistronic mRNA because the monocistronic mRNA is scarce in latently infected cells, but the fact that the monocistronic mRNA increases when lytic infection is induced (Fig. 4 in reference 23) complicates the judgment.

The letter (paragraph seven) mentions studies in which "in vitro synthesized mRNAs . . . were monitored in cell-free systems or examined after expression in cultured cells, and the RNAs were found to be intact." I don't know what this broad, undocumented statement means. Cellular IRES elements usually do not support translation efficiently in cell-free systems (32). In rare cases where a cellular IRES did allow efficient translation of the 3′ cistron in vitro, no attempt was made to show that the dicistronic mRNA remained intact (7, 47). The translatability of dicistronic mRNAs in vivo is what counts, and, for the reasons outlined above, the absence of unintended monocistronic transcripts certainly has not been proved in most cases.

The letter asserts that "Kozak is not alone in expressing concern" about the need for careful RNA analyses. Indeed, the new review by Hellen and Sarnow (25) has a nicely worded paragraph about the importance of determining that a vector produces only the intended dicistronic mRNA, but it is only lip service. Their table of cellular IRES elements makes no distinction between really careful studies (p58^{PITSLRE} [10]), studies that addressed the RNA issue via very, very faint Northern blots (Cat-1 and DAP5 [16, 26]), and studies that included no analysis of RNA structure at all (MYT2 and eIF4G [19, 36]).

The new review (25) extols a developmentally controlled IRES in fibroblast growth factor 2 mRNA, but that report (11) and similar studies with the c-myc IRES (12) did not establish that the dicistronic vector produces only dicistronic mRNA in mouse tissues that show translation of the 3′ cistron. Both reports included RNA analyses that documented the amount but not the form of mRNA. A tissue-specific or stage-specific IRES would be extremely interesting, but this should not be claimed until other explanations (tissue-specific splicing, tissue-specific promoters) have been ruled out. Is the "scholarly" review the one that repeats the premature claim or the one that explains why it is premature?

(iii) Other issues. In addition to the experimental problems discussed above, a major theoretical problem is posed by the absence of conserved sequences among cellular IRES elements. As the list of putative IRES elements grows, the problem only becomes more glaring. Given the complete absence of structural criteria, internal initiation is just a vague category into which every anomalous observation can be thrown.

Detailed structural studies carried out on the c-myc sequence raised hope that, at least in one case, a real structure might be implicated in IRES function. The problem is that, after carefully defining a double pseudoknot near the 5′ end of the mRNA (42), attempts to demonstrate the functional significance of the structure were disappointing. There was less than a two-fold reduction in internal initiation when the entire pseudoknot was deleted. Translation was reduced further, but still not abolished, when a downstream hairpin structure was also deleted. One of the loops has the sequence GGGAA (GGNRA), a stabilizing motif implicated in the function of many other folded RNAs; but substitution mutations in the c-*myc* hairpin loop actually augmented translation. The strongest decrease in translation was seen when an upstream AUG codon was inserted at various points in the c-*myc* sequence.

The putative IRES element derived from the 5′ end of c-myc mRNA is puzzling for other reasons. When transplanted to the midpoint of a dicistronic DNA vector, the c-myc sequence was shown to support efficient translation of the 3′ cistron, and RNA analyses (which I did cite) detected no exculpatory monocistronic mRNAs. The problem is that the dicistronic mRNA failed to support translation of the 3′ cistron when introduced through RNA transfection rather than the usual DNA transfection, implying the need for a nuclear experience. (The 5′ cistron was translated in those experiments, so there was a good internal control. The negative result is meaningful.) I suggested that the required nuclear event might involve splicing or a cryptic promoter, i.e., production of a monocistronic mRNA that simply was not detected by the RNA assays. The letter suggests instead that the c-myc IRES might require nuclear binding proteins. Others have proposed that adenine residues might have to undergo methylation in the nucleus in order for the dicistronic mRNA to support translation (32). The proponents of these ad hoc explanations seem unwilling to consider the simplest possibility—that the c-myc sequence might not be an IRES. The wisdom of Sherlock Holmes seems worth recalling. He advises that, when 9 out of 10 observations point in one direction, the one discrepancy should be considered the strongest clue.

The efficiency with which a new candidate IRES supports downstream translation is usually evaluated not by comparison to a normal monocistronic mRNA but by comparison to a picornavirus IRES. This sets the bar quite low, inasmuch as picornavirus sequences function poorly in some tests of internal initiation (33; Fig. 4 in reference 63). The usual rationale invoked for internal initiation is that the scanning mechanism cannot function efficiently when a 5′ leader sequence has upstream AUG codons or secondary structure, but that line of reasoning is undermined when the internal initiation mechanism also functions poorly. The letter asserts that "IRESs have been shown to represent a range of activities from weak to strong." The seemingly efficient cellular IRES elements, however, are mostly those for which RNA analyses are inadequate or missing (Table 1 in reference 39).

Initiation without Met-tRNA$_i$? The second section of my minireview concerned an unconventional mechanism of initiation postulated for some insect viruses. The experiments with *Plautia stali* intestine virus, which I cited only indirectly, showed that in vitro-synthesized capsid protein did not carry the usual N-terminal methionine (54). This was a provocative finding but it went no further. I focused on studies with CrPV because only in that case was a detailed mechanism proposed for initiating translation independently of Met-tRNA$_i$ (64). Much of that mechanism was based on toeprinting assays which to my eye did not show what was claimed regarding the position of the ribosome on the mRNA. The new review (25) recounts the "astounding" discoveries made with CrPV without responding to the questions I raised, point by point, about the toeprinting data. It would have been hard for Hellen and Sarnow (25) to address my concerns without even citing my review, which they did not.

In addition to toeprinting assays, the claim for a novel mechanism of translation rests on the fact that binding of CrPV mRNA to ribosomes was insensitive to standard inhibitors of initiation, such as L-methioninol and edeine. That insensitivity could mean either that initiation with CrPV mRNA follows a nonstandard pathway or that the observed mRNA-ribosome complexes are artifacts rather than functional intermediates in

initiation. This issue clearly concerned the authors, who argued that the edeine-resistant complexes detected in sucrose gradients are not artifacts because actual translation of luciferase, when directed by CrPV mRNA, was also resistant to edeine. This was true, however, only at very low concentrations. Figure 3K in reference 64 shows that CrPV translation was inhibited (80%) by 1 μM edeine, which is within the range (1 to 10 μM) normally used to inhibit initiation of translation in eukaryotes.

The letter tries to get around that result by arguing, in a confusing jumble of words, that 1 μM edeine inhibits the elongation phase of protein synthesis. The one study cited in support of that view used poly(U) as the template (4). Classical experiments showed that, with natural mRNAs, 1 μM edeine inhibits only the initiation step (31, 48). Those nontrivial experiments were conducted in a way that would have detected an effect on elongation, if such there were. Because edeine inhibits initiation and not elongation, it is frequently used to synchronize translation: after the first few minutes, 2 or 5 μM edeine is added to block further initiation so that various events that occur during elongation—such as ribosomal frameshifting or insertion of proteins into membranes—can be studied (29, 57, 59). Those experiments, carried out in many different laboratories, would not have worked if edeine inhibited elongation.

If one accepts that edeine inhibits only the initiation step, the argument regarding the authenticity of complexes detected by sucrose gradient analysis gets inverted. Because actual translation of luciferase directed by CrPV mRNA was sensitive to inhibition by 1 μM edeine (Fig. 3K in reference 64), the edeine-resistant complexes detected in sucrose gradients are likely to be artifacts. Nonfunctional sticking of CrPV mRNA to ribosomes could also explain the partial resistance to L-methioninol. The logic that applies when initiation complexes are sensitive to L-methioninol (40) does not hold when ribosome-mRNA complexes are resistant to the inhibitor.

The mechanism proposed for CrPV postulates that, without the usual binding of Met-tRNA$_i$ in the P-site, translation initiates with entry of Ala-tRNA into the A site. I suggested this should be tested directly by looking for binding of Ala-tRNA to CrPV mRNA-ribosome complexes. A positive result would support the claim that the complexes are functional. But that challenge received no response. Instead, because I used the word "aggregate" in that paragraph of the minireview, the letter jumps on me for questioning the oligomeric state of the RNA. As I used the word, "aggregate" clearly refers to nonfunctional complexes in which mRNA is merely adsorbed to the ribosome. I clearly was not questioning whether the mRNA per se was aggregated. This is an example of how the letter diverts attention to irrelevant side issues while ignoring the substantive concerns raised in the minireview.

The fact that CrPV capsid protein can be translated in vitro from genomic RNA could be an artifact, inasmuch as in vitro translation systems sometimes allow internal initiation that does not reflect what happens in vivo (2, 21, 24, 46, 56). In vitro translation of CrPV capsid protein via the putative IRES was inefficient (e.g., Fig. 6 in reference 65), which is grounds for questioning its authenticity. In vitro translation using mRNA from a related insect virus was also inefficient and inexact (Fig. 2 in reference 13). When cellular IRES elements function poorly, one can argue that cells might not need much of the protein, but viral capsid proteins are needed in large quantities. That is why so many other viruses in which the capsid protein is encoded at the 3′ end of the genome produce a subgenomic mRNA.

Perhaps I should have mentioned a study by Eaton and Steacie (14) in which no subgenomic mRNA was detected by labeling CrPV-infected cells with [^{3}H]uridine for 3 h, but I omitted the reference because the technique was not sensitive enough to prove the point. It is not fair to criticize a study from 20 years ago that used the best technique then available; it is fair to expect a key point to be reinvestigated using sensitive, modern techniques. The Northern blot proffered by Wilson et al. (65), which examined RNA from one unstated time point in the infection, is far from adequate to prove absence of a subgenomic mRNA. Wouldn't it be better to look carefully for a subgenomic mRNA than to claim the question was settled by "compelling and rigorous" experiments from 20 years ago?

I don't know why the low-level in vitro translation of these insect virus RNAs requires preservation of the pseudoknot. It could be something as trivial as targeting an RNase (15), or the structure really might be an IRES. In vitro translation clearly occurs in the absence of an AUG codon, which is surprising and interesting even if a more conventional mechanism turns out to operate, via a subgenomic mRNA, in vivo. I did not claim to have disproved that an unusual mechanism of initiation operates with these viruses. I said only that the experiments in reference 64 had serious deficiencies and therefore the postulated mechanism awaits proof.

Closing notes. The letter to the editor exposes no relevant issues that were ignored in my minireview. I did not discuss the literature on initiation factors because no candidate IRES of cellular origin has been shown to bind eIF4G or other initiation factors. Even if an initiation factor were shown to bind (several of the factors are general mRNA-binding proteins), it would prove little without functional tests, i.e., evidence that the prebound factor can mediate ribosome entry. That chase experiment has not yet been carried out with EMCV RNA to verify that the tight binding of eIF4G/4A (44) has functional consequences.

The letter condemns my failure to discuss putative IRES elements in viral mRNAs, but my short review was focused on cellular mRNAs. If a review is to be condemned for omissions, attention might be directed to lengthy reviews of viral translation (17, 50) that make no mention of the polycistronic mRNAs produced by adenoviruses, papovaviruses, retroviruses, coronaviruses, hepatitis B virus, brome mosaic virus, tobacco mosaic virus, etc. All these viruses produce polycistronic mRNAs in which the downstream cistrons are silent because of constraints imposed by the scanning mechanism. The silent cistrons are activated upon being relocated to the 5′ end of smaller transcripts. Reviews that ignore this remarkable body of literature while extolling the slightest hint of internal initiation give students a distorted view of how translation operates in eukaryotes.

I agree that the hairpin test mentioned in the letter would be a good alternative test for internal initiation, if it were accompanied by careful RNA analyses to ensure that the hairpin barrier is not circumvented by mRNA breakage or splicing or a downstream promoter. But the hairpin test is usually used as a shortcut—a substitute for carefully monitoring mRNA structure—and for that reason it is not determinative. Circularization of the mRNA would also be an excellent alternative test for internal initiation, but since that test has not been employed with any cellular IRES sequence, it seems unfair to fault me for not mentioning it. Indeed, the circularization test has not been attempted with any viral IRES other than EMCV.

The accusation that the minireview contains "numerous distortions of fact and of published data" would be serious, if true, but the letter misrepresents what I said in an attempt to prove the charge. In the discussion of vectors (see above), for exam-

ple, what I actually said about tests of the BiP IRES in no way resembles what the letter asserts. The letter accuses me of not presenting the data in reference 23 "fully and accurately," but because that paper concerns a viral IRES, it was not discussed at all in the minireview. It is the letter to the editor, not my minireview, that misrepresents established facts about edeine by invoking a result obtained with poly(U) that does not apply to natural mRNAs. The letter condemns my failure to cite various control experiments which, as explained above, simply did not prove what was claimed.

A scholarly review is not one that cites every paper but one that thoughtfully re-views what has been published. When the papers pertaining to internal initiation are stacked on one's desk, the pile looks overwhelming. But when the data are extracted and spread out in table form—how active was the sequence, what was the baseline, etc.—holes become apparent. Not every paper had the same flaw, but almost every paper had a major flaw or uncertainty (39). An overwhelming stack of papers does not equate with overwhelming proof. The letter defends these papers on the grounds that they were published in prominent journals. I wrote the minireview as a plea for stricter standards by those journals, whose editors now have a convenient list to check against when selecting referees.

When my minireview was submitted for publication, one of the referees who evaluated the manuscript wrote as follows: "I think the valid criticisms raised here are generally recognized by the major researchers in the translation field (although not always followed!). Therefore this audience will learn little or nothing new. For those outside the field, the review may inappropriately cause them to dismiss the possibility of alternate mechanisms of initiation, which would be a disservice to the scientific community." That referee's advice was disregarded perhaps because the editor believes, as I do, that people outside the field—people who attempt to put the "alternate mechanisms" to work—are entitled to know the problems.

I will save the curious reader the trouble of counting the names appended to the Letter to the Editor: there are 87 votes in favor of cellular IRES elements and associated phenomena. Some of the signers (e.g., Drs. Farabaugh, Filipowicz, Goldman, and Krug) work on subjects completely unrelated to the content of the minireview; they must have studied hard to qualify as judges. The letter was composed and circulated by Drs. Schneider and Sonenberg. Many of the signers have close links to Dr. Sonenberg, either as coauthors or members of the same institution.

It is obvious that the organizers worked hard to collect all those signatures, but to what end? A single voice suffices to present a logical argument. I might be alone in refusing to believe a story with so many flaws, but that does not mean I am wrong. Counting the votes determines the answer in politics (Florida excepted) but not in science.

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