SURVEY AND SUMMARY

A second look at cellular mRNA sequences said to function as internal ribosome entry sites

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ABSTRACT

This review takes a second look at a set of mRNAs that purportedly employ an alternative mechanism of initiation when cap-dependent translation is reduced during mitosis or stress conditions. A closer look is necessary because evidence cited in support of the internal initiation hypothesis is often flawed. When putative internal ribosome entry sequences (IRESs) are examined more carefully, they often turn out to harbor cryptic promoters or splice sites. This undermines the dicistronic assay, wherein IRES activity is measured by the ability to support translation of the 3′ cistron. Most putative IRESs still have not been checked carefully to determine whether the dicistronic vector produces only the intended dicistronic mRNA. The widespread use of the pRF vector is a major problem because this vector, which has *Renilla* luciferase as the 5′ cistron and firefly luciferase as the 3′ cistron, has been found to generate spliced transcripts. RNA transfection assays could theoretically circumvent these problems, but most candidate IRESs score very weakly in that test. The practice of calling even very weak results 'positive' is one of the problems discussed herein. The extremely low efficiency of putative IRESs is inconsistent with their postulated biological roles.

'. . .if it is a Miracle, any sort of evidence will answer, but if it is a Fact, proof is necessary'

—Mark Twain, *Letters from the Earth*

INTRODUCTION

Generalizations about when and how the internal initiation mechanism operates are meaningful only if credible examples underlie the generalizations. A popular idea is that translation of certain genes might be maintained via internal initiation when the overall translational capacity is reduced during mitosis, apoptosis and other special conditions. A recent compilation of cellular mRNAs said to harbor such regulable internal ribosome entry sequences (IRESs) (1) provides a convenient starting point—a defined set of examples—for evaluating this idea. Table 1 herein reproduces, with some added details, the list of IRESs compiled by Komar and Hatzoglou (1).

A few candidate IRESs listed in the original table have been omitted here for reasons explained in the first footnote to Table 1. The reason for omitting six mRNAs described by Qin and Sarnow (44) might require explication, as the problem is not unique to that case. Their dicistronic vector contained a big chunk of the EMCV IRES to which each candidate cellular IRES was appended. Although it was a defective version of the EMCV IRES (i.e. not active on its own), there was no good reason for it to be there and it might have stacked the deck. The same vector had been used previously to identify IRES activity in *pim-1* mRNA (55), and that claim did not hold up. When the *pim-1* sequence was re-tested without the EMCV appendage, it had no detectable IRES activity; cryptic promoter activity was found instead (56).

Table 1 lists 27 mRNAs. In none of these cases is the evidence for IRES activity convincing. Most can be dismissed either because the sequence harbors a transcriptional promoter or splice site, or because the demonstrated IRES activity is extremely weak, or for both reasons. The next section briefly discusses these and other deficiencies in the evidence for internal initiation. Some (not all) of these problems were pointed out in earlier reviews, but it is important to see how these issues apply to the set of mRNAs currently held to be translated via internal initiation.

OVERVIEW OF EVIDENCE AND REASONING

Problems with dicistronic test for IRES activity

The ability to demonstrate internal initiation should not depend on the choice of reporter genes, but it does. Hennecke

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Table 1. Cellular mRNAs said to harbor IRES elements that purportedly function during apoptosis (entries #1–7), mitosis (#7–12) or heat shock and other stress conditions (#13–19). Tissue-specific or developmental-specific IRES activity is claimed for entries #20–27[a]

mRNA[b]	Dicistronic vector[c]	Efficiency[d]	Promoter or splicing detected (or strongly suspected)	Comments[e]	Reference
(1) Apaf-1	pRF+	Weak		Inadequate or no RNA analysis.	(2–4)
(2) XIAP	pRF+	Weak (via RNA transfection)	Splicing detected.	Inadequate RNA analysis in earlier studies.[f]	(5)
(3) DAP5	Various	Weak		Inadequate RNA analysis.	(2,6)
(4) Protein kinase Cδ	pRF	Weak		Inadequate RNA analysis.	(7)
(5) Bcl-2	pRF	Weak (via RNA transfection)	Splicing detected.	Cryptic promoter ruled out.	(8)
(6) c-IAP1/HIAP2[g]	pRF	Weak (via RNA transfection)	Splicing detected.	Cryptic promoter ruled out.	(9)
(7) c-myc	pRF+	Strong only with pRF (see text)		Inadequate or no RNA analysis; *RNA transfection fails.*	(2,10,11)
(8) ODC	CAT-LUC	Weak		Inadequate RNA analysis.	(12)
(9) PITSLRE kinase	pRF	Weak		Cryptic promoter ruled out; no RNA analysis to rule out splicing (see text).	(13)
(10) hSNM1	RFP-GFP	Weak		No RNA analysis.	(14)
(11) Sp3 transcription factor[h]	pRF	?		Tested only *in vitro*; works better in reverse orientation.	(15)
(12) p27^{Kip1} (human)	pRF	Weak (see text)	Cryptic promoter detected (17).	Inadequate RNA analysis (16); *RNA transfection fails* (17).	(16,17)
(13) VEGF	Various	Weak (when promoter deleted)	Cryptic promoter detected (18).	Inadequate (19) or no RNA analysis (20).	(18–20)
(14) HIF-1α	pRF	Varies	Cryptic promoter possible in GC-rich 5'-UTR, but not yet verified.	Inadequate or no RNA analysis.	(21,22)
(15) Cat-1	CAT-LUC	Weak		Inadequate RNA analysis.	(23,24)
(16) AT$_1$R	pRF	Weak		Cryptic promoter ruled out; RNA analyses not adequate to rule out splicing.	(25)
(17) Bag-1	pRF	Varies with cell type		Inadequate RNA analysis; *RNA transfection fails.*	(26,27)
(18) BiP	Various	Weak		Inadequate RNA analysis.	(2,28,29)
(19) Rbm3	pRF	Strong	Splicing is probable based on mapping of IRES to a 22 nt sequence which resembles splice acceptor (Y$_{10}$CAG).	Cryptic promoter ruled out; no RNA analysis to rule out splicing.[i]	(30)
(20) FGF-2	pRF	Varies		Inadequate or no RNA analysis.	(31,32)

(21) FGF-1	pRF	Varies with cell type	Cryptic promoter ruled out only in MEF-3T3 cells; data said to 'rule out splicing' not shown. Inadequate RNA analysis.	(33)
(22) N-myc	pRF	Strong	Inadequate RNA analysis.	(34)
(23) NRF	pRF	Strong	Splicing probable. cDNA with long 5'-UTR (said to be IRES) is erroneous; it diverges from the correct cDNA (36) at what looks like splice junction. Inadequate RNA analysis.	(35,36)
(24) βPix-b$_L$[j]	RFP-GFP	Weak		(37)
(25) c-sis/PDGF2	CAT-LUC or pRF	Weak (38)	Cryptic promoter detected (33,39). *RNA transfection fails.*	(33,38,39)
(26) RUNX1/AML1	CAT-LUC	Weak	Splicing is probable because IRES derives from an intron and includes splice acceptor site (41). Inadequate RNA analysis.	(40,41)
(27) MYT2[j]	CAT-LUC	Weak	Splicing is probable based on presence of sequence (Y$_n$AG/GU) which resembles splice acceptor. No RNA analysis.	(42)

[a]The table of 'regulatable IRESs' compiled by Komar and Hatzoglou (1) is here reproduced with a few omissions. Survivin is omitted because it was never tested for IRES activity. The only IRES mentioned in the cited Ref. (43) comes from encephalomyocarditis virus (EMCV); i.e. survivin was expressed as the 5' cistron from a vector which used the EMCV IRES to drive expression of a selectable 3' reporter gene. Six entries proposed by Qin and Sarnow (44) are omitted because the candidate cellular IRESs were not tested independently; they were appended to a fragment of the EMCV IRES. Five entries from *Drosophila* (45–47) and one yeast gene (48) have also been omitted, thereby limiting the table to mammalian genes.

[b]mRNAs are abbreviated as follows: Apaf-1, apoptotic protease-activating factor 1; XIAP, X-linked inhibitor of apoptosis protein 1; ODC, ornithine decarboxylase; SNM1, sensitivity to nitrogen mustard; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible transcription factor; c-IAP1, cellular inhibitor of apoptosis protein 1; DAP5, death-associated protein 5; Cat-1, cationic amino acid transporter 1; AT$_1$R, angiotensin receptor; BiP, immunoglobulin heavy chain binding protein; Rbm3, RNA-binding motif protein 3; FGF, fibroblast growth factor; NRF, NF-κB repression factor; βPix, Pak-interacting exchange factor; PDGF2, platelet-derived growth factor 2; RUNX1, *runt*-related transcription factor; MYT2, myelin transcription factor 2.

[c]The plus sign means a vector other than pRF was used for some experiments. The RFP and GFP reporter genes encode red and green fluorescent proteins. Cryptic splice donor sites detected in the Rluc (5) and chloramphenicol acetyltransferase (CAT) (49) reporter genes might complicate interpretation of the dicistronic test.

[d]'Weak' means the candidate IRES augments translation of the 3' cistron ≤5-fold when compared with the empty dicistronic vector, or that the cellular IRES is ≤5% as active as the EMCV IRES. Unless otherwise indicated, the data are from DNA transfection experiments. In cases where DNA transfection results are not meaningful because splicing or cryptic promoter activity was detected, RNA transfection results are cited.

[e]'Inadequate RNA analysis' means the proffered evidence was not sufficient to prove the dicistronic DNA vector produces only the intended dicistronic mRNA. 'RNA transfection fails' means the 3' cistron was not translatable when cells were transfected directly with dicistronic mRNA. All entries pertain to expression in cultured cells, unless otherwise stated.

[f]One study with XIAP did include controls to rule out a cryptic promoter, but only a faint Northern blot was proffered as evidence against splicing [(50); βgal-CAT vector]. The XIAP sequence is now known to harbor a strong splice acceptor site (5) which explains its strong 'IRES' activity when tested by DNA transfection (2).

[g]The HIAP2 sequence was said to harbor an inducible IRES based on experiments with a βgal-CAT dicistronic vector; but the putative IRES activity measured via the CAT reporter gene did not match the response of the endogenous HIAP2 gene (51). Whereas over-expression of an 86-kDa fragment of DAP5 protein caused a 20-fold increase in CAT translation [Figure 4A in Ref. (51)], shown without RNA analysis], the endogenous HIAP2 protein barely increased [Figure 4B in Ref. (51)]. In contrast, treatment of cells with thapsigargin strongly stimulated production of the endogenous protein but barely stimulated expression of CAT from the dicistronic construct [≤2-fold; Figure 2A in Ref. (51)].

[h]The claim of internal initiation is untenable for Sp3, given that the putative IRES was tested only in conjunction with (i.e. appended to) a fragment of the EMCV IRES, and translation of the 3' cistron was more efficient when the putative Sp3 IRES was inverted. Cell-cycle regulation of the putative IRES was not tested in any way. The natural Sp3 gene produces a multiplicity of proteins (52) and a multiplicity of transcripts (53).

[i]The possibility of splicing was not ruled out by the faint northern blot shown in an earlier study (54), and the stated ability of the Rbm3 sequence to support translation of the 3' cistron *in vitro* [gels not shown, reaction conditions not revealed; (30)] is not sufficient to rule out splicing.

[j]Komar and Hatzoglou (1) include βPix-b$_L$ and MYT2 in their list of regulatable IRESs, but the cited references (37,42) show only that production of the endogenous protein might be developmentally regulated. IRES activity, such as it is, was not shown to be regulated.

et al. (57) found that the poliovirus IRES works when inserted into pRF but not when the order of reporter genes is reversed. The function of the c-myc IRES also depends strongly on the choice of vectors: the c-myc sequence supported efficient translation of the 3′ cistron when inserted into pRF (10,11); but when tested with a βgal/CAT vector, the c-myc IRES was 20- to 100-fold less active than the EMCV control (2). The discovery of a splice-donor sequence within the *Renilla* luciferase (Rluc) gene (5) might explain why detection of 'IRES' activity is so easy using the pRF vector. Whatever the reason, it is worrisome that so many claims of internal initiation are based on tests with one particular dicistronic vector (Table 1).

For some entries in the table, the published studies included no RNA analyses at all. In many other cases, the RNA analyses were not sufficient to prove that the dicistronic vector produces only the intended dicistronic mRNA. The table shows a remarkable number of cases in which followup experiments indeed uncovered splicing or cryptic promoter activity within a putative IRES.

For some other entries, the possibility of a cryptic promoter was ruled out but the possibility of splicing was not addressed. Splicing cannot be ruled out simply by demonstrating that the dicistronic mRNA supports low-level translation *in vitro*. A different artifact—namely, cleavage of the mRNA—might undermine *in vitro* tests, especially when the temperature is raised to 37°C (13,58,59) or the incubation is prolonged [e.g. 90 min in Ref. (9,24,51)]. Investigators sometimes point to lack of inhibition by cap analogs, or by a base-paired structure appended to the 5′ end of the mRNA, as evidence for internal initiation; but translation from broken mRNAs also would be resistant to those manipulations.

When translation of the 3′ cistron is inefficient (see next section), northern blots—proffered as evidence that the dicistronic mRNA is the sole transcript—mean nothing. Northern blotting sometimes fails to detect a low-abundance monocistronic mRNA which can be demonstrated via more sensitive assays (5,60). Thus, entries in Table 1 are marked 'inadequate RNA analysis' when the only attempted assay was northern blotting or when an RNase-protection assay, used to verify presence of the intended dicistronic mRNA, did not rule out the possibility that a monocistronic transcript might also be produced.

Picornavirus RNAs are often used as controls when testing other candidate IRESs, but these controls also need controls to check for splicing and cryptic promoters. Smaller-than-dicistronic mRNAs are produced sometimes even with putative IRESs from picornaviruses (61–63).

Weak IRES or no IRES?

When the 3′ cistron is translated very inefficiently, there are two reasons to worry. One concerns the sensitivity required for meaningful RNA analyses, as explained above. The other is whether the results of the dicistronic test should even be called positive.

An underlying problem is that the negative control—e.g. the 'empty' pRF vector with no special intercistronic insert—is never negative. There is always some translation of the 3′ cistron, and this unexplained background expression is not constant. Background translation of the 3′ firefly luciferase (Fluc) cistron varied >10-fold when three different negative controls were tested by RNA transfection [Figure 5C in Ref. (9)]. When tested *in vitro*, translation of the 3′ cistron increased 10-fold upon simply lengthening the intercistronic domain [Figure 9 in Ref. (64)]. Whatever the reason for this background, its variability would seem to set a lower limit on what level of expression constitutes a credible positive result when a candidate IRES is tested.

Tests for internal initiation often use the putative IRES from EMCV as a high-end control, but it really is not very high. When a dicistronic mRNA containing the EMCV sequence was tested by RNA transfection, the yield of Fluc from the 3′ cistron was only 3% of the Rluc yield (56). By convention, this counts as a positive control because it represents an 18-fold increase over Fluc production from the empty pRF vector; but readers need to understand what '18-fold increase over the empty vector' really means: even the paradigmatic EMCV IRES functions poorly. The putative IRES from hepatitis C virus (HCV) also is used often as a positive control, but it should not be: it is unsuitable for DNA transfection assays because the HCV sequence harbors a cryptic promoter (65), and it barely supports translation when dicistronic mRNAs are tested directly *in vitro* or via RNA transfection [see point (iv) below].

Many entries in Table 1 are marked 'weak' without providing details because the table would be unreadable if everything were written out. Here is what weak means in five cases (entries #1, #5, #6, #15 and #18).

(i) When the Apaf-1 sequence was tested by DNA transfection in HeLa cells using a dicistronic βgal-CAT vector, Apaf-1 was only ∼2% as active as the EMCV IRES (2). It was somewhat more active when tested with the pRF vector (4).

(ii) The BiP IRES was only ∼3% as active as EMCV when tested by DNA transfection using βgal-CAT constructs (2). When activity was judged by comparison with an empty dicistronic CAT-LUC vector, the BiP IRES elevated translation of the 3′ cistron only 2.5-fold [Figure 5 in Ref. (28)]. When translational efficiency was assessed *in vitro*, a 50-fold higher concentration of mRNA had to be used when the BiP IRES replaced the EMCV IRES (59).

(iii) The putative IRES from cat-1 mRNA was similar in efficiency to BiP when dicistronic constructs were tested by DNA transfection (23). When translation was tested *in vitro*, the yield of LUC from a dicistronic mRNA (CAT-LUC) containing the cat-1 IRES was only 1% of the yield from a monocistronic control mRNA [Figure 5C in Ref. (24)]. Elaborate explanations of how changes in secondary structure regulate the function of the cat-1 IRES amount to putting the cart before the horse: the 4- or 5-fold increase in translation of the 3′ cistron when the IRES is 'activated' is still so low, given the starting point, that calling it an IRES is not justified.

(iv) Because splicing was detected when the c-IAP1 sequence was tested by DNA transfection, those results are not usable. When tested by RNA transfection, the c-IAP1 sequence increased translation of the 3′ cistron only 2-fold, compared with a null control (9). When tested *in vitro*, the c-IAP1 sequence supported translation of the 3′ Fluc cistron as efficiently as the putative IRES from

HCV; but the HCV IRES barely worked. (The autoradiogram in Figure 2B in Ref. (9) was not quantified, but it is clear that the yield of Fluc from the dicistronic mRNA was much lower than from a monocistronic mRNA.)

(v) When the Bcl-2 sequence was tested by RNA transfection using a pRF-based vector, Bcl-2 was only 6% as active as the HCV IRES (8).

Two additional points about efficiency need to be mentioned. One is that a weak IRES such as HCV or BiP is sometimes made to look stronger by comparing it, not with the empty vector, but with a construct that has a highly structured intercistronic sequence (8,29,63). The proffered rationale—that the structured insert 'reduces readthrough'—makes no sense, given that eukaryotic ribosomes cannot readthrough (i.e. cannot reinitiate) following the translation of a full-length 5′ cistron. A more reasonable interpretation is that the structured insert precludes cleavage of the dicistronic mRNA. The 'IRES' looks strong only because it replaces this inhibitory element.

The second point is that, in many cases, the efficiency is concealed by setting at 1.0 (or 100%) the yield of protein obtained from the 3′ cistron in the presence of the candidate IRES—without reference even to the empty dicistronic vector—and then focusing on accessory proteins or growth conditions which slightly increase translation of the 3′ cistron [Figure 5 in Ref. (27); (58,66–69)]. Thoughtful readers will recognize that this tactic dodges the basic question of whether the 'IRES' functions well enough to be called an IRES.

A closer look at putative IRES elements said to function during mitosis

Two problems undermine the hypothesis that, when overall translation declines during mitosis, the mRNAs that remain polysome-associated might be translated via internal initiation. One problem is that retention on polysomes is not a reproducible criterion. Pyronnet *et al*. (12) reported that ODC and c-myc mRNAs are retained on polysomes in cells blocked at G2/M, but these mRNAs were not found on polysomes in a followup study (44). The bigger problem is that ODC and c-myc 5′-untranslated regions (5′-UTRs) do not score convincingly when IRES activity is tested directly. The sequence from ODC mRNA stimulated translation of the 3′ LUC cistron <2.5-fold [Figure 4D in Ref. (12)]. The putative IRES from c-myc mRNA was extremely weak [1–5% as active as EMCV, depending on cell type; Ref. (2)] except when the vector was pRF (10); and the experiments with pRF-based constructs lacked adequate controls to rule out splicing or a cryptic promoter.

A putative IRES derived from the coding domain of the PITSLRE kinase gene scored strongly when first tested: it mediated a 40-fold increase in translation of the 3′ βgal cistron under conditions where only a single form of mRNA could be detected (70). This nearly convincing story did not hold up, however. In a followup study with a different vector, the PITSLRE sequence stimulated translation only ~6-fold [nt 745–1125; Figure 1C in Ref. (13)]. The production of two protein isoforms from the natural gene is not in doubt: p110^{PITSLRE} accumulates throughout the cell cycle while p58^{PITSLRE} increases 20-fold in G2/M. What is not certain is whether both isoforms are translated from the same

mRNA. The natural gene does generate alternatively spliced transcripts (71). Additional studies are needed to determine the structure of transcripts produced when the PITSLRE sequence is transposed to a synthetic dicistronic vector. Control experiments eliminated the possibility of a cryptic promoter (13), but the possibility of splicing was not ruled out.

The p27^{Kip1} protein inhibits cyclin-dependent kinase activity, causing arrest in the G$_1$ phase. Expression of p27^{Kip1} appears to be regulated at the level of translation, as evidenced by a substantial shift of the mRNA to heavier polysomes during G$_0$ and G$_1$ (72); but the mechanism of regulation cannot be understood without additional information, such as checking for a possible change in structure of the 5′-UTR. Cho *et al*. (16) postulate that p27^{Kip1} mRNA harbors an IRES which is enhanced by polypyrimidine tract binding protein (PTB), and that changes in the concentration of PTB contribute to cell-cycle control of p27^{Kip1}. Neither the claim of internal initiation nor the putative involvement of PTB is supported by credible evidence. The results obtained by DNA transfection are not reliable because cryptic promoter activity has been detected in the p27^{Kip1} 5′-UTR (17). Thus, the claim of IRES activity must rest on RNA transfection and *in vitro* translation experiments. But those results [Figure 4 and Figure 6F in Ref. (16)] were presented in a way that conceals the efficiency of translation of the 3′ cistron: the Fluc/Rluc yield from a construct bearing the p27^{Kip1} sequence was simply set at 1.0. A 2-fold increase in Fluc/Rluc ratio when PTB was added to the system, or a 2-fold decrease when PTB was depleted by small interfering RNA (siRNA), is cited as evidence that PTB augments the function of the p27^{Kip1} IRES. In this way, attention is directed away from the basic question—whether p27^{Kip1} mRNA indeed possesses IRES activity—to showing that PTB helps. Demonstrating slight stimulation by a protein called an 'IRES *trans*-activating factor' (ITAF) is not a substitute for showing directly that the mRNA sequence functions as an IRES.

Are there regulable IRES elements?

Claims of tissue-specific IRES elements are premature because the most obvious alternative—a tissue-specific change in mRNA structure via splicing or alternative promoter usage—was not examined. Créancier *et al*. (73) claim the c-myc IRES is developmentally controlled in transgenic mice, i.e. translation of the 3′ Fluc cistron was detected in embryonic tissues but not in adults. The cursory RNA analyses were limited to adult tissues, however, leaving the essential point unexamined. Inadequate RNA analyses also undermine the claim that the FGF-2 IRES functions specifically in the brain of adult mice: only the amount of mRNA, not its (dicistronic?) structure, was examined in various tissues (31).

Four recent examples show how 'old problems' continue to undermine new reports of regulable IRESs.

(i) During hypoxia, the reduction in overall translation is modest [e.g. 50% reduction in Figure 2A of Ref. (21)]. Since cap-dependent translation is not fully shut off, an alternative mechanism of initiation is not really required to explain the continued translation during hypoxia of mRNAs such as Tie2 (74) and HIF-1α (21). When the Tie2 5′-UTR was tested for IRES activity via transposition to a synthetic dicistronic mRNA, it was only 3% as active as

the poliovirus IRES [Figure 6C in Ref. (74)]. It makes no sense to invoke this meager IRES activity (if such it is) to explain the sustained translation of Tie2 during hypoxia; the numbers do not add up. The HIF-1α sequence was not tested via RNA transfection, and its seemingly strong IRES activity in DNA transfection experiments might be explained by a cryptic promoter.

(ii) Questions about efficiency also undermine the claim of IRES activity in methionine synthase (MS) mRNA (75). The MS sequence was about as active as BiP, but use of BiP as the only positive control sets the bar very low. (The negative control was made to look worse by inserting, at the midpoint, a 400 nt chunk of *Drosophila* antennapedia cDNA. Simply removing this big, structured insert would increase translation of the 3′ LUC cistron, irrespective of whether the sequence that replaces it really is an IRES.) Most experiments sidestepped the question of efficiency by setting at '100%' the LUC yield from a dicistronic construct that contains the MS sequence. An increase to '140%' in the presence of vitamin B_{12} is the proffered evidence for regulation.

(iii) In contact-inhibited differentiated cells, wherein cap-dependent translation is said to be reduced, translation of connexin proteins Cx26 and Cx43 is postulated to occur via internal initiation (76). The basic finding is that accumulation of Cx26 and Cx43 proteins increases ∼10-fold in density-inhibited cancer cells. Because mRNA levels increased only 2- to 3-fold, the overexpression of connexins was said to be 'mainly translational.' But that conclusion is not justified on two counts: an increase in translation might be secondary to a change in structure of the 5′-UTR, which was not checked; and increased accumulation of connexin proteins might result from slower turnover rather than increased synthesis. The latter possibility was not ruled out by an experiment which monitored protein accumulation after labeling cells for 30 min with [^{35}S]methionine. The amount of protein that accumulates over 30 min is the sum of synthesis plus degradation; a much shorter pulse (e.g. 3 min) is required to measure the rate of protein synthesis *per se* (77).

The up-regulation was attributed to IRES-mediated translation based on two additional findings: elevated production of Cx26 and Cx43 proteins was insensitive to rapamycin, which impairs the function of eukaryotic initiation factor 4E (eIF4E); and the 5′-UTRs of Cx26 and Cx43 were able to support translation from a dicistronic vector. The first finding is meaningful only if rapamycin treatment of these cells indeed inhibits cap-dependent translation. The authors say this was established by showing that rapamycin strongly reduces expression of cyclin D1 (as it does); but manipulation of eIF4E is known to control the expression of cyclin D1 at the level of mRNA transport, not translation (78). Thus, cyclin D1 is an inappropriate control, and the point was not proven. As for the experiments with dicistronic vectors, suffice it to say that no RNA analyses were carried out. A control experiment ruled out the possibility of a cryptic promoter in the Cx26 sequence, but the Cx43 sequence was not tested for promoter activity, and the possibility of splicing was not examined in either case. The vector was pRF.

(iv) Miura *et al.* (79) postulate an inducible IRES to explain the increased translation of utrophin when muscle regeneration is induced by treating mice with cardiotoxin. When IRES activity was tested via a synthetic dicistronic mRNA, however, cardiotoxin caused only a 3-fold increase in translation of the 3′ CAT cistron [Figure 5B in Ref. (79)]. This contrasts with a 14-fold increase in translation of utrophin from the natural mRNA [Figure 2A in Ref. (79)]. Even if the magnitude of induction were greater in tests with the dicistronic mRNA, the point would not be proven. We have to know whether the absolute yield of (CAT) protein from the dicistronic construct is anywhere near the amount of protein produced normally from monocistronic utrophin mRNA; i.e. does the 'IRES' work well enough to function in the real world? This is unknowable because the only point of reference was the empty dicistronic vector.

The biggest fault with the utrophin story might be failure to examine the natural phenomenon carefully before attempting to reconstruct it. Several studies in mice documented increased production of utrophin protein under conditions where the amount of mRNA showed little or no change; but these studies did not check for a possible change in structure of the mRNA [e.g. shortening of the 5′-UTR], which could underlie the increase in translation.

Ideas borrowed (wrongly) from picornaviruses

Cellular mRNAs that remain polysome-associated during poliovirus infection are said to be probable candidates for internal initiation (55,80,81). Pim-1, eIF4G, BiP and c-myc were among the mRNAs found to be retained on polysomes; and experiments with dicistronic DNA vectors were said to confirm IRES activity in each case. But subsequent experiments uncovered cryptic promoter activity in the pim-1 and eIF4G sequences (56,60). When tested by RNA transfection, the pim-1 and c-myc sequences displayed no IRES activity (10,56), and eIF4G barely scored [e.g. the Fluc yield was ∼1% of Rluc in Figure 5 of Ref. (82)]. The BiP 5′-UTR scores very weakly in tests for internal initiation no matter which assay is used (see above).

The point here is not just that these cellular mRNAs arguably were misidentified as IRESs, but that the reasoning on which the original predictions were based is faulty. The popular belief that cleavage of eIF4G impairs cap-dependent translation is not upheld by the facts. In neuronal cells infected by poliovirus, eIF4G was cleaved extensively by 3.5 h post-infection and cleavage was complete by 5 h, but host protein synthesis continued unabated for at least 9 h (83). Reconstruction experiments confirm that the truncated form of eIF4G generated by the poliovirus-encoded protease can still support translation of capped mRNAs (84). Thus, translation under conditions where eIF4G gets cleaved is not grounds for postulating the involvement of an IRES.

ITAFs are another idea that originated with picornaviruses. These proteins are used as a crutch to explain whatever needs explaining; but it is a phantom crutch. To explain why starvation for 9 h is required to activate the putative IRES in cat-1 mRNA, Yaman *et al.* (69) postulate that an 'essential ITAF' has to be synthesized; but no evidence for such a protein has been uncovered. In other cases, although a protein has been

found to bind the IRES, claims about how the protein augments IRES function are based on weak, convoluted evidence are based on weak, convoluted evidence. An earlier review (85) explains what 'convoluted' means in two cases (86,87). A recent study which declares PTB to be a 'universal ITAF' (88) is flawed from the outset: none of the mRNA sequences shown to bind PTB was shown convincingly to harbor IRES activity. The proffered northern blots were not adequate to rule out splicing when the sequences were tested by DNA transfection using pRF-based vectors. And the claim that the candidate IRESs support translation *in vitro* is meaningless because the efficiency of translation of the 3′ Fluc cistron was never revealed: the Fluc/Rluc yield from a dicistronic mRNA containing the candidate IRES was set at 1.0 and shown to increase 2- or 3-fold upon addition of PTB [e.g. Figure 4G in Ref. (88)].

There simply is no meaningful evidence that RNA-binding proteins augment internal initiation. The p27^{Kip1}/PTB story discussed above typifies the findings: a protein which has no recognizable connection to translation (PTB is a splicing factor) produces a very small effect upon a target sequence which arguably is not an IRES. PTB is one of about a dozen proteins that bind weakly to the p27^{Kip1} mRNA leader sequence [Figure 1 in Ref. (16)], which raises the question of why the authors chose to focus on that particular protein. The reason they give is that PTB binds (strongly) to the EMCV IRES. What this reasoning-by-analogy ignores is that the binding of PTB to EMCV mRNA has no real functional consequences: although PTB stimulated translation using a laboratory-derived version of the 5′-UTR, translation was independent of PTB when the wild-type 5′-UTR was appended to the natural coding domain of EMCV (89).

With poliovirus, both PTB and La autoantigen are said to function as ITAFs. Binding of PTB is barely detectable even with the wild-type 5′-UTR, however, and the suggestion that inefficient binding of PTB might account for the attenuation of vaccine strains makes no sense (90). In cells depleted of La, production of viral proteins was reduced only late in the infection when a secondary burst of protein synthesis occurs from newly synthesized mRNAs (91). Thus, the effects of La protein might be on viral RNA synthesis rather than directly on viral translation. Only one experiment in that study directly tested IRES activity: expression from a dicistronic pRF-based vector was examined in cells co-transfected with siRNA against La. The effect of depleting La was small (a 3-fold reduction in Fluc translation) and the experiment was interpreted blindly (without RNA analyses).

CLOSING ARGUMENTS

When nature offers a clue, it should not be ignored. The fact that eukaryotic cells produce no natural dicistronic mRNAs (other than those explicable by leaky scanning) is strong grounds for questioning the internal initiation hypothesis. The dicistronic mRNAs produced by some plant and animal viruses only underscore the point: these transcripts are structurally dicistronic but functionally monocistronic, i.e. only the 5′ proximal cistron gets translated [reviewed in (92,93)]. Even picornaviruses do not employ dicistronic mRNAs: a single cistron encodes one large 'polyprotein' which is cleaved post-translationally to generate an array of viral proteins.

Another reason to doubt the internal initiation hypothesis is that the structural evidence does not cohere. There is no hint of a common structure among putative IRES elements from cellular mRNAs. 'Complex secondary and tertiary structure' (1) is not a definition. The cellular 5′-UTRs said to be IRESs bear no resemblance to picornavirus 5′-UTRs, and thus the suggestion that 'viruses captured their IRESs from cellular mRNAs' makes no sense (94).

Advocates of internal initiation protested loudly when deficiencies in the evidence were pointed out (95), but the deficiencies were so obvious that editors began asking for the missing controls. Unfortunately, this has not clarified the story because, even when control experiments reveal strong cryptic promoters or splice sites, advocates persist in calling the sequence an IRES as long as a very low level of activity can be demonstrated via RNA transfection or translation *in vitro*. 'Once an IRES, always an IRES' seems to be the rule. To get around this, editors will have to do more than ask whether cryptic promoters were ruled out. The section above entitled 'weak IRES or no IRES?' might merit re-reading.

Attempts to explain why IRES elements exist in cellular mRNAs are plagued by contradictions. One textbook tells us that internal initiation 'allows selected mRNAs to be translated *at a high rate* despite a general decrease in the cell's capacity to initiate protein synthesis' [(96), emphasis added]. The reality is that putative IRESs are extremely inefficient. Confronted by this uncomfortable fact, advocates now argue that internal initiation has to be inefficient to ensure that critical proteins are not over-produced (1). But that problem already has a solution: mRNAs that encode potent growth-regulatory proteins usually have AUG-burdened or highly structured 5′-UTRs which ensure that translation via scanning will be appropriately constrained (92,93,97,98). Internal initiation was supposed to be a way to circumvent this constraint, not a way to ensure inefficient translation. Advocates argue that even low-efficiency internal initiation can help by sustaining production of essential proteins when cap-dependent translation is impaired, as happens during mitosis. But readers should understand that 'impaired' means only that translation is reduced to ~30% of normal (99). The normal translation mechanism operating at 30% capacity is still far more efficient than the IRESs that purportedly function during mitosis.

These deficiencies in the rationale are of secondary importance. The main problem is that there is still no credible evidence for IRES elements in mRNAs of cellular origin. In many cases (e.g. p27^{Kip1}, connexins, c-myc, BiP) the gene in question really is up-regulated under certain conditions. The insistence on seeing this through the lens of the internal initiation hypothesis—even when no evidence supports that interpretation—only delays discovering how the regulation really is accomplished.

Because co-expression of genes is important in biotechnology, IRES elements are often tried, not always with success. A pervasive problem is that, even with the paradigmatic EMCV IRES, the yield of protein from the 3′ cistron is usually much lower than from the 5′ cistron. A recent study resorted to an alternative mechanism to achieve equimolar production of heavy and light antibody chains: the two cistrons were fused, so that translation of one large cistron produced a 'polyprotein' from which the heavy and light chains were

subsequently derived by proteolysis (100). This idea, borrowed from picornaviruses, actually works.

The fact that many people profess belief in internal initiation does not make internal initiation a fact. Many people also believe Abner Doubleday invented baseball. Examination of the evidence reveals contradictions galore.

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REFERENCES

1. Komar,A.A. and Hatzoglou,M. (2005) Internal ribosome entry sites in cellular mRNAs: mystery of their existence. *J. Biol. Chem.*, **280**, 23425–23428.
2. Nevins,T.A., Harder,Z.M., Korneluk,R.G. and Holčik,M. (2003) Distinct regulation of IRES-mediated translation following cellular stress is mediated by apoptotic fragments of eIF4G family members eIF4GI and p97/DAP5/NAT1. *J. Biol. Chem.*, **278**, 3572–3579.
3. Coldwell,M.J., Mitchell,S.A., Stoneley,M., MacFarlane,M. and Willis,A.E. (2000) Initiation of Apaf-1 translation by internal ribosome entry. *Oncogene*, **19**, 899–905.
4. Mitchell,S.A., Brown,E.C., Coldwell,M.J., Jackson,R.J. and Willis,A.E. (2001) Protein factor requirements of the Apaf-1 IRES: roles of polypyrimidine tract binding protein and upstream of N-ras. *Mol. Cell. Biol.*, **21**, 3364–3374.
5. Van Eden,M.E., Byrd,M.P., Sherrill,K.W. and Lloyd,R.E. (2004) Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedures. *RNA*, **10**, 720–730.
6. Henis-Korenblit,S., Strumpf,N.L., Goldstaub,D. and Kimchi,A. (2000) A novel form of DAP5 protein accumulates in apoptotic cells as a result of caspase cleavage and IRES-mediated translation. *Mol. Cell. Biol.*, **20**, 496–506.
7. Morrish,B.C. and Rumsby,M.G. (2002) The 5′ UTR of protein kinase Cδ directs translation by an IRES that is most active in densely growing cells and during apoptosis. *Mol. Cell. Biol.*, **22**, 6089–6099.
8. Sherrill,K.W., Byrd,M.P., Van Eden,M.E. and Lloyd,R.E. (2004) BCL-2 translation is mediated via internal ribosome entry during cell stress. *J. Biol. Chem.*, **279**, 29066–29074.
9. Van Eden,M.E., Byrd,M.P., Sherrill,K.W. and Lloyd,R.E. (2004) Translation of apoptosis protein 1 (c-IAP1) mRNA is IRES mediated and regulated during cell stress. *RNA*, **10**, 469–481.
10. Stoneley,M., Subkhankulova,T., Le Quesne,J.P.C., Coldwell,M.J., Jopling,C.L., Belsham,G.J. and Willis,A.E. (2000) Analysis of the c-myc IRES: a potential role for cell-type specific *trans*-acting factors and the nuclear compartment. *Nucleic Acids Res.*, **28**, 687–694.
11. Kobayashi,N., Saeki,K. and Yuo,A. (2003) GM-CSF and interleukin-3 induce cell cycle progression through the synthesis of c-Myc protein by IRES-mediated translation via phosphatidyl-inositol 3-kinase pathway in human factor-dependent leukemic cells. *Blood*, **102**, 3186–3195.
12. Pyronnet,S., Pradayrol,L. and Sonenberg,N. (2000) A cell cycle-dependent internal ribosome entry site. *Mol. Cell*, **5**, 607–616.
13. Tinton,S.A., Schepens,B., Bruynooghe,Y., Beyaert,R. and Cornelis,S. (2005) Regulation of the cell-cycle dependent IRES of the PITSLRE protein kinase: roles of Unr (upstream of N-ras) protein and phosphorylated eIF-2α. *Biochem. J.*, **385**, 155–163.
14. Zhang,X., Richie,C. and Legerski,R.J. (2002) Translation of hSNM1 is mediated by an IRES that upregulates expression during mitosis. *DNA Rep.*, **1**, 379–390.
15. Hernandez,E.M., Chan,C.H., Xu,B., Notario,V. and Richert,J.R. (2004) Role of an IRES in the translational control of the human transcription factor Sp3. *Int. J. Oncol.*, **24**, 719–724.
16. Cho,S., Kim,J.H., Back,S.H. and Jang,S.K. (2005) PTB enhances the IRES-dependent translation of p27^{Kip1} mRNA and modulates transition from G1 to S phase. *Mol. Cell. Biol.*, **25**, 1283–1297.
17. Liu,Z., Dong,Z., Han,B., Yang,Y., Liu,Y. and Zhang,J.-T. (2005) Regulation of expression by promoters versus internal ribosome entry site in the 5′ untranslated sequence of the human cyclin-dependent kinase inhibitor p27^{kip1}. *Nucleic Acids Res.*, **33**, 3763–3771.
18. Akiri,G., Nahari,D., Finkelstein,Y., Le,S.-Y., Elroy-Stein,O. and Levi,B.-Z. (1998) Regulation of VEGF expression is mediated by internal initiation of translation and alternative initiation of transcription. *Oncogene*, **17**, 227–236.
19. Stein,I., Itin,A., Einat,P., Skaliter,R., Grossman,Z. and Keshet,E. (1998) Translation of VEGF mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol. Cell. Biol.*, **18**, 3112–3119.
20. Bornes,S., Boulard,M., Hieblot,C., Zanibellato,C., Iacovoni,J.S., Prats,H. and Touriol,C. (2004) Control of the VEGF IRES activity and translation initiation by alternatively spliced coding sequences. *J. Biol. Chem.*, **279**, 18717–18726.
21. Lang,K.J.D., Kappel,A. and Goodall,G.J. (2002) HIF-1α mRNA contains an IRES that allows efficient translation during normoxia and hypoxia. *Mol. Biol. Cell*, **13**, 1792–1801.
22. Zhou,J., Callapina,M., Goodall,G.J. and Brüne,B. (2004) Functional integrity of NFκB, phosphatidylinositol 3′ kinase, and MAPK signaling allows TNFα-evoked Bcl-2 expression to provoke IRES-dependent translation of HIF1α. *Cancer Res.*, **64**, 9041–9048.
23. Fernandez,J., Yaman,I., Mishra,R., Merrick,W.C., Snider,M.D., Lamers,W.H. and Hatzoglou,M. (2001) Internal ribosome entry site-mediated translation of a mammalian mRNA is regulated by amino acid availability. *J. Biol. Chem.*, **276**, 12285–12291.
24. Fernandez,J., Yaman,I., Huang,C., Liu,H., Lopez,A.B., Komar,A.A., Caprara,M.G., Merrick,W.C., Snider,M.D., Kaufman,R.J. *et al.* (2005) Ribosome stalling regulates IRES-mediated translation in eukaryotes, a parallel to prokaryotic attenuation. *Mol. Cell*, **17**, 405–416.
25. Martin,M.M., Garcia,J.A., McFarland,J.D., Duffy,A.A., Gregson,J.P. and Elton,T.S. (2003) Translation of the human angiotensin II type 1 receptor mRNA is mediated by a highly efficient internal ribosome entry site. *Mol. Cell. Endocrinol.*, **212**, 51–61.
26. Pickering,B.M., Mitchell,S.A., Evans,J.R. and Willis,A.E. (2003) PTB and PCBP1 interact with the BAG-1 IRES and stimulate its activity *in vitro* and *in vivo*. *Nucleic Acids Res.*, **31**, 639–646.
27. Pickering,B.M., Mitchell,S.A., Spriggs,K.A., Stoneley,M. and Willis,A.E. (2004) Bag-1 IRES activity is promoted by structural changes mediated by PCBP1 and recruitment of PTB-1. *Mol. Cell. Biol.*, **24**, 5595–5605.
28. Kim,Y.K., Hahm,B. and Jang,S.K. (2000) Polypyrimidine tract-binding protein inhibits translation of Bip mRNA. *J. Mol. Biol.*, **304**, 119–133.
29. Macejak,D.G. and Sarnow,P. (1991) Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. *Nature*, **353**, 90–94.
30. Chappell,S.A. and Mauro,V.P. (2003) The IRES contained within the RNA-binding motif protein 3 (Rbm3) mRNA is composed of functionally distinct elements. *J. Biol. Chem.*, **278**, 33793–33800.
31. Créancier,L., Morello,D., Mercier,P. and Prats,A.-C. (2000) FGF-2 IRES activity *ex vivo* and in transgenic mice reveals a stringent tissue-specific regulation. *J. Cell Biol.*, **150**, 275–281.
32. Bonnal,S., Schaeffer,C., Créancier,L., Clamens,S., Moine,H., Prats,A.-C. and Vagner,S. (2003) A single IRES containing a G quartet RNA structure drives FGF-2 gene expression at four alternative translation initiation codons. *J. Biol. Chem.*, **278**, 39330–39336.
33. Martineau,Y., Le Bec,C., Monbrun,L., Allo,V., Chiu,I.-M., Danos,O., Moine,H., Prats,H. and Prats,A.-C. (2004) IRES structural motifs conserved among mammalian FGF-1 alternatively spliced mRNAs. *Mol. Cell. Biol.*, **24**, 7622–7635.
34. Jopling,C.L. and Willis,A.E. (2001) N-myc translation is initiated via an IRES that displays enhanced activity in neuronal cells. *Oncogene*, **20**, 2664–2670.
35. Oumard,A., Hennecke,M., Hauser,H. and Nourbakhsh,M. (2000) Translation of NRF mRNA is mediated by highly efficient internal ribosome entry. *Mol. Cell. Biol.*, **20**, 2755–2759.
36. Jianfeng,D., Feng,J., Chaoneng,J., Zhongzhou,Z., Shaohua,G., Qihan,W., Liu,W., Gang,Y., Yi,X. and Mao,Y. (2003) Cloning of the correct full length cDNA of NFκB-repressing factor. *Mol. Cells*, **16**, 397–401.
37. Rhee,S., Yang,S.J., Lee,S.J. and Park,D. (2004) βPix-b$_L$, a novel isoform of βPix, is generated by alternative translation. *Biochem. Biophys. Res. Commun.*, **318**, 415–421.
38. Bernstein,J., Sella,O., Le,S.-Y. and Elroy-Stein,O. (1997) PDGF2/c-sis mRNA leader contains a differentiation-linked IRES. *J. Biol. Chem.*, **272**, 9356–9362.

39. Han,B., Dong,Z. and Zhang,J.-T. (2003) Tight control of PDGF-B/c-sis expression by interplay between the 5′ UTR sequence and the major upstream promoter. *J. Biol. Chem.*, **278**, 46983–46993.

40. Pozner,A., Goldenberg,D., Negreanu,V., Le,S.-Y., Elroy-Stein,O., Levanon,D. and Groner,Y. (2000) Transcription-coupled translation control of AML1/RUNX1 is mediated by cap- and IRES-dependent mechanisms. *Mol. Cell. Biol.*, **20**, 2297–2307.

41. Levanon,D., Glusman,G., Bangsow,T., Ben-Asher,E., Male,D.A., Avidan,N., Bangsow,C., Hattori,M., Taylor,T.D., Taudien,S. *et al.* (2001) Architecture and anatomy of the genomic locus encoding the human leukemia-associated transcription factor RUNX1/AML1. *Gene*, **262**, 23–33.

42. Kim,J.G., Armstrong,R.C., Berndt,J.A., Kim,N.W. and Hudson,L.D. (1998) A secreted DNA-binding protein that is translated through an IRES and distributed in a discrete pattern in the central nervous system. *Mol. Cell. Neurosci.*, **12**, 119–140.

43. Fukuda,S., Foster,R.G., Porter,S.B. and Pelus,L.M. (2002) The antiapoptosis protein survivin is associated with cell cycle entry of normal cord blood CD34+ cells and modulates cell cycle and proliferation of mouse hematopoietic progenitor cells. *Blood*, **100**, 2463–2471.

44. Qin,X. and Sarnow,P. (2004) Preferential translation of IRES-containing mRNAs during the mitotic cycle in mammalian cells. *J. Biol. Chem.*, **279**, 13721–13728.

45. Ye,X., Fong,P., Iizuka,N., Choate,D. and Cavener,D.R. (1997) Ultrabithorax and Antennapedia 5′ untranslated regions promote developmentally regulated internal translation initiation. *Mol. Cell. Biol.*, **17**, 1714–1721.

46. Maier,D., Nagel,A.C. and Preiss,A. (2002) Two isoforms of the Notch-antagonist hairless are produced by differential translation initiation. *Proc. Natl Acad. Sci. USA*, **99**, 15480–15485.

47. Hernández,G., Vázquez-Pianzola,P., Sierra,J.M. and Rivera-Pomar,R. (2004) Internal ribosome entry site drives cap-independent translation of reaper and heat shock protein 70 mRNAs in *Drosophila* embryos. *RNA*, **10**, 1783–1797.

48. Komar,A.A., Lesnik,T., Cullin,C., Merrick,W.C., Trachsel,H. and Altmann,M. (2003) Internal initiation drives the synthesis of Ure2 protein lacking the prion domain and affects [*URE3*] propagation in yeast cells. *EMBO J.*, **22**, 1199–1209.

49. Burke,Z.D., Wells,T., Carter,D.A. and Baler,R. (2000) Use of a cryptic splice donor site in the CAT-SV40 small-t antigen cassette generates alternative transcripts in transgenic rats. *Transgenic Res.*, **9**, 67–70.

50. Holčik,M., Gordon,B.W. and Korneluk,R.G. (2003) The IRES-mediated translation of antiapoptotic protein XIAP is modulated by the hnRNPs C1 and C2. *Mol. Cell. Biol.*, **23**, 280–288.

51. Warnakulasuriyarachchi,D., Cerquozzi,S., Cheung,H.H. and Holčik,M. (2004) Translational induction of the inhibitor of apoptosis protein HIAP2 during endoplasmic reticulum stress attenuates cell death and is mediated via an inducible IRES element. *J. Biol. Chem.*, **279**, 17148–17157.

52. Sapetschnig,A., Koch,F., Rischitor,G., Mennenga,T. and Suske,G. (2004) Complexity of translationally controlled transcription factor Sp3 isoform expression. *J. Biol. Chem.*, **279**, 42095–42105.

53. Moran,K.M., Crusio,R.H.J., Chan,C.H., Grekova,M.C. and Richert,J.R. (2004) Human transcription factor Sp3: genomic structure, identification of a processed pseudogene, and transcript analysis. *Gene*, **341**, 235–247.

54. Chappell,S.A., Owens,G.C. and Mauro,V.P. (2001) A 5′ leader of *Rbm3*, a cold stress-induced mRNA, mediates internal initiation of translation with increased efficiency under conditions of mild hypothermia. *J. Biol. Chem.*, **276**, 36917–36922.

55. Johannes,G., Carter,M.S., Eisen,M.B., Brown,P.O. and Sarnow,P. (1999) Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. *Proc. Natl Acad. Sci. USA*, **96**, 13118–13123.

56. Wang,Z., Weaver,M. and Magnuson,N.S. (2005) Cryptic promoter activity in the DNA sequence corresponding to the *pim-1* 5′ UTR. *Nucleic Acids Res.*, **33**, 2248–2258.

57. Hennecke,M., Kwissa,M., Metzger,K., Oumard,A., Kröger,A., Schirmbeck,R., Reimann,J. and Hauser,H. (2001) Composition and arrangement of genes define the strength of IRES-driven translation in bicistronic mRNAs. *Nucleic Acids Res.*, **29**, 3327–3334.

58. Koloteva-Levine,N., Pinchasi,D., Pereman,I., Zur,A., Brandeis,M. and Elroy-Stein,O. (2004) The Apc5 subunit of the anaphase-promoting complex/cyclosome interacts with PABP and represses IRES-mediated translation. *Mol. Cell. Biol.*, **24**, 3577–3587.

59. Thoma,C., Bergamini,G., Galy,B., Hundsdoerfer,P. and Hentze,M.W. (2004) Enhancement of IRES-mediated translation of the c-*myc* and BiP mRNAs by the poly(A) tail is independent of intact eIF4G and PABP. *Mol. Cell*, **15**, 925–935.

60. Han,B. and Zhang,J.-T. (2002) Regulation of gene expression by internal ribosome entry sites or cryptic promoters: the eIF4G story. *Mol. Cell. Biol.*, **22**, 7372–7384.

61. Jimenez,J., Jang,G.M., Semler,B.L. and Waterman,M.L. (2005) An internal ribosome entry site mediates translation of lymphoid enhancer factor-1. *RNA*, **11**, 1385–1399.

62. Berg,M., DiFatta,J., Hoiczyk,E., Schlegel,R. and Ketner,G. (2005) Viable adenovirus vaccine prototypes: high-level production of a papillomavirus capsid antigen from the major late transcriptional unit. *Proc. Natl Acad. Sci. USA*, **102**, 4590–4595.

63. Thompson,S.R. and Sarnow,P. (2003) Enterovirus 71 contains a type I IRES element that functions when eukaryotic initiation factor eIF4G is cleaved. *Virology*, **315**, 259–266.

64. Gallie,D.R., Ling,J., Niepel,M., Morley,S.J. and Pain,V.M. (2000) The role of 5′-leader length, secondary structure and PABP concentration on cap and poly(A) tail function during translation in *Xenopus* oocytes. *Nucleic Acids Res.*, **28**, 2943–2953.

65. Dumas,E., Staedel,C., Colombat,M., Reigadas,S., Chabas,S., Astier-Gin,T., Cahour,A., Litvak,S. and Ventura,M. (2003) A promoter activity is present in the DNA sequence corresponding to the hepatitis C virus 5′ UTR. *Nucleic Acids Res.*, **31**, 1275–1281.

66. Kim,J.H., Paek,K.Y., Choi,K., Kim,T.-D., Hahm,B., Kim,K.-T. and Jang,S.K. (2003) HnRNP-C modulates translation of c-*myc* mRNA in a cell cycle phase-dependent manner. *Mol. Cell. Biol.*, **23**, 708–720.

67. Kim,Y.K., Back,S.H., Rho,J., Lee,S.H. and Jang,S.K. (2001) La autoantigen enhances translation of BiP mRNA. *Nucleic Acids Res.*, **29**, 5009–5016.

68. Kim,Y.K. and Jang,S.K. (2002) Continuous heat shock enhances translational initiation directed by internal ribosomal entry site. *Biochem. Biophys. Res. Commun.*, **297**, 224–231.

69. Yaman,I., Fernandez,J., Liu,H., Caprara,M., Komar,A.A., Koromilas,A.E., Zhou,L., Snider,M.D., Scheuner,D., Kaufman,R.J. *et al.* (2003) The zipper model of translational control: a small upstream ORF is the switch that controls structural remodeling of an mRNA leader. *Cell*, **113**, 519–531.

70. Cornelis,S., Bruynooghe,Y., Denecker,G., Van Huffel,S., Tinton,S. and Beyaert,R. (2000) Identification and characterization of a novel cell cycle-regulated IRES. *Mol. Cell*, **5**, 597–605.

71. Xiang,J., Lahti,J.M., Grenet,J., Easton,J. and Kidd,V.J. (1994) Molecular cloning and expression of alternatively spliced PITSLRE protein kinase isoforms. *J. Biol. Chem.*, **269**, 15786–15794.

72. Millard,S.S., Yan,J.S., Nguyen,H., Pagano,M., Kiyokawa,H. and Koff,A. (1997) Enhanced ribosomal association of p27^{Kip1} mRNA is a mechanism contributing to accumulation during growth arrest. *J. Biol. Chem.*, **272**, 7093–7098.

73. Créancier,L., Mercier,P., Prats,A.-C. and Morello,D. (2001) c-*myc* IRES activity is developmentally controlled and subjected to a strong translational repression in adult transgenic mice. *Mol. Cell. Biol.*, **21**, 1833–1840.

74. Park,E.-H., Lee,J.M., Blais,J.D., Bell,J.C. and Pelletier,J. (2005) Internal translation initiation mediated by the angiogenic factor Tie2. *J. Biol. Chem.*, **280**, 20945–20953.

75. Oltean,S. and Banerjee,R. (2005) A B$_{12}$-responsive internal ribosome entry site (IRES) element in human methionine synthase. *J. Biol. Chem.*, **280**, 32662–32668.

76. Lahlou,H., Fanjul,M., Pradayrol,L., Susini,C. and Pyronnet,S. (2005) Restoration of functional gap junctions through IRES-dependent synthesis of endogenous connexins in density-inhibited cancer cells. *Mol. Cell. Biol.*, **25**, 4034–4045.

77. Van Daalen Wetters,T., Macrae,M., Brabant,M., Sittler,A. and Coffino,P. (1989) Polyamine-mediated regulation of mouse ornithine decarboxylase is post-translational. *Mol. Cell. Biol.*, **9**, 5484–5490.

78. Rousseau,D., Kaspar,R., Rosenwald,I., Gehrke,L. and Sonenberg,N. (1996) Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eIF4E. *Proc. Natl Acad. Sci. USA*, **93**, 1065–1070.

79. Miura,P., Thompson,J., Chakkalakal,J.V., Holcik,M. and Jasmin,B.J. (2005) The utrophin A 5′ untranslated region confers internal ribosome entry site-mediated translational control during regeneration of skeletal muscle fibers. *J. Biol. Chem.*, **280**, 32997–33005.

80. Sarnow,P. (1989) Translation of GRP78/immunoglobulin heavy-chain binding protein (BiP) mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited. *Proc. Natl Acad. Sci. USA*, **86**, 5795–5799.

81. Johannes,G. and Sarnow,P. (1998) Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites. *RNA*, **4**, 1500–1513.

82. Byrd,M.P., Zamora,M. and Lloyd,R.E. (2005) Translation of eIF4GI proceeds from multiple mRNAs containing a novel cap-dependent IRES that is active during poliovirus infection. *J. Biol. Chem.*, **280**, 18610–18622.

83. Yanagiya,A., Jia,Q., Ohka,S., Horie,H. and Nomoto,A. (2005) Blockade of the poliovirus-induced cytopathic effect in neural cells by monoclonal antibody against poliovirus or the human poliovirus receptor. *J. Virol.*, **79**, 1523–1532.

84. Ali,I.K., McKendrick,L., Morley,S.J. and Jackson,R.J. (2001) Truncated initiation factor eIF4G lacking an eIF4E binding site can support capped mRNA translation. *EMBO J.*, **20**, 4233–4242.

85. Kozak,M. (2003) Alternative ways to think about mRNA sequences and proteins that appear to promote internal initiation of translation. *Gene*, **318**, 1–23.

86. Pilipenko,E.V., Viktorova,E.G., Guest,S.T., Agol,V.I. and Roos,R.P. (2001) Cell-specific proteins regulate viral RNA translation and virus-induced disease. *EMBO J.*, **20**, 6899–6908.

87. Mitchell,S.A., Spriggs,K.A., Coldwell,M.J., Jackson,R.J. and Willis,A.E. (2003) The Apaf-1 IRES attains the correct structural conformation for function via interactions with PTB and unr. *Mol. Cell*, **11**, 757–771.

88. Mitchell,S.A., Spriggs,K.A., Bushell,M., Evans,J.R., Stoneley,M., Le Quesne,J.P.C., Spriggs,R.V. and Willis,A.E. (2005) Identification of a motif that mediates PTB-dependent internal ribosome entry. *Genes Dev.*, **19**, 1556–1571.

89. Kaminski,A. and Jackson,R.J. (1998) The PTB requirement for internal initiation of translation of cardiovirus RNAs is conditional rather than absolute. *RNA*, **4**, 626–638.

90. Guest,S., Pilipenko,E., Sharma,K., Chumakov,K. and Roos,R.P. (2004) Molecular mechanisms of attenuation of the Sabin strain of poliovirus type 3. *J. Virol.*, **78**, 11097–11107.

91. Costa-Mattioli,M., Svitkin,Y. and Sonenberg,N. (2004) La autoantigen is necessary for optimal function of the poliovirus and hepatitis C virus IRES *in vivo* and *in vitro*. *Mol. Cell. Biol.*, **24**, 6861–6870.

92. Kozak,M. (2002) Pushing the limits of the scanning mechanism for initiation of translation. *Gene*, **299**, 1–34.

93. Kozak,M. (2005) Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene*, **361**, 13–37.

94. Belsham,G.J. and Sonenberg,N. (2000) Picornavirus RNA translation: roles for cellular proteins. *Trends Microbiol.*, **8**, 330–335.

95. Kozak,M. (2001) New ways of initiating translation in eukaryotes? *Mol. Cell. Biol.*, **21**, 1899–1907.

96. Alberts,B., Johnson,A., Lewis,J., Raff,M., Roberts,K. and Walter,P. (2002) *Molecular Biology of The Cell*. 4th edn.. Garland Science, NY, p. 448.

97. Ghilardi,N., Wiestner,A. and Skoda,R.C. (1998) Thrombopoietin production is inhibited by a translational mechanism. *Blood*, **92**, 4023–4030.

98. Kozak,M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.*, **115**, 887–903.

99. Fan,H. and Penman,S. (1970) Regulation of protein synthesis in mammalian cells. II. Inhibition of protein synthesis at the level of initiation during mitosis. *J. Mol. Biol.*, **50**, 655–670.

100. Fang,J., Qian,J.-J., Yi,S., Harding,T.C., Tu,G.H., VanRoey,M. and Jooss,K. (2005) Stable antibody expression at therapeutic levels using the 2A peptide. *Nat. Biotechnol.*, **23**, 584–590.