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REVIEW

Internal initiation of translation in eukaryotes: The picornavirus paradigm and beyond

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INTRODUCTION

All eukaryotic cellular mRNAs examined to date are monocistronic, and it is almost axiomatic that this will prove to be a universal rule in view of the way in which eukaryotic transcription units seem to be organized in the genome. However, an additional explanation is that the scanning ribosome mechanism only allows efficient translation of the 5′-proximal cistron of polycistronic mRNAs in which all the cistrons are large protein-coding ORFs (open reading frames): initiating ribosomes can only access the mRNA from the 5′-end and, after they have translated the first cistron, there is very little if any transfer of initiation-competent ribosomes to downstream cistrons. This has been amply demonstrated by laboratory-generated dicistronic constructs, as well as naturally occurring viral RNAs that are structurally polycistronic yet functionally monocistronic (e.g., tobacco mosaic virus and alphaviruses such as Sindbis virus). Only the 5′-proximal cistron, coding for nonstructural proteins required for viral RNA replication, is expressed from the full-length genomic RNA (Glanville et al., 1976; Hunter et al., 1976; Goelet et al., 1982; Kaariainen et al., 1987). The downstream cistrons of these viruses, coding for structural proteins, are only expressed from subgenomic monocistronic mRNAs, which are generated late in the infectious cycle and correspond to the 5′-distal part of the genomic RNA.

There are two types of deviation from this general default mechanism that eukaryotic ribosomes can only translate efficiently the 5′-proximal cistron of a polycistronic mRNA. The first concerns a small proportion of cellular mRNAs (but an interesting group coding for many proto-oncogenes, transcription factors, and components of transmembrane signaling pathways) that have long 5′-UTRs (untranslated regions) with one or more short ORFs preceding the main protein-coding ORF (Kozak, 1991). For reasons that are not yet fully understood, ribosomes that have translated specifically a short 5′-proximal ORF (usually less than 20 codons) may retain the potential to re-initiate translation of the same mRNA at initiation codons located further downstream. The paradigm for this class of mRNAs is yeast GCN4 mRNA, where it has been shown that the capacity to re-initiate at downstream AUG codons is dependent not only on the length, but also on the sequences around the termination codon of the short upstream ORF (Grant & Hinnebusch, 1994; Hinnebusch, 1994). In addition, the efficiency of re-initiation at a downstream AUG codon is determined by its distance from the upstream short ORF and by the intracellular concentration of eIF2/GTP/Met-tRNA$_i$ ternary complex. The interplay between these various parameters results in a very subtle regulation of GCN4 expression at the translational level (Hinnebusch, 1994). It seems likely, but remains unproven, that a similar scenario operates with those mammalian mRNAs that have short upstream ORFs. The expected diagnostic features of mRNAs subject to this type of regulation are that the length and the position of the short ORFs in any particular type of mRNA should be highly conserved among different species.

The other deviation is the more radical one of internal initiation of translation, and is characterized by the fact that insertion of specific *cis*-acting RNA sequences into the intercistronic spacer of a dicistronic construct with two large protein-coding ORFs promotes efficient expression of the downstream cistron. The minimal element required for this is commonly known as the IRES (for "internal ribosome entry segment"). Appropriate controls show that the product from the downstream cistron really is expressed from a dicistronic form of the mRNA rather than from monocistronic species that might have been generated if the IRES element had sites for cleavage by a specific ribonuclease, or, in the case of DNA transfections, if the IRES had a cryptic promoter element. The yield of translation product from the downstream IRES-dependent cistron

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is often much higher than that from the upstream cistron, particularly if the latter is deliberately downregulated by insertion of a hairpin loop near the 5′-end, or by the use of uncapped mRNAs in cell-free translation assays. This observation eliminates the possibility that the function of the IRES element is to promote the transfer of initiation-competent ribosomes from the termination codon of the upstream cistron to the initiation codon of the downstream cistron. Even more incisive evidence is provided by the recent demonstration that mammalian ribosomes can translate a covalently closed circular mRNA provided that it includes a functional IRES (Chen & Sarnow, 1995).

The unambiguous conclusion, therefore, is that the IRES element promotes direct internal ribosome entry to the initiation codon of the second cistron. Thus, the IRES can be considered as functionally equivalent to the Shine and Dalgarno motif in prokaryotic mRNAs, which is usually present upstream of the initiation site of each cistron of a polycistronic mRNA and acts as an "identifier" for independent initiation at each cistron. It remains an open question whether this functional equivalence implies a similar mechanism, and that IRESes therefore promote internal initiation by base pairing with rRNA. Internal initiation was first discovered in the animal picornaviruses, but has since been extended to other RNA viruses and a few cellular mRNAs. Nevertheless, the picornaviruses are still the most studied and best understood example, and have thus become the paradigm. This review first examines what is known about the picornavirus paradigm, and then considers how far the other examples of internal initiation conform to this model. For a historical perspective and for more detailed bibliographies, the reader is referred to the following reviews that have appeared over the past five years: Jackson et al. (1990, 1994), Agol (1991), Meerovitch and Sonenberg (1993), Kaminski et al. (1994b), Ehrenfeld and Semler (1995), and Hellen and Wimmer (1995).

PICORNAVIRUSES

The animal picornavirus family comprises a great many different species (Table 1) that give rise to a plethora of different diseases. In all species, the genome is a positive-strand RNA about 7,500–8,000-nt long, and encodes a single large polyprotein containing at least one, and in many species two, protease domains that cleave the polyprotein into the four mature viral capsid proteins and several nonstructural proteins. The picornavirus RNAs came under suspicion of not conforming to the scanning ribosome mechanism because their 5′-UTRs have several properties that seem incompatible with efficient translation by this mechanism: (1) the 5′-UTRs are long (610 to more than 1400 nt, depending on the species) and are believed to be highly structured; (2) at the 5′-end of the virion RNA, there is a small covalently linked and virus-encoded protein (VPg), which, however, is rapidly cleaved off following uncoating of the virus in the infected cell, with the result that the genome is translated as an uncapped RNA; and (3) there are many AUG triplets in the 5′-UTR, most of which are poorly conserved, not just between closely related species, but even between different strains or different isolates of the same serotype (Pöyry et al., 1992). It is particularly this lack of conservation of the upstream AUG codons that distinguishes IRESes from those mRNAs, such as yeast GCN4 mRNA, which have upstream short ORFs yet are translated by the conventional scanning ribosome mechanism.

By testing deletions of the picornavirus 5′-UTRs in the dicistronic mRNA assay, the boundaries of the IRES for each main species have been delineated. In all cases, the IRES is about 450-nt long, but does not appear to include the extreme 5′-proximal sequences, which are thought to be principally RNA replication signals (Andino et al., 1993). On the basis of their sequence and structure, the IRESes fall into two large groups or superfamilies and one minor group: (1) hepatitis A virus (the minor group); (2) the cardio- and aphthoviruses; and (3) the entero- and rhinoviruses. Within each group, there is moderate conservation of primary sequence of the IRES and even stronger conservation of deduced secondary structure, but there is almost no conservation between the groups apart from a pyrimidine-rich tract that starts some 25 nt before the

TABLE 1. Classification of picornaviruses based on IRES sequence homologies.[a]

Entero-/rhinoviruses		
Humanrhinoviruses	(>100 serotypes)	HRV
Enteroviruses		
Poliovirus group	Polioviruses (3 serotypes)	PV
	Coxsackie A21 virus	
	Enterovirus 70	
Coxsackie B group	Coxsackie B viruses	CBV
	Coxsackie A9 and A16 viruses	
	Most echoviruses	EV
Bovine enterovirus		BEV
Cardio-/aphthoviruses		
Cardioviruses		
	Encephalomyocarditis virus and mengovirus	EMCV
	Theiler's murine encephalomyelitis virus	TMEV
	Echovirus 22	
Aphthoviruses		
	Foot and mouth disease virus (9 serotypes)	FMDV
Hepatoviruses		
	Hepatitis A virus	HAV

[a] The above classification, based on IRES sequence homologies, is simpler than classifications based on coding region sequences, because there is more divergence of coding sequences than IRESes (or more convergence of IRES sequences). Thus, echovirus 22 has an IRES typical of cardio-/aphthoviruses, yet its coding sequences are very different (Hyppia et al., 1992); a similar relationship holds with respect to the position of enterovirus 70 in the poliovirus group and to coxsackie A16 virus in the coxsackie B virus group.

3′-end of the IRES (Agol, 1991; Jackson et al., 1994; Kaminski et al., 1994b).

There is also a difference in the position of the IRES relative to the authentic initiation codon for viral polyprotein synthesis. In the cardio-/aphthovirus group, as well as with HAV (abbreviations in Table 1), the IRES is the 450 nt immediately preceding this initiation codon, i.e., the authentic initiation codon is at the 3′-boundary of the IRES. In contrast, in the entero-/rhinoviruses, the 3′-boundary of the IRES, as defined by testing deletions in the dicistronic mRNA assay, is ~40-nt upstream of the viral polyprotein initiation site in the rhinoviruses and ~160-nt upstream in the enteroviruses.

A final difference between the groups is that, whereas cardio- and aphthovirus IRESes function efficiently in a wide variety of vertebrate cells or cell-free translation systems, including rabbit reticulocyte lysates, the entero-/rhinovirus IRESes as well as the HAV IRES function inefficiently in reticulocyte lysates. Addition of cytoplasmic extracts from HeLa cells (or L-cells or Krebs II ascites cells) to the reticulocyte lysate strongly stimulates internal initiation dependent on the entero- and rhinovirus IRESes (Brown & Ehrenfeld, 1979; Dorner et al., 1984; Svitkin et al., 1988; Borman et al., 1993), but has no effect on HAV IRES utilization, which, however, can be stimulated by supplementation with mouse liver cytoplasmic extracts (Glass & Summers, 1993). Thus, there are differences in which *trans*-acting cellular factors are required for the function of the IRESes of the various subgroups of picornaviruses, differences that seem more dependent on cell-type than on the mammalian species from which the factors are derived.

Despite these differences, internal initiation promoted by the IRESes of at least the two major groups of picornaviruses can be described by a single unified model (Jackson et al., 1994; Kaminski et al., 1994b), which embraces both major classes of IRES with only slight variations. This model is depicted in Figure 1, and has two fundamental propositions:

1. Most of the 450-nt IRES is required not so much for its primary sequence, but for its secondary (and presumably also tertiary) structure, the purpose of which is to present several quite short primary sequence motifs (denoted by thickened shaded lines in Fig. 1), most of them in unpaired loops and bulges, in the correct three-dimensional spatial array.

2. The actual ribosome entry site in all picornavirus IRESes is at an AUG triplet at the 3′-end of the IRES, some 25-nt downstream from the start of the conserved oligopyrimidine tract. Where the viruses differ is in what happens following entry at this AUG. In the cardioviruses, virtually all entering ribosomes use this as a functional initiation site. In contrast, none of the ribosomes utilize the AUG at the putative entry site at the 3′-end of the IRES of entero-/rhinoviruses, but all initiation occurs at the next AUG downstream, which is probably reached by scanning from the actual entry site. Aphthoviruses represent an intermediate case, with some of the internally entering ribosomes initiating synthesis at the AUG at the 3′-end of the IRES, and some utilizing the next downstream AUG (Fig. 1).

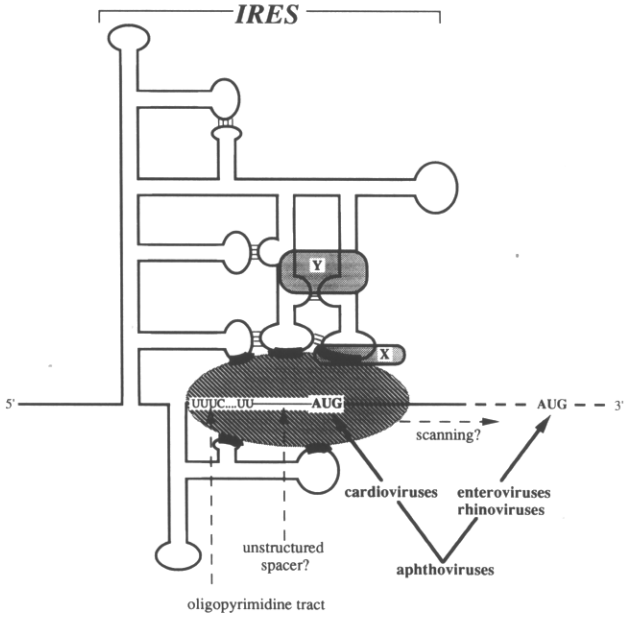

FIGURE 1. General model for internal initiation of translation on picornavirus IRESes. The IRES consists of several base paired stem-loops, with possible tertiary structure interactions between the loops and bulges. This structure presents a number of quite short unpaired primary sequence motifs, denoted by thickened lines, in the appropriate three-dimensional spatial organization for internal ribosome entry. These motifs may be binding sites for specific RNA-binding proteins (e.g., protein X), which are then recognized by the initiating ribosome (denoted by the shaded oval), or they may be recognized directly by the ribosome and associated initiation factors. The actual ribosome entry site is at the AUG triplet located at the 3′-end of the IRES, some 25-nt downstream of the start of a conserved pyrimidine-rich tract, with the intervening sequence probably serving as an unstructured spacer of defined length. The AUG at the 3′-end of the IRES is the authentic initiation site for viral polyprotein synthesis in the cardioviruses. In the case of the entero-/rhinoviruses, this AUG is likewise the ribosome entry site, but is not used as an initiation site; the ribosomes are transferred, probably by a scanning mechanism, to the authentic initiation site, which is the next AUG codon further downstream. The aphthoviruses represent an intermediate case between the cardioviruses and the entero-/rhinoviruses (see text). Two possible roles for essential *trans*-acting factors are shown. Protein X binds to a specific sequence in an unpaired loop and directs internal initiation via specific interactions with the ribosome. Protein Y binds to specific sequences or structures and thereby promotes or stabilizes the appropriate three-dimensional structure of the IRES. *Trans*-acting factors identified to date are more likely to fulfil the role of protein Y than protein X (see text).

RELATIONSHIP BETWEEN IRES STRUCTURE AND FUNCTION

The importance of RNA secondary (and presumably also tertiary) structure for IRES function emerges from a comparison of the sequences and secondary struc-

tures of different IRESes within each of the two main groups (Fig. 2). With the wisdom of hindsight, it was fortunate that the picornaviruses became the first focus of interest in internal initiation. The large size of this family of viruses, with many different distinct species and a high degree of genetic drift between different strains of any one species, or even between different isolates of the same serotype (Pöyry et al., 1992), as a result of the high error rate in RNA replication, has provided a rich phylogenetic database, especially for the entero-/rhinovirus group. Thus, even though the primary sequences are moderately divergent, the phylogenetic comparisons allow a common secondary structure to be unambiguously deduced (Fig. 2A), which has been confirmed by biochemical probing. All entero-/rhinovirus IRESes conform to this model with only minor variations, such as slightly more base pairing in the large central stem in the case of coxsackie B viruses and especially rhinoviruses, and somewhat less base pairing at the top of the 5′-proximal stem. The only radical variation within the group is that a centrally located stem-loop is entirely deleted in a viable poliovirus type 1 mutant (Dildine & Semler, 1989), and is very vestigial in all strains of bovine enterovirus (Fig. 2A), which leads to the conclusion that it is not required for internal initiation.

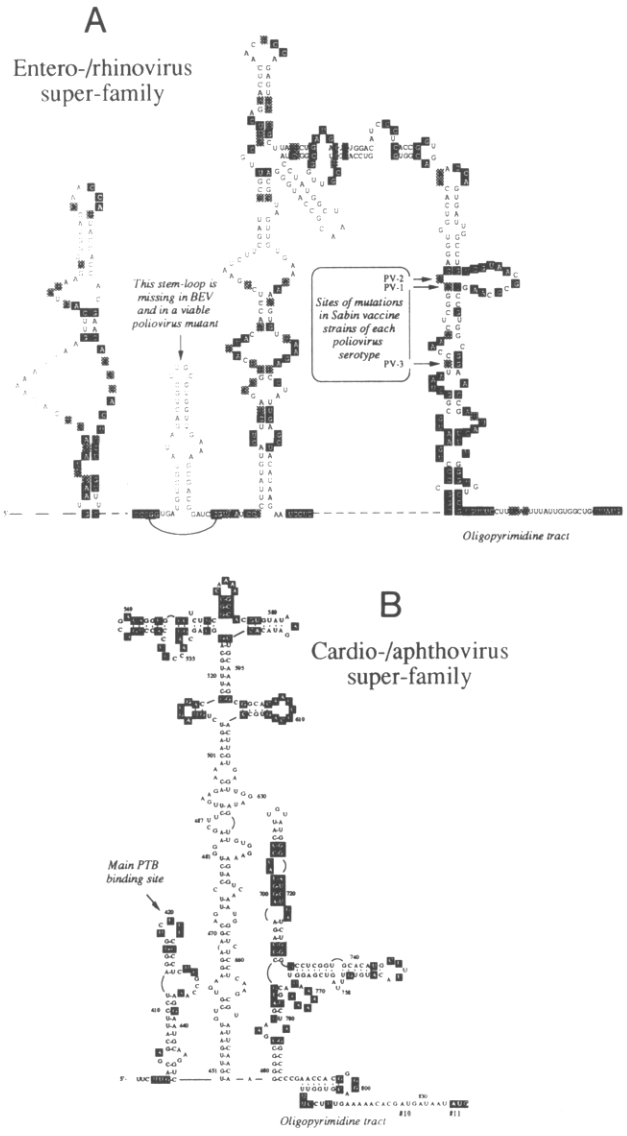

FIGURE 2. Conservation of the sequence and secondary structure of IRESes within (**A**) the entero-/rhinovirus group and (**B**) the cardio-/aphthovirus group. The sequence shown in A is that of poliovirus type 1 and the structure is based on an amalgamation of those presented in Pilipenko et al. (1989b) and Pöyry et al. (1992); residues that are absolutely conserved in all species are blacked out, and residues that are not absolutely invariant yet are conserved among more than 80% of the members of this superfamily are shaded. The sequence shown in B is that of EMCV (strain R) and the structure is that proposed by Pilipenko et al. (1989a), with residues that are absolutely conserved in all members of the cardio-/aphthovirus superfamily blacked out.

Another advantage of the picornaviruses, which the community has only just begun to exploit, is that it is possible to obtain infectious RNA from full-length cDNA clones of picornavirus genomes. Thus, mutations generated at the cDNA level can be transfected into cells as mutant RNAs and, provided the mutation is not completely lethal, the high error frequency of RNA replication generates phenotypic revertants, which can provide useful information about the relationship between IRES structure and function. Thus, some debilitating mutations in putatively base paired residues give rise to phenotypic revertants that retain the original mutation but have compensatory second-site suppressor mutations that would restore base pairing (Kuge & Nomoto, 1987; Haller & Semler, 1992). This type of result not only confirms the accuracy of the secondary structure map in the region of the mutation, but also attests to the overall importance of the base paired secondary structure for IRES function.

One interesting mutation destabilizing the IRES structure is found in the attenuated Sabin poliovirus vaccine strains (Fig. 2A). Although these strains have accumulated many mutations during the course of their development, neurovirulence tests with Sabin/wild-type chimerics show that it is the mutation in the IRES that is the main determinant of the attenuated phenotype. This mutation significantly lowers infectivity in some cell types and also in vitro translation efficiency (LaMonica & Racaniello, 1989), whereas in other cells the infectivity is only marginally affected unless the IRES also carries another mutation that, on its own, likewise has only a marginal influence (Kuge & Nomoto, 1987; Iizuka et al., 1989).

As for the question of which primary sequence motifs are important for IRES function, here again the phylogenetic comparisons can provide much useful insight. If we compare the IRES sequences of just the polioviruses together with the obviously related coxsackie A21 virus and enterovirus 70 (Table 1), we find about

66% of the positions absolutely conserved and 79% highly conserved (defined as conserved in at least 80% of the sequences under scrutiny). Apart from a clustering of variations in a spacer loop near the 5′-end of the IRES, and in the last (3′-proximal) ~20 nt (Fig. 2A), most of the primary sequence variations are in base paired stems, particularly in the middle of such stems, and are compensatory such that the base pairing is conserved. A similar pattern is seen if we examine just the IRESes of the coxsackie B virus group: 67% of positions are absolutely conserved and 77% highly conserved. However, if the two families are amalgamated, the conservation falls to about 53% absolutely and 65% highly conserved residues, and, if the data for human rhinoviruses and bovine enteroviruses are also incorporated into this compilation, it falls to 31% residues absolutely and 41% highly conserved, as shown in Figure 2A. Given that these compilations do not include some recently published data from previously unsequenced species, it is likely that the proportion of absolutely conserved residues may be as low as 25%. As is clear from Figure 2A, the residues that are absolutely conserved in the whole entero-/rhinovirus superfamily tend to be located in unpaired loops and bulges, and, apart from the last (3′-proximal) ~20 nt of the IRES, their density increases toward the 3′-end. Moreover, a similar pattern of conserved residues clustering in unpaired regions with a more frequent occurrence toward the 3′-end is seen when the same exercise is carried out on the IRESes of the cardio-/aphthovirus superfamily (Fig. 2B).

Are all the absolutely conserved unpaired residues in loops and bulges essential for IRES function? The systematic mutagenesis necessary to answer this question has so far been carried out only on a rather limited scale with the poliovirus IRES, and there has been only one such study of a cardiovirus IRES (Hoffman & Palmenberg, 1995). Surprisingly, there are some absolutely conserved unpaired residues that can be mutated in the poliovirus IRES with no apparent effect on translation efficiency or infectivity (Pelletier et al., 1988; K.M. Kean & R.J. Jackson, unpubl. results). However, for the majority of such positions examined so far, it is quite remarkable that single-site mutation results in a drastic decrease (>90%) in translation efficiency and loss of infectivity. Nevertheless, phenotypic revertant viruses are usually recovered from transfections of cells with full-length RNA bearing such a mutation. The outcome so far is that only direct (primary site) revertants have been recovered (K.M. Kean & R.J. Jackson, unpubl. results), and there has not yet been any case where the phenotypic revertant carried a remote second-site suppressor mutation that might be indicative of long-range RNA tertiary structure interactions. However, because only a rather limited number of sites have been examined so far, it is too early to rule out that some of the conserved residues unpaired in the secondary structure map might be required to maintain higher-order RNA structure.

Aside from the possibility of involvement in tertiary structure interactions, most of the essential conserved residues in loops and bulges highlighted in Figure 2A are presumed to play a direct role in the internal initiation mechanism. One possibility is that they are recognized directly by the initiating 40S ribosomal subunit and its associated initiation factors, either through protein/IRES RNA interactions or by direct IRES RNA/rRNA interactions, a sort of highly disperse equivalent to the interaction between the 16S rRNA and the mRNA Shine and Dalgarno motif in prokaryotic systems. An alternative, illustrated by hypothetical protein X in Figure 1, is that *trans*-acting cellular proteins might bind specifically to the conserved motifs in the loops of the IRES, and that it is these bound proteins that are actually recognized by the initiating ribosome, either through protein/protein interactions or via interaction between the 18S rRNA and the specific IRES-binding protein. However, as discussed below, there is no evidence that any of the *trans*-acting factors identified to date as being required for internal initiation actually function in this way. On the contrary, it seems more likely that the role of such cellular factors is to aid the correct folding of the IRES structure, as illustrated by protein *Y* in Figure 1.

THE ACTUAL RIBOSOME ENTRY SITE IS AT AN AUG TRIPLET AT THE 3′-END OF THE IRES

Cardioviruses

Although it is very likely that the ribosome makes contact with sequences dispersed throughout the IRES (Fig. 1), the only useful definition of the actual ribosome entry site is the operational one of what is the most 5′-proximal position within the viral 5′-UTR at which internal initiation can occur. How far can the authentic initiation codon be displaced toward the 5′-end without compromising internal initiation? If in-frame AUG codons are naturally present upstream of the authentic initiation codon, or are introduced by point mutation, are any of these upstream AUGs used as functional internal initiation sites? By these criteria, the actual ribosome entry site in all picornavirus IRESes is at the 3′-end of the IRES, some 25-nt downstream from the start of the universal oligopyrimidine tract.

This conclusion emerged originally and most obviously from the cardioviruses, where the IRES is the ~450 nt immediately preceding the authentic initiation site. Deletions that move this initiation site further upstream toward the 5′-end totally abrogate internal initiation (Jang & Wimmer, 1990; Hunt et al., 1993). Moreover, in the most commonly studied strain of EMCV (strain R), but not in the majority of EMCV

strains nor in any strain of TMEV, there is an out-of-frame AUG triplet (AUG-10, numbering from the 5′-end) that is only 8-nt upstream of the authentic initiation codon, AUG-11 (Fig. 2B). By studying the translation of constructs in which AUG-10 was made in-frame by a frame-shift insertion located ~60-nt further downstream in the reporter cistron, it is possible to test whether this upstream AUG is used as a functional initiation codon. When most of the upstream IRES sequences were deleted to give an RNA that would be translated by the scanning ribosome mechanism, AUG-10 was used in preference to AUG-11 as expected, but, in the background of the full-length IRES, when internal initiation is operative, the frequency of initiation at AUG-10 was less than 1% of that at AUG-11 (Kaminski et al., 1990). This argues that the actual internal ribosome entry site is very close to AUG-11 and certainly downstream of AUG-10.

Surprisingly, the sequence between the oligopyrimidine tract and the authentic initiation site is not strongly conserved between different species and strains (Fig. 2B). Indeed, this seems to be a hot spot for strain-dependent variation, at least in FMDV (Sangar et al., 1987) and the 11 strains of TMEV sequenced by Pritchard et al. (1992), where the only really conserved feature of this region is its invariant length and the paucity of G residues, which suggests that it might serve the role of an unstructured spacer of defined length (Fig. 1). This in turn suggests that the selection of AUG-11 over AUG-10 in the case of the EMCV IRES may be on the basis of distance from the oligopyrimidine tract. When this length was shortened, initiation at AUG-11 and AUG-10 no longer occurred and only AUG-12 (five codons downstream of AUG-11) was utilized, consistent with the hypothesis that the length of the spacer determines the position of the actual ribosome entry site. When the length of the spacer was increased by 8 nt over the wild-type length, the relative frequency of initiation at AUG-10 rather than AUG-11 increased by a factor of more than 30-fold, but a complete switch in preference to AUG-10 was not achieved (Kaminski et al., 1994a). Further expansion of the spacer length resulted in decreased overall efficiency of internal initiation rather than a greater switch in preference from AUG-11 in favor of AUG-10.

Thus, it appears that the distance from the start of the oligopyrimidine tract and other IRES elements located further upstream is one parameter influencing the selection of the ribosome entry site, but that some other determinants also have an influence, causing AUG-11 to be still favored even when it is at a greater distance than ideal. One possible explanation not yet fully tested is that it is the coding sequences just downstream of AUG-11 that cause it to be favored, even though a direct test shows that viral coding sequences are not absolutely required for efficient internal initiation (Hunt et al., 1993).

Differences between entero-/rhinoviruses and cardioviruses

In the case of the entero-/rhinovirus IRESes, there is likewise an AUG triplet some 25-nt downstream of the start of the oligopyrimidine tract, but this does not seem to be used as a functional initiation site. No translation product initiated at this site has ever been detected in infected cells, and it is extremely doubtful that any such product could have biological significance because the length of the following reading frame varies from 4 codons in enterovirus 70, via 18 codons in poliovirus type 3, to 64 codons in PV-1, where it extends past the polyprotein initiation site, but in a different reading frame. The reason no initiation occurs at this site is thought to be that the context is poor according to Kozak's rules (Kozak, 1986); initiation at this site occurs in vitro if the context is improved, and it can be assumed to occur in vivo because infectivity is reduced (Pestova et al., 1994).

The authentic viral polyprotein initiation site is actually some 65-nt downstream of the start of the oligopyrimidine tract in the human rhinoviruses and ~185-nt in the enteroviruses (Agol, 1991). Nevertheless, there are strong indications that the actual ribosome entry site is at or near the position of the silent (nonfunctional) AUG triplet 25-nt downstream of the oligopyrimidine tract, rather than at the actual authentic initiation codon further downstream, because viable enterovirus mutants have been isolated with deletions of ~160 nt, which remove the silent AUG codon and most of the sequence between it and the authentic initiation site. Although this type of deletion considerably reduced infectivity in the case of coxsackie B1 virus and the Sabin attenuated strain of poliovirus type 1 (Kuge & Nomoto, 1987; Iizuka et al., 1989, 1991), the infectivity of two such deletion mutants of neurovirulent PV-1 was almost indistinguishable from that of wild-type virus (Iizuka et al., 1989; Haller & Semler, 1992). These deletions displace the authentic initiation codon to a position about 25-nt downstream of the oligopyrimidine tract, just as in the cardiovirus IRESes. Larger deletions, which displaced the authentic initiation site even further toward the 5′-end, were inviable (Iizuka et al., 1989, 1991).

In the wild-type background, mutation of the silent AUG codon results in a small plaque phenotype and a ~70% reduction in translation efficiency (Pelletier et al., 1988; Meerovitch et al., 1991; Nicholson et al., 1991), suggesting that, even if it is not a functional initiation site, this AUG is an important but not absolutely essential determinant of the actual ribosome entry site. As in the cardio-/aphthovirus IRESes, the spacing but not the sequence between the oligopyrimidine-rich tract and the silent AUG seem important, for when insertions were made into this region to increase the spacing, infectivity was very severely compromised.

The revertants of these insertion mutants were mainly of two types: deletions of an equivalent length to the original insertion, which thus restored the wild-type spacing between the pyrimidine-rich tract and the cryptic AUG codon; or point mutations, which retained the insertion but generated a new AUG triplet about 25-nt downstream of the start of the pyrimidine-rich tract (Pilipenko et al., 1992).

If the actual entry site is at this cryptic AUG codon at the 3′-end of the IRES, how does the ribosome get transferred from this site to the authentic initiation codon, a distance of some 40-nt further downstream in the rhinoviruses and ~160-nt in the enteroviruses? The most likely answer is that the transfer is by scanning, although others have argued for a direct nonlinear transfer akin to a ribosomal shunt (Hellen et al., 1994). Several lines of evidence favor the scanning hypothesis. First, even though this region in polioviruses, particularly the ~100 nt just before the authentic initiation site, is hypervariable between different strains and even between different isolates of the same strain, it never has an AUG triplet (Pöyry et al., 1992). Second, initiation at the correct site and viability of the virus is not compromised by deletions of the intervening sequence, or by most types of insertions (Kuge & Nomoto, 1987). When a 72-nt insert with an AUG codon in a poor Kozak context background was made in this region of poliovirus type 1, it conferred a small-plaque phenotype, irrespective of whether the AUG was in- or out-of-frame with the downstream authentic initiation site (Kuge et al., 1989a). Significantly, of 46 large-plaque revertants, no fewer than 44 had retained the insert but carried a single point mutation, abrogating the AUG codon in the insert, thus demonstrating that it was the AUG in the insert rather than the insertion itself that was compromising infectivity (Kuge et al., 1989b). Finally, insertion of a stable hairpin loop in the intervening sequence of PV-2 was found to inhibit initiation at the authentic site (Pelletier & Sonenberg, 1988). All these observations argue strongly that the ribosome is transferred from the cryptic AUG entry site to the authentic initiation codon by a process of scanning.

Thus, the only substantive difference between internal initiation driven by the cardiovirus IRES and the entero-/rhinovirus IRESes is what happens following ribosome entry at the AUG triplet at the 3′-end of the IRES. FMDV represents an intermediate between these two extremes, with about one-third of the ribosomes utilizing the AUG as a functional initiation codon, and the others initiating at the next AUG codon located 84-nt downstream in most strains (Sangar et al., 1987). Again, it seems likely that this behavior is dictated by the context of the AUG at the 3′-end of the IRES, and that those ribosomes that do not initiate translation at this AUG are transferred to the downstream site by a scanning mechanism (Belsham, 1992).

Apart from differences in the particular *trans*-acting factors required, which will be discussed below, the only other difference between the cardiovirus IRES on the one hand, and the entero-/rhinovirus IRESes on the other, is the question of whether the oligopyrimidine tract is required per se as a primary nucleotide sequence determinant, or whether it is just part of the unstructured spacer. Complete substitution of this tract in EMCV by purines reduced in vitro translation efficiency by only 30% (Kaminski et al., 1994a) and gave a viable virus, whereas infectious virus was likewise obtained when the tract was mutated in the related TMEV (Pilipenko et al., 1994). On the other hand, in the enterovirus IRES, the retention of the conserved 5′-proximal UUUC(C) motif seems absolutely essential, even though mutations elsewhere in the pyrimidine-rich tract have a rather small effect (Iizuka et al., 1989, 1991; Nicholson et al., 1991; Pestova et al., 1991).

The question of whether the sequences immediately downstream of the actual ribosome entry site (which are the sequences coding for the N-terminus of the viral polyprotein in the cardioviruses) play a role is of interest in comparison with internal initiation dependent on the hepatitis C virus IRES, to be discussed below. In the case of the cardiovirus IRESes, efficient internal initiation was not dependent on whether specifically virus-coding sequences followed the initiation codon, although efficiency was reduced if there were G-rich sequences immediately following this AUG (Hunt et al., 1993). Deletion of the six residues immediately following the putative ribosome entry site (the cryptic AUG at the 3′-end of the IRES) in polioviruses reduced in vitro translation by about twofold, but single point mutations had no effect (Meerovitch et al., 1991). In addition, the large ~160-nt deletions discussed above remove the cryptic AUG at the 3′-end of the IRES plus the immediate downstream sequences, replacing it with the authentic initiation codon and the start of the viral polyprotein coding sequences, yet infectivity is not compromised, at least in the neurovirulent strains of PV-1 (Iizuka et al., 1989; Haller & Semler, 1992). All of these results suggest that any role played by the sequences just downstream of the AUG entry site can only be very minor.

ARE THE CANONICAL INITIATION FACTORS NECESSARY AND SUFFICIENT FOR INTERNAL INITIATION?

There is remarkably little difference between the requirements for canonical initiation factors to sustain internal initiation as opposed to scanning-dependent initiation (Staehelin et al., 1975; Scheper et al., 1992). Surprisingly, this conclusion also holds for eIF4F, because addition of this factor to partially fractionated cell-free systems (or even to unfractionated systems under certain conditions) stimulates internal initiation (Anthony & Merrick, 1991; Scheper et al., 1992; Pause

et al., 1994b). The only difference observed is that translation of capped mRNAs by the scanning mechanism absolutely requires the intact eIF4F holoenzyme complex, whereas internal initiation can operate with an incomplete complex (Liebig et al., 1993; Ohlmann et al., 1995; Ziegler et al., 1995). The holoenzyme consists of three polypeptide chains (Fig. 3): eIF4E, which is the only translation initiation factor capable of direct binding to the 5′ cap structure; eIF4A, which has an ATP-dependent RNA helicase activity; and eIF4G, which seems to serve as a bridge between the other two factors (Lamphear et al., 1995) and was previously known as eIF-4γ or p220, although the cDNA clone shows that its actual size is 154 kDa (Yan et al., 1992). Experiments with dominant negative eIF4A mutants have indicated that the activity of eIF4A in promoting translation, whether by internal initiation or by the scanning mechanism, is only expressed efficiently when this factor is in the eIF4F complex rather than as singular eIF4A (Pause et al., 1994b).

Infection with enteroviruses, rhinoviruses, or FMDV results in the cleavage of the eIF4G component into an N-terminal one-third fragment with bound eIF4E, and a C-terminal two-thirds fragment with bound eIF4A and the capacity for association with eIF3 (Fig. 3; Lamphear et al., 1995). The cleavage seems to occur in a flexible exposed hinge region, because the FMDV L-protease and the entero-/rhinovirus 2A proteases, which are quite different classes of protease with different specificity, both cleave in the same region, although at different sites (Kirchweger et al., 1994). The result of this cleavage is to separate the cap-binding function associated with the N-terminus from the eIF4A helicase function associated with the C-terminal fragment. As a consequence, the cleaved factor is inactive in promoting initiation on capped mRNAs, whereas IRES-dependent translation may actually be stimulated (Buckley & Ehrenfeld, 1987; Liebig et al., 1993; Ohlmann et al., 1995; Ziegler et al., 1995). Under these circumstances, it is believed that the internal initiation is being driven by the C-terminal cleavage product of eIF4G with its associated eIF4A, which is thought to be more effective than intact eIF4F holoenzyme complex in catalyzing IRES-dependent initiation. Nevertheless, despite these intriguing, subtle differences, it is important not to lose sight of the overall similarity of the initiation factor requirements for scanning-dependent and internal initiation, which implies that the two mechanisms cannot be fundamentally very different.

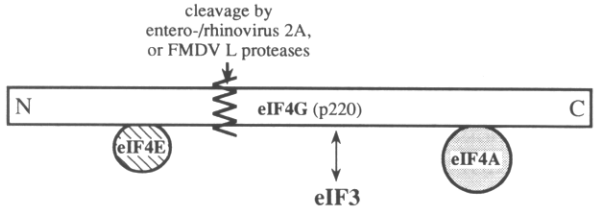

FIGURE 3. Architecture of the eIF4F holoenzyme complex. The cap-binding factor eIF4E binds to the N-terminal part of eIF4G (formerly known as eIF-4γ or p220), and eIF4A, which has RNA helicase activity, binds to the C-terminal portion of eIF4G. The association of eIF3 with eIF4G is weaker than the other interactions and is roughly at the position shown. The diagram is based on the results of Lamphear et al. (1995).

No viral or cellular IRES has been reported to function in the wheat germ system, even though this system is fully competent for initiation by the scanning ribosome mechanism. This is often taken as evidence that specific *trans*-acting factors, absent from wheat germ extracts, are required for internal initiation. However, it has not so far proved possible to confer internal initiation on a wheat germ system by the addition of ribosome-free crude extracts of mammalian cells, for example post-ribosomal supernatant or ribosomal salt-wash proteins. Thus, the inability of the wheat germ system to carry out internal initiation may not be due to just the lack of a particular IRES binding protein, but may reflect some more fundamental defect such as an intrinsic inability of wheat germ ribosomes to recognize IRES determinants.

A more significant indicator that internal initiation dependent on at least some picornavirus IRESes requires specific *trans*-acting cellular proteins is the fact mentioned previously that only cardio- and aphthovirus IRESes function efficiently in unsupplemented rabbit reticulocyte lysates. Internal initiation dependent on the entero-/rhinovirus IRESes requires supplementation with cytoplasmic extracts of HeLa cells (or mouse L-cell or Krebs II ascites cells), and hepatitis A IRES function is greatly stimulated by addition of mouse liver cytoplasm (Brown & Ehrenfeld, 1979; Dorner et al., 1984; Svitkin et al., 1988; Borman et al., 1993; Glass & Summers, 1993).

At the risk of stating the obvious, the fact that picornavirus IRESes promote internal initiation in dicistronic mRNAs with standard reporter cistrons shows that no virus-coded proteins are essential for internal initiation, and, although there are reports that expression of poliovirus 2A may stimulate IRES-dependent translation (Hambridge & Sarnow, 1992), this may possibly be explained by the recent indications that internal initiation is more efficient with cleaved eIF4F than the intact factor (Ohlmann et al., 1995; Ziegler et al., 1995).

THE QUEST FOR CELLULAR *TRANS*-ACTING FACTORS REQUIRED FOR PICORNAVIRUS IRES FUNCTION

A common approach in the search for cellular proteins that might be required for internal initiation is to carry out UV-crosslinking reactions with ^{32}P-labeled viral 5′-UTR and cytoplasmic extracts from the relevant cell type, although it needs to be born in mind that not ev-

ery protein that binds to the IRES may necessarily be crosslinkable by UV-irradiation. Nevertheless, many cellular proteins are labeled in these assays: in general, the cardio- and aphthovirus IRESes give strong labeling of a limited number of cellular proteins, whereas with the entero- and rhinovirus IRESes, there is less intense labeling of a wider variety of cellular proteins (Borman et al., 1993). All these IRES-binding proteins are more abundant in HeLa cell extracts than in reticulocyte lysates, and this is particularly true of those that are specific for the entero-/rhinovirus IRESes. Only two of these crosslinkable proteins have been positively identified by a permutation of purification, sequencing, and immunoprecipitation of the crosslinked protein. One is the autoantigen La, which has been shown to bind to sequences at the 3′-end of the poliovirus IRES (Meerovitch et al., 1993). The other is polypyrimidine tract binding protein (PTB), a protein first isolated by virtue of its binding to the pyrimidine-rich tract in pre-mRNA introns (Gil et al., 1991; Patton et al., 1991) and is now thought to act as a negative regulator of pre-mRNA splicing (Lin & Patton, 1995; Singh et al., 1995). PTB is the same as hnRNP I, and under this guise was found predominantly in the nucleus, but with some in the cytoplasm (Ghetti et al., 1992), which is significant because picornaviruses replicate exclusively in the cytoplasm. All picornavirus IRESes can be crosslinked to PTB, and the binding, or at least the crosslinking, is more efficient with the cardio-/aphthovirus than the entero-/rhinovirus IRESes (Pestova et al., 1991; Borman et al., 1993; Hellen et al., 1993). It is somewhat surprising that the highest affinity binding site for PTB in the EMCV IRES is not the oligopyrimidine tract near the 3′-end of the IRES, but a stem-loop near the 5′-end of the IRES, as shown in Figure 2B (Jang & Wimmer, 1990; Kaminski et al., 1995).

A serious criticism of this work is that the binding of a particular protein to an IRES provides absolutely no evidence that the interaction has functional significance, and thus the numerous publications cataloguing all the crosslinkable proteins and attempting to delineate their binding sites on the IRES seem somewhat fruitless. What is needed is functional assays for the possible activity of such proteins. In the case of the cardio- and aphthovirus IRESes, which work efficiently in all mammalian cell-free systems tested, such a functional assay requires specific depletion of the protein in question. Immunodepleting HeLa cell extracts of PTB resulted in a loss of capacity to translate mRNAs with a picornavirus IRES, but had little effect on the translation of globin mRNA by the scanning mechanism (assayed separately rather than in the more rigorous type of co-translation control, or, even better, translation of a dicistronic mRNA). However, the capacity for IRES-dependent initiation could not be restored by addition of recombinant PTB (Hellen et al., 1993), which raised some doubt as to whether the active entity is a complex of PTB with some other unknown protein, or whether it is only the putative PTB-associated protein that is relevant. More recently, an affinity column procedure was used to deplete reticulocyte lysate of PTB, and the depleted lysate was found to be incapable of internal initiation dependent on the EMCV IRES, but retained good activity for translation of the upstream cistron by the scanning mechanism. In this case, full activity could be restored by addition of recombinant PTB at concentrations similar to those found in the parent untreated lysate (Kaminski et al., 1995). A study of PTB deletion mutants showed that virtually the whole protein, with its four RNA-binding domains (Ghetti et al., 1992), is required for full activity, although truncated versions with just the two C-terminal RNA-binding motifs could bind to the EMCV IRES with an affinity comparable to that of the full-length protein, and could inhibit the stimulatory activity of the full-length PTB. This provides a salutory warning that high-affinity binding of a protein to the IRES may not in itself be sufficient to potentiate internal initiation.

Surprisingly, internal initiation dependent on the IRES of TMEV, another cardiovirus closely related to EMCV, was quite efficient in the PTB-depleted lysate and was not stimulated by recombinant PTB, even though PTB binds and is crosslinked to the TMEV IRES as efficiently as to the EMCV structure (Kaminski et al., 1995). This observation, plus the fact that PTB is not required for hepatitis C virus IRES function (see below), implies that PTB is not an essential catalyst specifically of internal initiation (in the same way as, say, eIF2 is an essential catalyst of all initiation). It is more consistent with the idea that the binding of PTB may (like protein *Y* in the model depicted in Fig. 1) aid in folding the IRES into the correct conformation. According to this "chaperone" hypothesis, the EMCV IRES would only adopt the appropriate conformation when PTB is bound to it, whereas the default conformation of the TMEV IRES even in the absence of bound PTB is appropriate for promoting internal ribosome entry. In any case, these observations sound yet another strong warning that binding of a particular protein to the IRES, even high-affinity binding to a specific site, does not automatically signify that it has a functional role in internal initiation.

The fact that internal initiation promoted by enterovirus IRESes and, more especially, rhinovirus IRESes is very inefficient in reticulocyte lysates unless HeLa cell extracts are added, provides an obvious functional assay for the isolation of *trans*-acting factors required for the function of this class of IRESes. By using a dicistronic mRNA with the HRV IRES to assay the purification of such factors, the translation of the upstream (scanning-dependent) cistron provides an internal control for general inhibitors or stimulators of translation (Borman et al., 1993). Fractionation of the HeLa cell ex-

tract leads to the separation of two activities, each capable on its own of specifically stimulating HRV IRES utilization in reticulocyte lysates (Borman et al., 1993). One of these has been identified as PTB on the basis of its purification properties and the fact that recombinant PTB has the same specific stimulatory activity (S.L. Hunt & R.J. Jackson, unpubl. results). The other has been purified recently to apparent homogeneity and has been partially sequenced. However, the amino acid sequence does not correspond to a known protein, and thus the final proof that this factor is needed for efficient internal initiation must await de novo isolation of the corresponding cDNA clone and testing of the recombinant protein.

It has been reported that the autoantigen La specifically stimulates poliovirus RNA translation in rabbit reticulocyte lysates (Meerovitch et al., 1993; Svitkin et al., 1994), and thus mimics the effect of adding crude cytoplasmic extracts or ribosomal salt wash from HeLa cells or Krebs II ascites cells. However, the quantities of recombinant La required for this stimulation are very high indeed (approximately 2 μM, at least 100-fold molar excess over the viral RNA template), which does not seem compatible with the reported dissociation constant for La-IRES binding of 5 nM (Svitkin et al., 1994). The results clearly show that La can stimulate internal initiation driven by the poliovirus IRES, but there are serious grounds for doubting whether this is physiologically significant and whether, when stimulation is effected by adding HeLa or Krebs II ascites cell extracts, the activity is ascribable solely to the endogenous La in such extracts. If indeed the main function of *trans*-acting factors is to promote the correct folding of the IRES, as argued above, then one could reasonably imagine that high concentrations of La might mimic the action of the physiologically relevant RNA-binding protein.

The search for *trans*-acting factors required for internal initiation might be more effective if it could be done by a genetic screen rather than by the more laborious functional biochemical assays. Thus, the recent report of internal initiation in a yeast cell-free system takes on considerable significance (Iizuka et al., 1994), although it remains to be proven whether it occurs in vivo and whether the mechanisms in yeast and mammalian cells are sufficiently similar that yeast genetics can aid in the identification of essential *trans*-acting factors from mammalian cells.

INTERNAL INITIATION PROMOTED BY THE IRES OF HEPATITIS C VIRUS AND THE PESTIVIRUSES

After the picornaviruses, the best-studied examples of internal initiation against which one can judge the generality of the picornavirus paradigm are hepatitis C virus (HCV) and the fairly closely related pestiviruses. It is likely that the recently characterized GB viruses are more distantly related members of the same superfamily (Muerhoff et al., 1995). HCV is basically a single species, although there are at least six distinct subspecies or strains (Bukh et al., 1992; Simmonds et al., 1993). It turns out that the 5′-UTR of HCV (341 nt in most strains), plus the first few codons of coding sequences, is the region of the virus genome most highly conserved between different strains and isolates, a fact that is extremely useful for PCR-based detection methods, but gives much less phylogenetic information than is available for the picornaviruses. In contrast, there are three distinct species of known pestiviruses (but relatively few distinct strains of each of these three species): bovine viral diarrhoea virus (BVDV), classical swine fever virus (CSFV), and border disease virus. The 5′-UTR of the pestiviruses is ~370 nt, slightly longer than that of HCV, and shares blocks of nucleotide sequence homology with the HCV 5′-UTR, interspersed by blocks that show no obvious homology (Brown et al., 1992). Nevertheless, the HCV and pestivirus 5′-UTRs can adopt similar RNA secondary structures as judged by biochemical probing experiments and phylogenetic analysis of co-variances (Brown et al., 1992; Deng & Brock, 1993). Another significant difference between HCV and the pestiviruses lies in the order of the genes within the virus-encoded polyprotein: the HCV polyprotein starts with the Core protein (Choo et al., 1991), whereas the pestivirus polyprotein starts with a self-excising ~20-kDa protease that is then followed by the Core protein (Wiskerchen et al., 1991).

Despite one negative report that can be explained by the inappropriate design of the dicistronic constructs used to test for internal initiation (Yoo et al., 1992), there is general agreement that HCV has a moderately efficient IRES (Tsukiyama-Kohara et al., 1992; Wang et al., 1993; Fukushi et al., 1994; Reynolds et al., 1995; Rijnbrand et al., 1995), and this has recently been extended to two pestiviruses, BVDV and CSFV (Poole et al., 1995; S.P. Fletcher & R.J. Jackson, unpubl. results). There is also general agreement that the extreme 5′-proximal sequences of the HCV 5′-UTR do not form part of the functional IRES, and most reports place the 5′-boundary at around nt 40. A more interesting question is the position of the 3′-boundary of the HCV IRES. In our hands, up to 10 codons of HCV coding sequences are required (in addition to 5′-UTR sequences from nt 40) for efficient internal initiation, and thus the IRES extends into the coding region (Reynolds et al., 1995). This requirement was observed both in cell-free translation assays, and in transfected cells in vivo, and was independent of the nature of the downstream reporter cistron. Moreover, a similar, although less stringent requirement for coding sequences was observed in the case of the IRES of the pestivirus CSFV: the efficiency of internal initiation with constructs that retained 5 codons or less of viral coding sequences was only 10–15% of that seen if 17 or more codons were re-

tained (S.P. Fletcher & R.J. Jackson, unpubl. results). The strong coding sequence requirement for internal initiation, especially in the case of the HCV IRES, is in sharp contrast with the picornavirus IRESes, as discussed above.

Sequencing numerous worldwide isolates of HCV has shown that the high conservation in the 5′-UTR extends into the start of the coding region (Bukh et al., 1992; Simmonds et al., 1993). This conservation in the coding region cannot be simply a reflection of constraints on the encoded amino acid sequence, because it includes even the wobble positions, which implies that there are constraints on the nucleotide sequence itself. This is in sharp contrast to the sequences of different worldwide isolates of poliovirus, for example, which show variations in virtually all wobble positions over the regions examined (Rico-Hesse et al., 1987), reflecting the typical high genetic drift in RNA viruses as a result of error-prone RNA replication (Holland et al., 1982).

It is possible that the constraints on the nucleotide sequence at the start of the coding region are due to their contribution to the IRES. Because the sequences in question code for the N-terminus of the Core protein in the case of HCV and the N-terminus of a protease in the case of pestiviruses, there is no strong nucleotide-sequence conservation between the two classes of virus, even though the region is somewhat A-rich in both cases. This suggests that the coding sequences are not needed by virtue of their primary nucleotide sequence, and leads to the question of whether their role might be to pair with sequences upstream in the 5′-UTR, which would serve to fix the position of the AUG initiation codon in the three-dimensional structure relative to other determinants in the 5′-UTR. Although initiation and subsequent elongation by the ribosome would be expected to disrupt any such pairing, it would probably reform quickly by snap-back given that it is intramolecular pairing. One indication that the ribosome is very strongly directed by the IRES to bind in the vicinity of the initiation codon is provided by the fact that initiation efficiency is remarkably unperturbed by substitution of non-AUG codons such as CUG or AUU (Reynolds et al., 1995), whereas such a substitution in the EMCV IRES, for example, severely reduces the overall efficiency of internal initiation, most of which occurs at the next AUG codon downstream (A. Kaminski & R.J. Jackson, unpubl. results).

Other differences between the IRESes of HCV and picornaviruses include the fact that there is no oligopyrimidine tract located ~25 nt before the authentic initiation codon in HCV or the pestiviruses. It is true that near the center of the 5′-UTR of most strains of HCV, there is an oligopyrimidine tract followed by an AUG at the appropriate distance, but this AUG is not absolutely conserved among all strains (Bukh et al., 1992), and mutagenesis has shown that neither the pyrimidine-rich tract nor the AUG triplet is important for HCV IRES function (Wang et al., 1994; J.E. Reynolds & R.J. Jackson, unpubl. results). Yet another distinction from at least some picornavirus IRESes is that PTB binds only weakly to the HCV IRES and is not required for its activity: the efficiency of internal initiation dependent on the HCV IRES is not affected if reticulocyte lysates are depleted of PTB by the affinity column procedure, and is not stimulated by addition of recombinant PTB to such depleted lysates (Kaminski et al., 1995).

Nevertheless, internal initiation dependent on the HCV and pestivirus IRESes resembles at least the cardiovirus paradigm in one respect: the actual ribosome entry site is at or very near to the authentic initiation codon. In all pestiviruses, there is a conserved out-of-frame AUG triplet with a good local sequence context just seven residues upstream of the authentic initiation codon (Brown et al., 1992; Deng & Brock, 1993). In our hands, this upstream AUG is not used to a detectable extent as a functional initiation codon in constructs that have the complete CSFV IRES, including at least 17 codons of coding sequences (S.P. Fletcher & R.J. Jackson, unpubl. results). Although HCV doesn't have this upstream AUG triplet, if one was introduced 21-nt upstream of the authentic initiation site by a single point mutation, it too was not used under conditions where internal initiation is operative, although it was functional if the upstream cistron and most of the HCV 5′-UTR was deleted to convert the dicistronic construct to a monocistronic mRNA translated by the scanning ribosome mechanism (J.E. Reynolds & R.J. Jackson, unpubl. results).

Another, although less precisely defined, feature held in common between the IRES of HCV and the various picornavirus IRESes is the critical importance of RNA secondary structure. This has been amply demonstrated by the fact that mutations that severely debilitate HCV IRES function and are located in one side of a putatively base paired stem, can be fully suppressed by compensating mutations in the other side of the base paired region (Wang et al., 1994). Recent evidence from this type of approach has even indicated that there is a structure in the HCV 5′-UTR, akin to a pseudo-knot, that is essential for HCV IRES function (Wang et al., 1995).

IRESes IN CELLULAR mRNAs

A number of cellular mRNAs with IRESes have been discovered through individual testing in the dicistronic mRNA assay: first, mammalian BiP mRNA (immunoglobulin heavy-chain binding protein), then the *antennapedia* mRNA of *Drosophila melanogaster*, and more recently, mammalian fibroblast growth factor-2 (Macejak & Sarnow, 1991; Oh et al., 1992; Vagner et al., 1995). Comparatively little is known about these IRESes. The

boundaries determined by deletion analysis have not been published, except for limited information on the *antennapedia* IRES. Nor has the exact position of internal ribosome entry been determined by testing whether internal initiation still occurs if the initiation codon is moved in a 5′-direction, or whether AUG codons inserted or created by point mutation upstream of the authentic initiation site are used as functional initiation codons. There is no indication that any of these IRESes binds PTB or La in UV-crosslinking assays, and there is no obvious homology in the primary nucleotide sequence between the 5′-UTRs of these mRNAs, or between any of them and the IRESes of either the HCV/pestivirus group, the cardio-/aphthoviruses or the entero-/rhinoviruses. It would thus appear that functional IRESes can be generated out of a wide variety of primary nucleotide sequences. Although this conclusion may seem surprising at first sight, it is actually implicit in the model of Figure 1, and is well illustrated by the fact that, apart from the oligopyrimidine tract near the 3′-end of the IRES, there is no strong homology between the entero-/rhinovirus and the cardio-/aphthovirus IRESes, yet a common model can be derived for internal initiation promoted by both types of picornavirus IRES.

The IRESes in BiP and *antennapedia* mRNAs were discovered by inserting just the 5′-UTR of these mRNAs between standard reporter cistrons, and thus these IRESes do not extend into coding sequences. However, in view of the strong coding sequence requirements for HCV and pestivirus IRES function (Reynolds et al., 1995), it would seem prudent that future tests for IRESes in cellular mRNAs should be designed to cover the possibility that coding sequences are important. Any negative results obtained in the past on the basis of tests confined to 5′-UTRs must now be considered as inconclusive until repeated with the inclusion of some coding sequences.

WHAT ARE THE ADVANTAGES OF INTERNAL INITIATION?

Given the obvious efficacy of the scanning ribosome mechanism of initiation of translation of eukaryotic cellular and capped viral mRNAs, a number of obvious questions arise as to why internal initiation has evolved and persisted, and as to how it evolved in the first place.

For the picornaviruses, the obvious advantage of internal initiation is that it allows the viruses to shut-off host cell mRNA translation, through the cleavage of the eIF4G component of translation initiation factor eIF4F by virus-encoded proteases. This cleavage not only abolishes the activity of this factor in catalyzing competing translation initiation of capped cellular mRNA, but may also enhance its activity for internal initiation (Buckley & Ehrenfeld, 1987; Liebig et al., 1993; Ohlmann et al., 1995; Ziegler et al., 1995). However, although this explains the advantage of internal initiation for the entero-/rhinovirus subgroup of the picornaviruses, it is not relevant to all picornaviridae. Apart from the entero-/rhinoviruses, only FMDV encodes a protease that cleaves eIF4G. Significantly, the FMDV L-protease is a completely different class of protease and is located in a different position in the viral polyprotein than the entero-/rhinovirus 2A protease (Kirchweger et al., 1994), which implies that the acquisition of this protease by FMDV was an independent event in evolution from the acquisition of a 2A protease by the entero-/rhinoviruses. The cardioviruses, which are most closely related to FMDV, do not have such a protease, yet they have an IRES similar to that of FMDV. This carries the implication that IRESes and the mechanism of internal initiation arose *before* the cardio- and aphthoviruses diverged, and before FMDV acquired its L-protease that would make internal initiation so advantageous.

Even though cardioviruses do not have a protease capable of cleaving eIF4G, they replicate quite rapidly and efficiently in cultured cells, largely for two reasons: (1) the output of positive-stranded RNA seems very high; and (2) the perturbation of monovalent cation balance as a result of virus-induced damage to the plasma membrane favors translation of the viral RNA at the expense of cellular mRNA (Alonso & Carrasco, 1982; Lacal & Carrasco, 1982). This latter property is actually ascribable to the IRES: initiation dependent on cardiovirus IRESes (and FMDV) exhibits an unusually high optimum monovalent salt concentration.

On the other hand, hepatitis A virus replicates relatively slowly and there is no indication of any shut-off of host cell mRNA translation, or any selective advantage of viral RNA translation. With hepatitis C virus, the inability of this virus to grow in cultured cells limits any direct test, but the low titer of circulating virus in infected patients suggests that this virus also replicates very slowly. Thus, there is no indication that internal initiation offers any selective advantage to HAV or HCV RNA translation over host cell mRNA translation.

Another possible explanation for IRESes in RNA viruses is related to strategies for RNA replication. The extreme 5′-proximal region of picornavirus genomes, extending over at least 80 nt, seems to be highly structured (Andino et al., 1993) and, in hepatitis C virus, there is a smaller but very stable hairpin loop at the extreme 5′-end (Han et al., 1991; Brown et al., 1992). At least with the entero-/rhinovirus subgroup of the picornaviruses, there is good evidence that the extreme 5′-proximal structure is needed for RNA replication (Andino et al., 1993), and it seems likely that this may be the explanation for the structures in the other viruses because it is almost axiomatic that RNA replication signals must be located at the extreme ends of the genome. Such structures would be likely to impede

translation by the scanning ribosome mechanism, and this consideration leads to an alternative explanation for internal initiation of translation of positive-strand viral genomes: it allows efficient translation despite the presence of RNA replication signals with complex secondary structure at the extreme 5′-end. However, it should be noted that RNA replication doesn't necessarily and universally require complex structures at the 5′-end; the replication of tobacco mosaic virus RNA, to name but one example, results in efficient production of capped RNA despite the fact that the 5′-UTR is unstructured (Sleat et al., 1988).

The question of whether internal initiation of translation confers any advantage to those cellular mRNAs that have an IRES is even more complex. Obviously, such mRNAs would continue to be translated efficiently under conditions in which the function of eIF4E or eIF4F holoenzyme were impaired. Such circumstances include infection by many picornavirus species, when eIF4G is cleaved by proteolysis, and by adenovirus, when eIF4E is dephosphorylated late in the infectious cycle (Schneider, 1995), but there is no indication yet that any cellular anti-viral defense mechanism involves translation of a host cell mRNA via internal initiation. As for uninfected cells, conditions that are believed to result in low eIF4E/4F activity include heat shock, mitosis, and the quiescent growth-arrested state (reviewed in Rhoads, 1993). However, the evidence that the activity of these factors is reduced under these conditions is less conclusive than one would wish, and the mechanism of any such regulation is still obscure. In general, there is a correlation between protein synthesis rates and the phosphorylation of eIF4E and eIF4G, and there is some suggestive but perhaps not yet conclusive evidence that this phosphorylation does increase the activity of the eIF4F holoenzyme complex (Rhoads, 1993). In addition, there is the recently discovered mechanism whereby the binding of a small protein, PHAS-I, to eIF4E effectively sequesters the factor and prevents its entry into the eIF4F complex (Pause et al., 1994a). Phosphorylation of PHAS-I, as occurs in response to insulin and mitogens, decreases its affinity for eIF4E and hence can result, indirectly, in an increase in eIF4F holoenzyme concentration.

As for those cellular mRNAs in which an IRES has been identified, it is far from clear what advantages would accrue from the possibility that these particular mRNA species (BiP, *antennapedia*, and fibroblast growth factor-2) could be translated more efficiently than most cellular mRNAs during heat shock, mitosis, or growth arrest, or indeed any other condition in which eIF4E, 4F, and PHAS-I are in the dephosphorylated state. But perhaps we first need to discover more cellular mRNAs with IRESes before any pattern will be discernible. Nevertheless, it does seem clear that, even though it might seem advantageous if heat-shock mRNAs were translated by an IRES-dependent mechanism, this is almost certainly not the case. All the evidence suggests that these mRNAs are translated by the conventional 5′-end-dependent scanning mechanism, but, because their 5′-UTRs are A-rich and probably unstructured, their translation requires only very low concentrations of eIF4F (reviewed in Jackson et al., 1995; Rhoads & Lamphear, 1995).

HOW DID IRESes EVOLVE?

If we confine our attention to the origin of the viral IRESes, two alternative pathways of evolution can be suggested. One possibility is that, starting with an mRNA translated by the scanning mechanism, an IRES evolved by a process of progressive expansion of a shorter 5′-UTR and mutation of this longer structure toward the present-day IRES structure, both stages being accelerated by the high rate of genetic drift in RNA viruses (Holland et al., 1982). At the risk of reiterating all the discredited arguments against Darwinism, the problem with this idea is how the evolution broke through the intermediate stages in which the developing secondary structure of the 5′-UTR would be an impediment to the scanning mechanism, and yet the signals for internal ribosome entry were not yet optimal. The alternative possibility is that the viruses acquired their IRESes from a cellular mRNA translated by internal initiation, through an error in the normal recombination process that occurs, certainly in the picornaviruses, by copy choice during RNA replication. That acquisition of cellular RNA sequences is a real possibility is shown by recent reports of poliovirus assimilating a short sequence of ribosomal RNA (Charini et al., 1994), and a pestivirus, BVDV, acquiring ubiquitin mRNA coding sequences (Meyers et al., 1991).

As for the evolution of IRESes in cellular mRNAs, there are too few examples identified so far for a pattern to be discernible that would allow useful speculation on this question. What is needed is a strategy for generating cDNA libraries that are either selected for, or at least specifically enriched in, copies of mRNAs that have the potential for translation via the internal ribosome entry mechanism.

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