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RNA-Protein Interactions in Regulation of Picornavirus RNA Translation

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INTRODUCTION

The picornaviruses are a large family of animal viruses (termed the *Picornaviridae*), which are widespread in nature. Certain members of this family are well known since they are important in human health terms, e.g., poliovirus (PV) (causing poliomyelitis) and the rhinoviruses (causing the common cold). Foot-and-mouth disease virus (FMDV), responsible for the world's most economically important disease of farm animals, is also one of the picornaviruses. A detailed overall review of their biology can be found elsewhere (130). The family *Picornaviridae* is currently divided into five genera (Table 1), namely, the enteroviruses (including PV), rhinoviruses, cardioviruses (including encephalomyocarditis virus [EMCV]), aphthoviruses (foot-and-mouth disease viruses), and hepatoviruses (hepatitis A viruses). Picornaviruses are small icosahedral particles containing a single copy of a positive-sense RNA genome (about 7,500 to 8,300 nucleotides). The virion RNA has a virus-encoded peptide, VPg, attached at its 5′ terminus, but this protein is rapidly lost within the cell and most of the viral transcripts (all genome length) consequently lack the protein. The picornavirus RNAs also lack the cap structure (m^7GpppN, where N is any nucleotide and m is a methyl group) found on all eukaryotic cellular (except organellar) mRNAs. The viral RNA encodes a single large polyprotein which undergoes a series of processing events, mediated by virus-encoded proteases, to produce the mature virus proteins (about 11 different mature polypeptides plus many different partially processed products, depending on the virus). Four of these proteins (60 copies each) constitute the virus capsid, while the others are involved in virus replication. Although there is considerable similarity in the organization of the genome among the picornaviruses (Fig. 1), there are also significant differences between the different genera (for reviews, see references 8, 111, 130, and 137).

With the exception of hepatitis A virus, infection of susceptible cells by the picornaviruses has profound and rapid effects on the cell. These include inhibition of host cell transcription and translation, modification of intracellular membrane structures, cell rounding, and, finally, cell lysis and release of mature virions. Up to 100,000 virus particles may be produced from a single infected cell within 6 h. This requires that concomitant with the inhibition of host cell functions, the virus-induced processes be efficiently instigated. This review will focus on the mechanisms by which the translation of picornavirus RNA occurs while the translation of cellular mRNAs is essentially abolished. It is, of course, true that at the initial stage of infection both virus RNA and cellular mRNAs are translated simultaneously and only later is the host cell protein synthesis inhibited.

We shall first address the question how picornavirus infection modifies the intracellular environment, since this clearly has implications for the strategies which are used by the viruses to produce their own proteins. The prototype picornavirus is PV, and hence much of this review will concentrate on studies of this virus. However, other virus genera have distinct prop-

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TABLE 1. Characteristics of different picornaviruses

Picornaviridae genus	Poly(C) tract	eIF4G cleavage	RRL IVT[a] activity	Representative species[b]
Enterovirus	No	Yes (2A)[c]	+	PV, CV-A, CV-B, EV
Rhinovirus	No	Yes (2A)[c]	+	HRV
Aphthovirus	Yes	Yes (L)[c]	+++	FMDV
Cardiovirus	Yes/no[d]	No	+++	EMCV, MV, TMEV
Hepatovirus	No	No	±	HepA
Orphanovirus[e]	No	No	+++	EV-22, EV-23

[a] RRL IVT, in vitro translation activity of viral RNA within rabbit reticulocyte lysate; ±, very low; +, low; +++, high. The translation of hepatitis A virus, enterovirus, and rhinovirus RNA can be stimulated in this system by supplementation with additional cellular factors (see the text).

[b] Abbreviations: CV-A, coxsackievirus A; CV-B, coxsackievirus B; EV, echovirus; HRV, human rhinoviruses (over 100 serotypes); MV, mengovirus; HepA, hepatitis A virus; EV-22 and EV-23, echoviruses 22 and 23.

[c] The viral proteases (2A or L) required for eIF4G cleavage are indicated.

[d] TMEV lacks a poly(C) tract.

[e] The name *Orphanovirus* has been suggested by the picornavirus taxonomy group for these two novel picornaviruses but has yet to be confirmed by the International Committee for the Taxonomy of Viruses.

erties in this area, and hence studies on the cardioviruses, usually EMCV, and FMDV will be discussed when this is helpful.

EFFECT OF PICORNAVIRUS INFECTION ON HOST CELL PROTEIN SYNTHESIS

Infection of cells by PVs, rhinoviruses, and aphthoviruses results in a rapid (1- to 3-h) inhibition of host cell protein synthesis (30, 36, 37). This inhibition is accompanied by the cleavage of eukaryotic initiation factor 4G (eIF4G) (formerly termed p220 or eIF-4γ), a subunit of eIF4F (the cap-binding complex). eIF4F contains two other associated proteins, namely, eIF4E (which binds to the cap structure) and eIF4A (which possesses ATP-dependent RNA helicase activity). eIF4G may serve as a scaffold within the eIF4F complex. Recently, specific binding sites for eIF4E, eIF4A, and eIF3 on eIF4G have been identified (78, 93). The eIF4F complex attaches to mRNAs at the cap structure. It is then believed to proceed along the mRNA, unwinding RNA secondary structure in conjunction with eIF4B to facilitate 40S ribosomal subunit association. The 40S subunits are thought to scan along the 5′ untranslated region (UTR) until the AUG initiation site is encountered. At this point, attachment of the 60S ribosomal subunit occurs and polypeptide chain formation begins (for reviews, see references 73, 100, and 101). The cleavage of eIF4G following picornavirus infection results in the inactivation of the eIF4F complex with respect to its ability to recognize capped mRNAs and hence in a severe inhibition of cap-dependent translation.

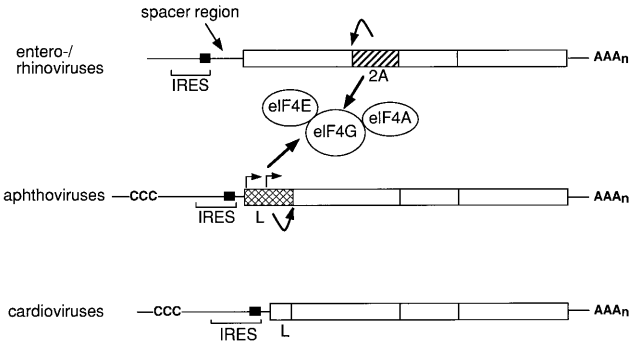

FIG. 1. Genome organization of different genera of picornaviruses. The locations of the PV and rhinovirus 2A protein and the FMDV L protein which cleave the eIF4G subunit of eIF4F are shown. The L protein of cardioviruses lacks protease activity, and no cleavage of eIF4G occurs in cardiovirus-infected cells. ■, polypyrimidine tract at the 3′ end of the IRES (see the text for details). The presence of two translation initiation sites on FMDV RNA and the cleavage sites of L and 2A proteases are indicated by arrows.

The cleavage of eIF4G induced by rhinoviruses and PV (36, 37, 74) is dependent on the viral 2A protease (Fig. 1). Viable PV mutants with mutations in 2A, which inefficiently inhibit host cell protein synthesis and fail to induce the eIF4G cleavage, have been identified (12, 110). Initial biochemical evidence indicated that the cleavage activity (termed p220ase) was separable from the bulk of PV 2A (86). Furthermore, anti-2A antiserum failed to block eIF4G cleavage, although it could block 2A-mediated polyprotein processing. Consequently, it was suggested that the 2A protease modifies a host enzyme, resulting in the generation of a novel proteolytic activity. Further studies indicated that recombinant PV 2A requires eIF3 to initiate eIF4G cleavage (157). However, no modification of eIF3 has been detected, and the induced p220ase with an apparent molecular mass of 55 to 60 kDa was separable from eIF3 (158).

The FMDV leader (L) protease (Fig. 1), which is unrelated to the PV and rhinovirus 2A, is responsible for eIF4G cleavage in FMDV-infected cells (30, 95). Initial evidence suggested that the cleavage sites within eIF4G were substantially different in FMDV-infected bovine cells from in PV-infected human cells (72, 85). However, this was shown merely to reflect a species difference, since eIF4G cleavage products which were indistinguishable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained in transient-expression studies within a single cell type when using either the PV 2A protease or the FMDV L protease (95).

Recently, the picture has changed. Evidence which appears to directly conflict with the view that the virus-induced eIF4G cleavage is indirect has been presented. The rhinovirus and coxsackievirus B4 2A proteases have been expressed in *Escherichia coli* and have been shown to cleave the eIF4G subunit directly and independently of eIF3 (79). Furthermore, the FMDV L protease has also been reported to cleave eIF4G directly (71). The cleavage products from eIF4G generated by both the rhinovirus 2A and FMDV L activities were sequenced, and the cleavage sites were shown to lie just 7 amino acids apart on the C-terminal side of residues 486 and 479, respectively (a result consistent with the observations of Medina et al. [95]). The N-terminal domain of eIF4G liberated by this process contains the sequences identified as the binding site for eIF4E (78, 93), consistent with the retention of cap-binding activity by part of the modified eIF4F complex (81). Since the studies by Wyckoff et al. (157, 158) had failed to observe cleavage by PV 2A of purified eIF4G unless eIF3 was added, a direct conflict apparently exists. It is possible that the rhinovirus and coxsackievirus 2A proteases operate in a different mode from that of the PV 2A, but this seems rather unlikely. A helpful discussion of this issue has been produced by Ehrenfeld (35). The studies by Wyckoff et al. (157, 158) used a partially purified eIF4G preparation (derived by phosphocellulose and calmodulin affinity chromatography). In contrast, Lamphear et al. (79) and Kirchweger et al. (71) used an intact eIF4F complex purified on a cap affinity column. It was suggested that isolated eIF4G is misfolded and requires the presence of other proteins with which it interacts, e.g., eIF3 or eIF4A and eIF4E, to adopt a native structure and act as a substrate for the 2A proteases (35).

The correlation between the cleavage of eIF4G and inhibi-

tion of host cell protein synthesis holds good until about 70% inhibition occurs, when almost all eIF4G has been cleaved (15, 121). PV replication in cells is blocked by guanidine, but complete eIF4G cleavage can still be observed in the presence of this inhibitor (15). However, incomplete inhibition of host protein synthesis occurs under these conditions. This suggests that eIF4G cleavage is necessary but not sufficient for PV-induced inhibition of host cell protein synthesis. Thus, it seems that PV uses additional mechanisms to block host protein synthesis, since the inhibition normally observed during PV infection is almost absolute. Translation of any cellular mRNAs under these conditions requires these mRNAs to be translated by a cap-independent mechanism. Indeed, the identification of a cellular polypeptide whose synthesis continued in cells infected with a replication-defective PV (132) led to the discovery of the first cellular mRNA (encoding BiP [immunoglobulin heavy-chain binding protein] [92]) which uses a mechanism analagous to that used by the picornaviruses for initiation of translation (see below).

These observations have led to the search for other modifications of initiation factors that are induced by picornavirus infection and which may be responsible for inhibiting host cell translation. It has been shown that in PV-infected cells, the initiation factor eIF2α is phosphorylated (14, 110). However, this is a late event and probably not significant in the regulation of host cell protein synthesis. This phosphorylation probably occurs in response to the presence of double-stranded RNA in the infected cells and the activation of the double-stranded RNA-dependent protein kinase of which eIF2α is a substrate (reviewed in reference 69). The phosphorylation of eIF2α blocks the recycling of eIF2 (by eIF2B) and results in a general inhibition of protein synthesis. There is no indication that PV protein synthesis is resistant to this inhibitory effect. Hence, there is no selective advantage for the virus, and the effect may be the result of a host defense mechanism (69). Indeed, it appears that PV infection induces degradation of double-stranded RNA-dependent protein kinase (13, 14), thereby limiting eIF-2α phosphorylation.

In contrast to the enteroviruses, rhinoviruses, and FMDV, the cardioviruses do not induce cleavage of eIF4G (105). It has been proposed that inhibition of host cell protein synthesis following infection by cardioviruses may reflect competition between the cellular and viral RNAs for the translation machinery (33, 80, 142). It was also suggested that cardioviruses induce a change in ion concentration within the cell to bias the translational capacity of the cell toward the viral RNA (3), and such a mechanism has been proposed for PV infection too (23). No inhibition of host cell protein synthesis has been detected in hepatitis A virus- or echovirus-22-infected cells (138), and eIF4G is not cleaved under these conditions either (27).

Recently, another mechanism for the regulation of host cell cap-dependent protein synthesis has been identified. Two small proteins, of about 12 kDa (although they migrate at about 20 kDa according to SDS-PAGE), bind to eIF4E and block its activity (113). Phosphorylation of one of these eIF4E-binding proteins, termed 4E-BP1 (for eIF4E-binding protein 1), results in its dissociation from eIF4E and hence stimulation of cap-dependent protein synthesis (Fig. 2). Such a mechanism is believed to underlie the stimulation of protein synthesis by insulin and growth factors (83, 113). The interaction of 4E-BP1 with eIF4E does not significantly affect cap-independent translation directed by picornavirus 5′ UTRs (113) (see below). Thus, stimulation of 4E-BP1 activity, by dephosphorylation, could potentially inhibit cellular cap-dependent protein synthesis without blocking picornavirus protein synthesis. Evidence that such a mechanism does indeed operate in picornavirus-infected cells has recently been obtained (42). There is a strong temporal correlation between the inhibition of cellular protein synthesis in EMCV-infected Krebs-II ascites cells and the dephosphorylation of 4E-BP1. Therefore, this may be the major mechanism operating for the regulation of cap-dependent protein synthesis by cardioviruses. Dephosphorylation of 4E-BP1 has also been seen in PV-infected cells, but in this case the change in 4E-BP1 activity occurs after the onset of inhibition of cellular protein synthesis resulting from eIF4G cleavage (42). The effect on 4E-BP1 activity requires PV replication and is blocked by guanidine. In contrast, the eIF4G cleavage occurs in the presence of this inhibitor. The mechanism by which the dephosphorylation of 4E-BP1 is achieved in picornavirus-infected cells has not yet been determined. It is apparent that at present the complete basis for the inhibition of host cell protein synthesis by picornaviruses is not resolved.

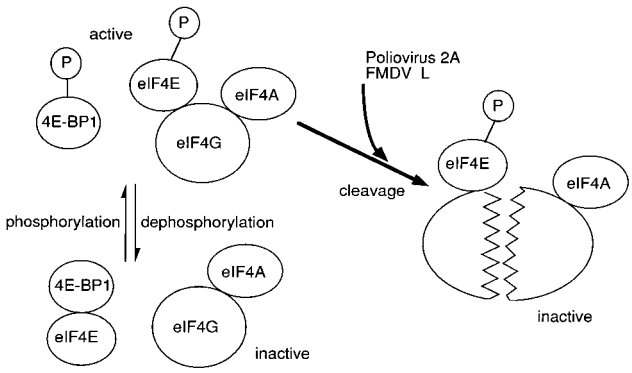

FIG. 2. Mechanisms involved in the inhibition of cap-dependent protein synthesis by picornaviruses. The activity of the cap-binding complex, eIF4F, in cap-dependent protein synthesis can be blocked by two distinct mechanisms. The cleavage of eIF4G results in loss of eIF4E (the cap-binding protein) from the complex, while interaction of eIF4E with 4E-BP1 blocks the association of eIF4E with eIF4G.

THE PROBLEM OF PICORNAVIRUS RNA TRANSLATION

Picornavirus RNAs contain long 5′ UTRs. These range from about 650 nucleotides (rhinoviruses) to around 1,300 nucleotides (FMDV). Within these regions are multiple AUG codons, most of which are poorly conserved between strains and do not appear to be recognized by the translational machinery (117). As mentioned above, the 5′ terminus of the viral RNA is not capped. The 5′ UTR is predicted to contain extensive secondary structure (Fig. 3), and RNA structure-probing studies are consistent with these models. Much of the secondary structure is highly conserved even in the absence of high sequence conservation (see below). These features of the picornavirus 5′ UTRs conflict with the recognized characteristics of mRNAs which are efficiently translated (58, 73), and, of course, the translation of these RNAs occurs when the translation of cellular mRNAs is abolished. A priori, these considerations strongly argue for a different mechanism for the initiation of translation.

The importance of understanding picornavirus translation is amply demonstrated by the finding that a major determinant of PV neurovirulence is located within the 5′ UTR (108, 129, 153). This determinant affects the translational efficiency of the viral RNA. PVs differing only by single nucleotide substitutions within the 5′ UTR cause disease with markedly different se-

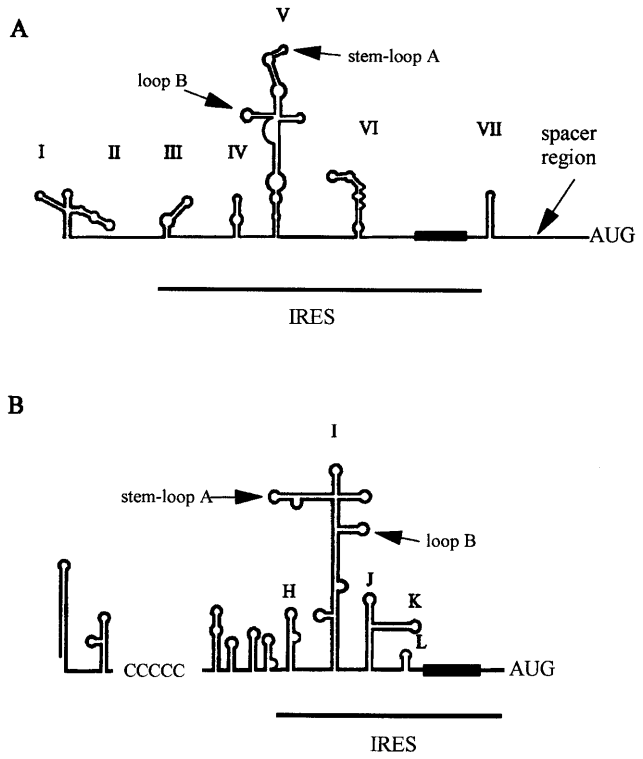

FIG. 3. (A) Secondary-structure prediction for the enterovirus and rhinovirus IRES elements. (B) Secondary-structure prediction for the cardiovirus and aphthovirus IRES elements. Conserved elements, namely, the polypyrimidine tract (▬), the GNRA tetraloop (stem-loop A), and loop B are indicated (60).

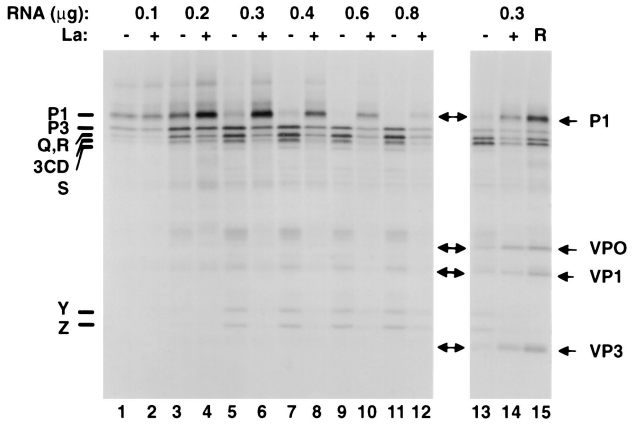

FIG. 4. Stimulation and correction of PV RNA translation in vitro by La in rabbit reticulocyte lysate. Indicated amounts of PV RNA were translated in the absence or presence of recombinant La protein (1 μg) or Krebs-2 cell ribosomal salt wash fraction (15 μg) (R) for 1 h (lanes 1 to 12) or 3 h (lanes 13 to 15). The positions of the P1 capsid precursor and authentic processed products VP0, VP1, and VP3 are indicated on the right. Note the presence of aberrant initiation products, particularly Y and Z (as defined previously [31]) synthesized at high RNA concentrations in the absence of La and the increased synthesis of P1 in the presence of La. Reproduced from reference 144.

verity (91). The attenuating mutations in the Sabin vaccine strains of PV have been identified, and it has been demonstrated that a key determinant of neurovirulence in each of the PV serotypes is located between residues 472 and 481, within domain VI of the PV 5′ UTR (Fig. 3) (108, 129, 153). Attenuated viruses fail to replicate efficiently in neural tissue or in neuroblastoma cells (1, 77) but replicate efficiently in the gut and in HeLa cells, whereas wild-type (wt) viruses replicate efficiently in all these cells. It has been demonstrated that the translational efficiency of the viral RNAs in cell-free systems also correlates with their neurovirulence (141, 143, 146). These studies indicate that the tropism of the virus can be determined by differential recognition of the 5′ UTR, presumably by cell type-specific proteins which affect the translational efficiency of the virus.

MECHANISM OF PICORNAVIRUS RNA TRANSLATION

The key experiments in demonstrating that picornavirus RNA contains an internal element which directs the initiation of translation required a novel approach. Pelletier and Sonenberg (119) showed that the insertion of the PV 5′ UTR as the intergenic spacer within an artificial bicistronic mRNA directed efficient translation of the second open reading frame. In the absence of the PV sequence, little synthesis from the second open reading frame occurred, consistent with the fact that mRNAs are generally functionally monocistronic in eukaryotic cells. Analogous results were obtained with the EMCV 5′ UTR (62). These studies indicated that the picornavirus 5′ UTRs were capable of directing the initiation of protein synthesis independently of the 5′ terminus of the mRNA and independently of the expression of the upstream open reading frame. This mechanism of cap-independent initiation of protein synthesis is referred to as internal initiation. To show that an RNA element directs internal initiation requires the translation of artificial bicistronic mRNAs, with the element under study as the intergenic spacer and efficient translation of the second open reading frame (for a review, see reference 67). Subsequent studies have confirmed and extended the characterization of the picornavirus 5′ UTR elements. It is now established for representatives of each of the picornavirus genera (9, 18, 22, 62, 64, 76, 118, 119, 149) that a region of about 450 bases directs cap-independent internal initiation of protein synthesis. The region of RNA directing internal initiation of protein synthesis is now generally referred to as an internal ribosome entry site (IRES), although initially the term "ribosome landing pad" was used (119). Recently, it has been demonstrated that the EMCV IRES can efficiently direct initiation of translation within a circular RNA (26), confirming the ability of the protein synthesis machinery to initiate translation in the complete absence of any free RNA terminus. The initial studies characterizing these elements have already been extensively reviewed (8, 35, 52, 58–61, 63, 67, 99), and we will not repeat this. The key properties of internal initiation of protein synthesis directed by a picornavirus IRES are as follows: (i) IRES-directed translation is independent of the location within an mRNA; (ii) internal initiation is cap independent (it functions in the presence of cap analogs [118, 149] and in PV-infected cells or cell extracts [118] or in cells expressing the PV 2A protease [139] or the FMDV L protease [9, 95], which induce the cleavage of the eIF4G component of the cap-binding complex); (iii) IRES-directed translation is independent of the expression of other ORFs within the same mRNA; and (iv) the picornavirus IRES elements function without a requirement for any picornavirus proteins (although this does not exclude the possibility that certain virus proteins may influence IRES activity [see below]).

The properties listed above are true for all the picornavirus elements described. However, it has been established that the cardiovirus and aphthovirus RNAs are very efficient templates in vitro in a rabbit reticulocyte lysate whereas, in contrast, the PV (and other enterovirus and rhinovirus) RNAs translate

rather poorly and inaccurately (i.e., many aberrant products are generated, [Table 1; Fig. 4] [21, 31]). Addition of HeLa cell lysate to the reticulocyte translation system both stimulated the total amount of synthesis and corrected the translation (i.e., reduced the level of aberrant products) from PV RNA (21, 31). The high fidelity of PV RNA translation in HeLa cell extract has permitted the generation of infectious PV from the de novo-synthesized components (104). These studies provide evidence for two separate classes of picornavirus IRES elements. Furthermore, they indicate that cellular proteins, in addition to the canonical translation factors present in rabbit reticulocyte translation extracts, are required for the PV IRES to function efficiently.

STRUCTURE OF THE IRES

Secondary-structure predictions for the regions of the viral RNAs which constitute the picornavirus IRES elements have been derived (Fig. 3). The enterovirus and rhinovirus group of viruses have a common predicted structure (Fig. 3A) (125, 136), while the cardiovirus and aphthovirus group shares a second type of RNA structure (Fig. 3B) (60, 124). There is little similarity between these two major types of structures. The hepatitis A virus IRES is predicted to form a third type of structure (22) with some similarity to the cardiovirus and aphthovirus element. A closer similarity between the echovirus-22 IRES and the cardiovirus and aphthovirus IRES has also been described (52). The two principal groups correspond to the previously defined classes of IRES elements on the basis of their ability to direct efficient translation in rabbit reticulocyte lysate (see above). Numerous picornavirus RNA sequences have been determined. This database has been valuable in assessing the validity of the secondary-structure models. The presence of many compensatory base substitutions is strong evidence in support of the secondary structures. Within a group, the same secondary-structure model can be derived even though sequence identity with other members of the group can be less than 50%. Most mutations introduced into the IRES either are inhibitory or produce no phenotype. However, two examples of enhanced IRES activity have been documented for FMDV (94) and hepatitis A virus (43). Absolute conservation of specific regions of the IRES may indicate sequences involved in interactions with proteins or other RNAs or interactions within the viral RNA. The recent review by Jackson et al. (60) discusses these analyses in detail.

Location of the IRES

A further distinction between the two classes of IRES elements is indicated by their locations relative to the initiation codon from which translation commences (Fig. 1). The 3′ ends of the cardiovirus and aphthovirus IRES elements are adjacent to the initiation codon, albeit with a caveat that in FMDV a second initiation codon some 84 bases downstream is also used (7, 131). In the enteroviruses and rhinoviruses, the 3′ terminus of the IRES is up to 150 bases from the initiation codon. This distance can also be greatly modified with little effect on the virus; for example, an insertion of 72 bases into this region was tolerated in PV unless the insertion also included an AUG (75). This is compatible with the idea that a process analagous to "scanning" occurs from the 3′ end of the enterovirus and rhinovirus IRES to the initiation codon. This view is also consistent with data obtained on initiation on the FMDV RNA. Insertion of two additional AUG codons between the two authentic initiation sites led to the recognition of these sites, compatible with a scanning mechanism operating after a single point of ribosome entry onto the mRNA upstream of the first initiation codon (7). This entry point must be close to the initiation codon, since in EMCV an AUG codon, just 8 bases upstream from the authentic initiation codon, is not used by the virus although it is capable of acting as a start site in the absence of the IRES (66). Thus, on EMCV RNA, little or no scanning would in fact occur. Hellen et al. (51) have suggested that a discontinuous transfer of ribosomes from the 3′ end of the PV IRES to the initiation codon occurs, but proof that sequences within this spacer sequence are "skipped" is lacking. However, the role of this spacer region, which is conserved in size but not in sequence in PV RNA, is not fully understood.

At the 3′ end of all picornavirus IRES elements is a polypyrimidine tract, first noted by Kuhn et al. (76). In both groups of IRES elements, there is an AUG codon at a conserved distance (20 to 25 nucleotides [nt]) from this tract. In the cardioviruses and aphthoviruses, the AUG is (one of) the initiation codon(s), whereas in PV it is one of the conserved AUG codons in the 5′ UTR. The importance of this cryptic AUG codon in the PV 5′ UTR was shown by mutagenesis of each of the upstream AUG codons. Only when the AUG codon associated with the polypyrimidine tract was mutated was a phenotype (small plaque) observed in the virus (117). This mutation resulted in inhibition of translational efficiency of the PV 5′ UTR within PV-infected HeLa cell translation extracts (117). Extensive analysis of the requirement for this cryptic AUG codon and its spacing relative to the polypyrimidine tract has been performed within the context of the virus (127). PV has a strong preference for the spacing to be approximately 20 nucleotides. Modification of this structure results in a virus which grows poorly, and selection for rearranged sequences occurs in which the optimal spacing between the polypyrimidine tract and the cryptic AUG has been regenerated (127).

In EMCV (and presumably other cardioviruses and aphthoviruses), there is also a preference for a specific spacing (about 20 nucleotides) between the polypyrimidine tract and the initiation codon (AUG-11) (65). In particular, shortening of the distance is highly deleterious. Modification of the authentic initiation codon in EMCV indicated that translation could occur with reasonable efficiency at a downstream site (28). However, there does appear to be something highly significant about the environment of the correct AUG codon in EMCV. Another AUG codon (AUG-10), in a different context, even when positioned at the correct spacing from the polypyrimidine tract, is utilized much less well (65), while the authentic initiation codon (at a suboptimal distance from the polypyrimidine tract) continues to be used; however, overall there was reduced total translational efficiency. In the absence of the IRES, AUG-10 efficiently acts as an initiation codon (66). The situation in FMDV with an 84-base spacer between the two functional initiation sites is more complicated (7). An AUG codon at the correct position relative to the 3′ end of the IRES is present even when not used. Thus, initiation on FMDV RNA may be viewed either as an intermediate between the strategies used by PV and EMCV or, alternatively, as a combination of them.

Polypyrimidine Tract

Initial studies on the polypyrimidine tract (12 to 15 residues) suggested that this is an essential element of the IRES for FMDV, EMCV, and PV (64, 76, 97, 107, 122). In these studies, the polypyrimidine tract was modified by partial deletion or the substitution of nucleotides with purines, especially G's. In PV, a minimal sequence of UUUCC, at the 5′ end of the tract, is required (107). In contrast, recent studies on the EMCV and

Theiler's murine encephalomyelitis virus (TMEV) (another cardiovirus) IRESes have demonstrated a remarkable insensitivity of the polypyrimidine tract to mutation (65, 127). Indeed, in the most drastic change, all the pyrimidines in the EMCV motif were changed to A's and the mutant IRES still exhibited about 70% of wt efficiency both in vitro and in vivo (65). It may be that the specific mutants that have been made in the earlier studies were defective whereas other modifications may be tolerated. However, these results may also indicate a real difference in the importance of the polypyrimidine tract for the two types (enterovirus versus cardiovirus) of IRES elements. An important test to distinguish between these possibilities is the modification of the PV polypyrimidine tract to a poly(A) tract as in the mutant EMCV IRES described above (65). Indeed, recent results have shown that the modification of just the core PV (type 1, Mahoney) polypyrimidine tract sequence UUUCC to UAAAC reduced the translational efficiency to about 2% of wt (123). These results suggest that there is a real functional difference in the sequence requirement for these tracts between different picornaviruses.

Other Conserved Motifs

Sequence comparisons among all known picornavirus IRES elements do not reveal any other extensive regions of sequence identity. However, two short motifs, termed stem-loop A and loop B, have been identified by Jackson et al. (60) as being conserved in both types of IRES element (Fig. 3). The loop of stem-loop A fits the GNRA tetraloop consensus (where N is any nucleotide and R is a purine), which is statistically overrepresented at this position of an RNA loop (156). It is believed that these loops are involved in long-range tertiary interactions with helical regions, a suggestion compatible with recent structural analyses of RNA molecules (128). Modification of the GNRA motif in the EMCV IRES or the PV IRES greatly reduces the ability of each of these IRES elements to direct internal initiation (60). The loop B motif is C rich, but its functional significance is unknown.

Analysis of RNA-RNA Interactions within the IRES

The secondary-structure models give no clues about interactions between different regions of the IRES; for example, motifs at the 5′ end may interact directly with sequences near the 3′ end. Eventually, the three-dimensional structure of an IRES may be determined by physical methods, although currently rather few RNA structures have been solved and these are significantly smaller, e.g. tRNA (about 80 nt). An approach which has been used to obtain such information is the generation of point mutations in the IRES and the introduction of these mutants into a full-length infectious cDNA of the virus. If the RNA transcripts retain infectivity but are defective, it can be possible to obtain pseudo-wt revertant viruses which contain second-site mutations which compensate for the loss of function. Such mutations can reveal previously unknown interactions. To date, studies of this type have focused on certain discrete regions, for example, nt 471 to 538 within the PV 5′ UTR, which contain a major determinant of neurovirulence (89, 103, 136) and have generally resulted in direct reversion or compensatory mutations in predicted base pairs. However, evidence for an interaction between the IRES and PV 2A has been obtained from these studies (90) (see below). Studies of this type were also used for determining that the spacing and association between the polypyrimidine tract and the cryptic AUG codon are important (45, 127). This approach should be extended to other regions of the IRES and to other IRES elements.

The initial studies identifying the limits of the IRES essentially determined the 5′ and 3′ boundaries of the linear sequence, but this did not exclude the possibility that certain internal elements are dispensable. Indeed, domain IV (Fig. 3A) within the PV 5′ UTR is totally dispensable for IRES function (47, 107, 120, 139). This is consistent with the absence of this sequence from bovine enterovirus (34). Deletion of domain VII (Fig. 3A) results in a reduction of IRES function, but a significant level of activity remains (47a, 120, 139). Indeed, a viable PV lacking both of these regions within the IRES has been produced (47). In contrast to these studies on the PV IRES, none of the principal elements of the FMDV IRES can be individually deleted without abolishing its function (32). Similarly, major modification, e.g., linker insertion, of each of the major domains of the EMCV IRES has a severely detrimental effect on its translational activity (33).

It is generally believed that the IRES is a *cis*-acting element, and this is probably generally correct. However, it has been possible to show that defective IRES elements within bicistronic mRNAs can be complemented in *trans* by the coexpression within cells of the parental wt IRES by using the vaccinia virus-T7 RNA polymerase expression system. This process has been shown to occur in both the PV IRES (139) and the second family of IRES elements represented by FMDV (32) and EMCV (152). The complementation is highly sequence specific and efficient and appears to be independent of any recombination event, since certain mutations cannot be complemented even though recombination could repair the defect (32, 152). Two models have been proposed to explain this effect. In one, it is proposed that a functional initiation complex is assembled on the wt IRES and then transfers to the site on the mRNA from which translation begins (perhaps the starting window defined by Pilipenko et al. [126]). Although this process will usually occur in *cis*, it is conceivable that under conditions of high RNA concentration (as occurs in a picornavirus infection), the process may occur in *trans*. This process is then reminiscent of the discontinuous scanning process described for cauliflower mosaic virus, termed a ribosome "shunt" (39). A second explanation could be that during cosynthesis of the two RNA molecules, association occurs to generate a pseudo-wt structure from two different molecules. The possibility that such an interaction occurs was demonstrated by Borovjagin et al. (19), who showed that by melting and annealing two nonfunctional RNA molecules in vitro, a functional IRES element could be generated. However, the recent studies indicating that defective EMCV IRES elements containing only point mutations can be complemented makes this second model seem less likely (152). The complementation assay may prove useful in determining regions of the IRES which interact by identifying features required for an element either to be complemented or to complement another element.

EVIDENCE FOR PROTEIN INTERACTIONS WITH THE ENTEROVIRUS AND RHINOVIRUS IRES

As indicated above, early experiments on the translation of picornavirus RNAs indicated that the enterovirus and rhinovirus RNAs required factors for efficient and accurate translation in excess of those found in the rabbit reticulocyte lysate. Clearly, many mRNAs are translated efficiently in reticulocyte lysate, indicating either that these IRES containing mRNAs have a greater requirement for canonical translation factors than do most mRNAs or that additional protein factors present in HeLa cells but deficient in rabbit reticulocyte lysate are required for their efficient translation. The hepatitis A virus IRES also functions inefficiently in rabbit reticulocyte

translation extracts, but its activity is not enhanced by HeLa cell factors. However, it is stimulated (up to 12-fold) by liver cell proteins (44).

The differential translation of wt and attenuated Sabin strains of PV in different cell types indicates that cellular factors influencing translational activity may be differentially expressed, consistent with the idea that factors distinct from the standard translation initiation factors determine picornavirus translation efficiency. These observations led to the search for cellular proteins which interact with the IRES.

DIRECT IDENTIFICATION OF CELLULAR PROTEINS INTERACTING WITH ENTEROVIRUS AND RHINOVIRUS IRES ELEMENTS

Essentially two different experimental approaches have been used to provide evidence for specific protein interactions with IRES elements: mobility shift assays and UV-induced cross-linking of RNA to protein. Mobility shift assays can be used with small regions of the IRES, but most studies have used UV cross-linking of ^{32}P-labelled RNA probes to proteins in cell extracts.

A mobility shift assay was performed with a labelled RNA probe corresponding to residues 559 to 620 of the PV IRES, using a HeLa cell extract (98). This probe was selected since it contains both the single AUG codon whose modification confers a distinct phenotype on PV (AUG-7 in type II Lansing [117]) and the polypyrimidine tract. A distinct, specific complex was generated with a protein present mainly in the S100 fraction of the cell extract. A much weaker signal was obtained with rabbit reticulocyte lysate. UV cross-linking of the HeLa cell complex indicated that the RNA-binding protein was a 52-kDa polypeptide. This protein was purified on the basis of the mobility shift assay, and a partial amino acid sequence (22 amino acids) was obtained. Surprisingly, the sequence was identical to a region of the human La autoantigen (96). The identity of the 52-kDa protein as La was confirmed with anti-La monoclonal antibodies which "supershifted" the RNA-p52 complex (96). The La protein is known as an RNA-binding protein, containing a characteristic RNA recognition motif (termed an RRM [70] or RNA-binding domain [6]). It binds to the 3′ termini of RNA polymerase III transcripts and is believed to function in transcription termination and/or the maturation of these RNAs (46). However, some doubt has been cast on this assignment, since recent studies have shown that in *Drosophila melanogaster* the La protein is not present in all cells (5). The location of the protein is predominantly within the nucleus, but upon PV infection the localization changes (96), so that a significant fraction is found within the cytoplasm (where the picornaviruses replicate and translation occurs).

Other proteins have also been shown to interact with the PV 5′ UTR in the gel shift assay. A 50-kDa protein was found to interact with nt 178 to 224 within the PV IRES (106), but the significance of this protein for IRES function is doubtful, since this region of the IRES is absent in bovine enteroviruses (34). Also, this region can be deleted without loss of function from the PV IRES (47, 47a, 107, 120).

DelAngel et al. (29) obtained an array of protein-RNA complexes formed between cellular proteins and two distinct regions of the PV 5′ UTR: nt 1 to 247 and nt 320 to 629. These complexes were not fully characterized but were recognized (and gel shifted) by a monoclonal antibody specific for eIF2α, an observation which is compatible with the mobility shift of the PV nt 559–624 probe with eIF2 (98). It is noteworthy that evidence for interaction between eIF2 and the EMCV IRES has also been obtained (38, 133). In further analysis of the nt 320 to 629 region complexed to protein, footprinting indicated that the region of the PV 5′ UTR from 550 to 629 was protected from chemical cleavage (29). A number of different RNA-protein complexes (as judged by gel mobility patterns) were formed between the nt 320 to 629 probe and ribosomal salt wash proteins, perhaps indicating that several different proteins are involved. It is likely that La would have been one component, since the PV sequences used included that studied by Meerovitch et al. (96, 98).

UV-cross-linking assays have detected numerous additional proteins which interact with the PV IRES. Haller et al. (47) observed 13 different species cross-linking to the wt 5′ UTR. These included major species with molecular masses of 39, 48, 52 and 64 kDa. The 52-kDa protein may well correspond to La, as was also suggested for the 54-kDa protein observed by Gebhard and Ehrenfeld (41) to bind between nt 457 and 657 of the PV 5′ UTR. These authors also detected species of 48 and 38 kDa which cross-linked to the PV 5′ UTR within nt 286 to 456 and may be the same as the smaller species identified by Haller et al. (47). In each case, the proteins appeared to specifically interact with PV sequences. The identities of the 64-, 48-, and 38-kDa species have not been determined.

A protein of 57/58 kDa (this doublet will be referred to as p57) has been reported to cross-link to the PV and rhinovirus IRES (17, 122). The p57 species has been identified as the polypyrimidine tract-binding protein (PTB) (17, 53). PTB is localized predominantly within cell nuclei and was postulated to function in RNA splicing (40, 112), but its precise function in this process is not fully established. Recently, it has been shown that PTB can regulate splice site selection by inhibiting the selection of 3′ splice sites adjacent to PTB-binding sites (82, 135). Multiple binding sites for PTB have been identified in the PV 5′ UTR (50). The sites were included in three noncontiguous sequences, 70 to 286, 443 to 539, and 630 to 730. The region 443 to 539 includes the region implicated in determining neurovirulence; however, no difference in interaction between PTB and RNA transcripts containing either the wt or attenuated virus sequence could be detected (50). This suggests that the interaction between PTB and the PV 5′ UTR does not determine PV neurovirulence. The 630 to 730 region is not required for IRES function, and this is also true for parts of the 70 to 286 region; hence, the significance of these sites is not known. Interestingly, it has been recently demonstrated that a mutant PV, containing mutations in the pyrimidine-rich region, did not interact with PTB but displayed wt growth (48), suggesting that the interaction of the PV IRES with PTB is not essential for PV translation. However, as discussed below, the protein can stimulate EMCV RNA translation.

In a distinct approach, a functional assay for the ability of fractionated HeLa cell lysate to enhance rhinovirus IRES-directed translation in rabbit reticulocyte lysate has been combined with analysis by UV cross-linking to the rhinovirus 5′ UTR (17). This study showed that two separate stimulatory activities could be resolved. One type contained the bulk of the p57 (PTB) cross-linking activity. The second activity, with an apparent molecular mass of more than 400,000 Da, contained at least two proteins, p97 and PTB (but only a minor portion of the total), which could be UV cross-linked to the rhinovirus 5′ UTR. It was suggested that some synergism between fractions containing p97 and PTB occurred in the stimulation of the rhinovirus IRES-directed translation. The total protein composition of the stimulatory fractions was not indicated. However, further fractionation has shown that the second stimulatory activity copurifies with just two proteins, described as 96- and 38-kDa proteins. The identity and biochemical functions of these proteins are not yet known (58), but these proteins

also apparently stimulate PV IRES function in rabbit reticulocyte lysate at low concentrations (1 to 5 μg/ml). A similar approach has been taken by Toyoda et al. (148), who fractionated HeLa cell lysate and assayed the ability of the fractions to enhance PV IRES activity. The stimulatory activity had a molecular mass of more than 240,000 Da, and these fractions contained both PTB and La. It was suggested that a large complex including these two components represented the active component. However, further fractionation resulted in a loss of stimulatory activity.

EVIDENCE FOR A FUNCTIONAL ROLE FOR THE INTERACTION OF La WITH THE PV IRES

The La autoantigen is a target for autoimmune recognition in patients with systemic lupus erythematosus and Sjögren's syndrome (reviewed in reference 147) and its role in internal initiation has been recently reviewed (10). Since La is known to be an RNA-binding protein and is localized predominantly to the nuclei of cells, it was clearly important to verify that the interaction observed in vitro has functional significance. The key experiment was the demonstration that purified, recombinant La (expressed in *E. coli*) both enhanced and corrected (i.e., reduced aberrant initiation) translation of PV RNA in a rabbit reticulocyte translation extract (Fig. 4), an effect previously detected with HeLa cell lysate or extracts from Krebs-2 cells (96, 144). This result has been criticized on the basis that the amount of La used constituted an unphysiological concentration (58, 61). Indeed, the level of recombinant La used was about 10-fold higher than the level of La in a HeLa cell-supplemented rabbit reticulocyte translation extract in which activation and correction are observed (96). A number of factors may well explain this discrepancy. First, it is likely that some activity is lost during purification. Second, it is possible that a significant portion of the *E. coli*-expressed La is partially misfolded. Although the purification procedure selects for a functional RNA-binding domain (present within the N-terminal portion of the molecule), this does not ensure that the carboxy-terminal region of La (which is required for the enhancement and correction activity [see below]) is also in a native conformation. More interestingly, the high levels of La required in rabbit reticulocyte translation extracts may indicate that other factors normally act in concert with La and that these may also be limiting in such extracts. Consistent with this idea, immunodepletion of La from HeLa cell lysate with specific antisera inhibited PV RNA translation. However, it was not possible to restore translational activity by the addition of recombinant La (140). Finally, it is possible that some form of covalent modification, e.g., phosphorylation, used in mammalian cells but not used appropriately in *E. coli* regulates the activity of La. It is known that La is a phosphoprotein (147).

In support of a physiological role for La in internal initiation, there is considerable specificity in the enhancement of PV RNA translation by protein factors. The initiation factors eIF4A, eIF4B, eIF4F, and eIF3 failed to stimulate or correct PV RNA translation. The factors eIF2 and eIF2B (GEF) did stimulate overall translation but failed to correct the aberrant translation (144). It should be noted that neither eIF4B nor the 1 to 194 region of La, both of which contain a functional RRM, displayed any stimulatory activity, indicating that the effect is not merely dependent on RNA binding. The carboxy-terminal region of La is required for the effects of La on PV translation and may therefore function as an effector domain. It is of interest that the phosphorylation sites within La reside within this C-terminal portion of the protein (24, 25). There is also specificity with regard to which RNAs are responsive to La. No stimulation of the translation of EMCV, brome mosaic virus, or tobacco mosaic virus RNAs was observed (96, 144). However, La has been shown to stimulate the translation of mRNAs containing the human immunodeficiency virus TAR element in their 5′ noncoding region (145). The diverse range of activities attributed to La may suggest that it functions as an RNA chaperone; i.e., it may serve to stabilize particular RNA structures which alone are unfavored (99, 145).

DIRECT IDENTIFICATION OF CELLULAR PROTEINS INTERACTING WITH THE CARDIOVIRUS/APHTHOVIRUS IRES ELEMENTS

Studies with EMCV and FMDV RNA established that a p57 protein cross-linked to their IRES elements (64, 87, 88). As with studies on the enterovirus and rhinovirus IRES, the p57 species has been identified as the PTB (17, 53). Two binding sites were identified on the FMDV IRES, one close to the 5′ end (stem-loop H [Fig. 3B]) and a second around the polypyrimidine tract at the 3′ terminus of the IRES. However, binding to this latter site was observed only at low salt concentrations (88). The major site of association between p57 and the EMCV IRES is in stem-loop H (Fig. 3B) (64), although evidence for a second site in a region further upstream in the 5′ UTR was also obtained. Subsequently, evidence has been obtained that PTB also binds near the polypyrimidine tract at the 3′ end of the EMCV IRES (154, 155). A correlation was obtained between binding of the p57 to mutants in stem-loop H and translational activity (64). However, there is some controversy about the requirement for the stem-loop H region of the IRES for translation. By using in vitro translation assays, it was shown for EMCV (33) and FMDV (76) that loss of much of this stem-loop structure (including the major p57-binding site), in the context of a monocistronic transcript, reduced translation by only 50% or less in vitro. However, assays performed with bicistronic mRNAs indicated a strict requirement for the integrity of this stem-loop structure for efficient IRES activity (9, 32, 64). It is possible that when the p57-binding site is located at the 5′ terminus of the RNA transcript, there is a reduced requirement for this factor. Perhaps the presence of p57 stabilizes a particular structure when upstream sequences (as in the virion RNA and the bicistronic mRNAs) are attached. This is analagous to the postulated RNA chaperone role described above for La (see the previous section).

EVIDENCE FOR A FUNCTIONAL ROLE FOR PTB

Following the demonstration that a p57 protein binds to stem-loop H of the EMCV and FMDV IRES (64, 87, 88), evidence was sought to determine whether this interaction was functional. A correlation was observed between the ability of mutants with mutations in stem-loop H to interact with p57 (PTB) and their ability to direct internal initiation; however, it was not established that this was a direct link (64). As with La, immunodepletion of PTB blocked the ability of rabbit reticulocyte translation extracts to translate EMCV IRES-containing mRNAs, but the translation could not be restored by the addition of recombinant PTB (53). It could be that PTB is complexed to other essential factors (148) or that the immunodepletion is not sufficiently specific. A different approach was used by Borovjagin et al. (20), who used a region from the 5′ terminus of the EMCV IRES, which contains a PTB-binding site, as a competitor in in vitro translations. This RNA inhibited EMCV RNA translation but had no effect on tobacco mosaic virus RNA translation. The competition could be relieved by addition of PTB. Recently, in an extension of this

strategy, an RNA affinity column containing the 5′-terminal region of EMCV IRES (including stem-loop H [Fig. 3B]) as the ligand has been used to deplete rabbit reticulocyte translation extracts of PTB (68). Depletion of PTB by this procedure prevented EMCV IRES-directed translation, and, in contrast to the immunodepletion studies, the addition of recombinant PTB restored translation (68). These results indicate that PTB is indeed required for EMCV IRES-directed translation and suggests that the immunodepletion experiments were insufficiently specific. The PTB-depleted reticulocyte translation extracts were little impaired in their ability to translate mRNAs by a cap-dependent mechanism, and they also efficiently translated TMEV IRES and hepatitis C virus (a member of the family *Flaviviridae*) IRES-containing mRNAs (68). The lack of effect on TMEV-directed translation is surprising, since TMEV is, like EMCV, a member of the *Cardiovirus* genus and the IRES element is predicted to have a very similar structure to that of EMCV and can be efficiently UV cross-linked to PTB (55, 68). Consistent with the postulated RNA chaperone role for PTB described above, the EMCV IRES needs the presence of PTB to stabilize a particular structure but the TMEV IRES must be able to achieve this state without the presence of PTB. Very recent results have identified circumstances in which EMCV IRES function is also PTB independent, further supporting an RNA chaperone model (61a). The result with the hepatitis C virus IRES also contrasts with recent data from Ali and Siddiqui (2), who demonstrated PTB cross-linking to the hepatitis C virus IRES. Furthermore, immunodepletion of PTB prevented hepatitis C virus RNA translation, but, as described above, caution about the significance of this result must be exercised since the addition of PTB failed to restore function (2).

INVOLVEMENT OF CANONICAL INITIATION FACTORS IN IRES-DIRECTED TRANSLATION

Meyer et al. (102) have recently identified an 80-kDa protein which can be UV cross-linked to the FMDV IRES as initiation factor eIF4B. The binding site was located at the 3′ end of the IRES. The interaction of eIF4B with the IRES was dependent on the presence of ATP, while interactions with other proteins, i.e., p57 (PTB), p38, and p95 (cf. the proteins stimulating enterovirus and rhinovirus RNA translation described by Jackson [58]), observed in the same analysis were unaffected by the presence of ATP. The known functional interaction between eIF4B and eIF4A and the fact that eIF4A is an ATP-dependent RNA helicase suggest that the interaction of eIF4B with the IRES may also involve eIF4A, a protein which cross-links poorly to RNA (see below). No experiments concerning the consequence of eIF4B interaction with the IRES have been reported.

The technique of UV cross-linking will not necessarily identify all the proteins that are interacting with the IRES, since, for example, in most studies only a single labelled nucleotide is used to prepare the labelled RNA probe, and it is therefore necessary that bases of this type be present within the region of RNA that is cross-linked to the protein and protected from RNase digestion. Lack of evidence for interactions with the canonical initiation factors (except eIF2 and eIF4B) is suggestive of this. Hence, it is by no means certain that the critical protein interactions involved in IRES function have been identified. Other experimental approaches have also given evidence for the involvement of certain initiation factors in IRES function.

Mutants of the initiation factor eIF4A (an ATP-dependent RNA helicase [see reference 116 for a review]) have been constructed, and some of these mutants have a dominant negative effect on translation of capped mRNAs in vitro (115). These studies provided evidence consistent with a model in which eIF4A recycles through the eIF4F complex in each round of initiation. It was proposed that the dominant negative mutants blocked initiation by forming a stable, inactive eIF4F complex, essentially reducing the availability of eIF4F for initiation. It was shown that translation of monocistronic mRNAs containing either the PV or the EMCV IRES was strongly inhibited by these dominant negative mutants. These studies have been extended with bicistronic mRNAs (containing an IRES) both in vitro and in vivo, and it is apparent that the IRES-directed translation is more sensitive to the dominant negative mutants than is cap-dependent translation (114). It does not seem likely that extensive unwinding of RNA secondary structure is required for IRES function, because the ATP requirement for IRES-directed initiation is much reduced compared with that for cap-dependent translation (57). However, some localized unwinding to permit ribosome attachment may be necessary. It is of interest that these dominant negative eIF4A mutants block PV IRES-directed translation in PV-infected HeLa extract when cap-dependent translation is abolished and the eIF4G component of eIF4F is cleaved. Thus, the dominant negative effect of the mutant eIF4As does not require intact eIF4F (114). This indicates that for IRES-directed translation, a modified eIF4F is sufficient for function or that eIF4A plays a role in internal initiation distinct from that within the eIF4F complex. Taken together, the results suggest that IRES-directed translation can function with the cleaved eIF4G component of eIF4F and hence without eIF4E but that the concentration of eIF4F (intact or modified) required is greater than for cap-dependent protein synthesis. Earlier data, which are consistent with this idea, demonstrated that eIF4F stimulates PV and EMCV IRES function in vitro (4, 134). Recently, Ohlmann et al. (109a) demonstrated that the C-terminal domain of eIF4G is sufficient to support cap-independent translation.

VIRAL PROTEINS THAT AFFECT IRES FUNCTION

Although it is apparent that no picornavirus proteins are absolutely required for IRES activity, there is evidence that some virus-encoded proteins facilitate IRES function. Evidence for a role of the PV 2A protease (which cleaves eIF4G) has been obtained by analysis of revertant PV obtained by selection from the temperature-sensitive (*ts*) mutants generated by mutagenesis of the IRES. Suppression of the *ts* phenotype was achieved by several distinct mutations within the 2A protein-coding sequence (90). Mutations in 2A enhanced virus protein synthesis even when complete inhibition of host protein synthesis had been achieved. This suggests that PV 2A plays a direct role in the IRES directed translation. However, no evidence for a direct interaction, for example, by cross-linking, has been obtained. It is difficult in this system to separate a variety of different effects that could be exerted by such mutations. Other studies (49, 139) have provided evidence that PV 2A enhanced PV directed translation, but it was not possible to clearly distinguish between a direct effect of 2A and an effect mediated by the eIF4G cleavage products or loss of competition from capped mRNAs. Similar difficulties in interpretation are encountered with the report that the translational activities of the rhinovirus and enterovirus IRES elements (but not the EMCV or FMDV IRES elements) were stimulated by the FMDV L protease (159).

FUTURE DIRECTIONS

It is apparent that mammalian cells have all the machinery required for recognition of an IRES element. It is most probable that the picornaviruses have usurped a device already used by cells. There are circumstances when internal initiation by ribosomes may be useful to the cell. For example, during mitosis, cap-dependent translation is inhibited (16, 54) but the translation of mRNAs containing an IRES can continue (16). The 5′ UTR of BiP (immunoglobulin heavy-chain binding protein, or glucose-regulated protein [GRP78]) mRNA was the first example of a cellular IRES (92). This indicates that recognition of an IRES is a physiological function. Subsequently, additional cellular IRES elements have been identified in the basic fibroblast growth factor mRNA (151) and also recently, rather intriguingly, in the 5′ UTR of eIF4G (39a). Examples of nonmammalian IRES elements in *D. melanogaster* (*antennapedia* [109]) and *Saccharomyces cerevisiae* (*hap4*, *tfIID*) (56) mRNAs have also been reported. The opportunity to use yeast genetics to study IRES function may have considerable utility in identifying functional *trans*-acting factors.

As already indicated, viruses distinct from the picornavirus family, e.g., hepatitis C virus (150), certain retroviruses (11), and the coronavirus infectious bronchitis virus (84), also contain IRES elements, but there is little apparent similarity between these elements and the picornavirus or cellular IRES elements. It may be that there are many different ways in which RNA sequences can exhibit IRES function, and there may be a significant number of cellular IRES elements that have been used as the basis for a virus to acquire this function.

The identification of proteins required for IRES function is not yet complete; in particular, methods which allow the identification of proteins interacting with the IRES in vivo will have to be developed. The role of all the canonical initiation factors in IRES-directed translation is not yet established, but some progress, as indicated above, is being made in this direction.

The current interest in IRES elements should lead to an increase in the understanding of cellular as well as viral protein synthesis.

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