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Internal initiation of translation of picornaviruses, hepatitis C virus and pestiviruses

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ABSTRACT

In the life cycle of picornaviruses, hepatitis C virus and pestiviruses, a special mechanism plays a key role for the initiation of translation which is significantly different from the standard scheme of translation in eukaryotic cells. Internal ribosome entry site (IRES) elements, located far downstream from the 5´-end of the viral RNA, serve as binding sites for proteins that facilitate the internal entry of ribosomes. This strategy allows these viruses to induce a general shut-down of the cap-dependent cellular translation while maintaining translation from their own RNAs. To support internal translation initiation, these viruses use essentially the entire basic set of eukaryotic initiation factors but apply some modifications. In addition to the standard translation initiation factors, they recruit other cellular proteins that bind to the IRES elements. Such proteins may enhance translation efficiency, confer tissue specificity on the translational level, or mediate the balance between translation of the infecting positive-strand viral RNA and its replication during the viral life cycle.

INTRODUCTION

Essentially all eukaryotic cellular mRNAs are monocistronic (1). Depending on the 5´-terminal cap structure (2,3) and mediated by a cap-binding complex of eukaryotic initiation factors (eIFs), the small 40S ribosomal subunit binds near the 5´-end of the mRNA (4). After binding, the ribosome processively scans along the RNA to find its initiator AUG codon (5-7) and starts translation if an appropriate context of this AUG is given (8,9).

From this general rule, there are two major exceptions. The first mechanism is regulation of the translation rate by reinitiation. The paradigm for this type of regulation is the 5´-untranslated region of the mRNA for the yeast transcription factor GCN4. Four small open reading frames (uORFs) that precede the main GCN4 ORF regulate reinitiation at the GCN4 AUG. After translating uORF1, some 40S subunits continue scanning the mRNA. When amino acids are abundant, the level of the ternary complex of eIF2·GTP·Met-tRNAi is high. The scanning 40S subunits are reloaded rapidly with ternary complexes, translate the other uORFs and dissociate from the mRNA after translation of uORF4, resulting in low expression of the downstream GCN4 ORF. Under starvation conditions, the levels of ternary complexes are low. This allows reloading of several 40S subunits with ternary complexes only after they have passed uORF4 by scanning without translation, resulting in enhanced translation of the downstream GCN4 ORF (10).

The second important exception is internal initiation of translation. Challenging the long-standing hypothesis that eukaryotic ribosomes are not able to initiate translation from internal sites in RNAs (11), the Internal Ribosome Entry Site (IRES) elements of the picornaviral RNAs were demonstrated to mediate the internal entry of ribosomes on an RNA (12-18), even on a circular closed RNA (19). This phenomenon extends also to some members of the distantly related Flaviviridae, namely hepatitis C virus

(HCV) (20,21), the members of the pestivirus group (22,23), and the recently discovered hepatitis G virus (24). The synthesis of the picornaviral polyprotein is initiated cap-independently from the IRES which is located far downstream from the 5´-end of the positive-strand viral RNA (25,26). This strategy allows picornaviruses to induce a general shut-down of the cap-dependent cellular translation. They either proteolytically clip off the N-terminal domain of the eukaryotic initiation factor (eIF) 4G that interacts with the cap-binding protein eIF-4E, or they inactivate eIF-4E by inducing dephosphorylation of its inhibitor, eIF4E-binding protein 1 (27).

It is an obvious assumption that eukaryotic cells have not developed specialized mechanisms that are useful only for the propagation of viruses infecting the cells. Accordingly, also cellular mRNAs were found to contain IRES elements (28), e.g., the mRNAs for the immunoglobulin heavy-chain binding protein (BiP) (29), eIF4G (30) and the N. crassa Albino-3 gene (31). Interestingly, also several mRNAs of genes involved in cellular growth regulation were found to be translated by internal initiation, like the D. melanogaster antennapedia and ultrabithorax mRNAs (32,33), the proto-oncogene c-myc (34), and the mRNAs of some growth factors as human basic fibroblast growth factor (35), human insulin-like growth factor II (36), platelet-derived growth factor B (PDGF2) (37) and vascular endothelial growth factor (VEGF) (38). The considerable number of growth factor mRNAs containing IRES elements and the finding that cap-independent translation appears to play an important role in oocytes (39) point to the idea that the translation of genes involved in growth regulation and development is often mediated by IRES elements for reasons yet unknown.

ORGANIZATION OF VIRAL IRES ELEMENTS

After infection of the cell and uncoating of the viral particle, the positive-strand RNAs of picornaviruses, HCV and pestiviruses serve not only as template for viral replication, but they are directly used as messenger RNAs for translation of the viral gene products. They contain a single, large open reading frame (ORF) that encodes a polyprotein which is processed co- and posttranslationally into the mature gene products by cellular and viral proteases (40). Picornaviral RNAs are uncapped (41) and carry a small protein covalently attached to their 5´-ends which is involved in their replication. The polyprotein ORFs are preceded by long 5´-untranslated regions (5´-UTRs). In picornaviruses, the length of these 5´-UTRs is usually 600-1200 nucleotides (nts), and they may contain several AUG codons. However, these AUGs are not used for translation. The internal initiation of translation on the viral RNA is facilitated by the IRES elements which are located within the 5´-UTRs directly preceding the polyprotein ORFs, in some cases also extend into the coding region.

According to their primary and secondary structures, the viral IRES elements are classified in four groups, three groups of picornaviruses and one including HCV and the pestiviruses. The overall structure of the different picornavirus IRES elements is roughly similar, with a large central domain consisting of a long stem and a cross-shaped upper part (Fig. 1). Upstream of this large central domain, there are different numbers of smaller domains with structures that differ between the picornavirus groups. A mid-size domain is located distal of the central domain and consists of a stem-loop with bulges in the upper part in entero-/rhinoviruses and hepatitis A virus (HAV), or a characteristic Y-shaped structure in cardio-/aphthoviruses. In contrast, in the HCV/pestivirus group, one large domain appears to replace the central domain and the more distal domains of picornaviruses.

Although the three groups of picornaviral IRES elements are quite different in detail, they are separated from the HCV/pestivirus IRES elements by another distinct feature which is common for all picornavirus IRES elements. This is a characteristic tandem of cis-elements at their 3´-borders, an oligopyrimidine tract followed by an AUG triplet. In cardio-/aphthoviruses translation is initiated usually at this AUG. However, in entero-/rhinoviruses the conserved AUG at this position is not used for translation, instead the actual polyprotein start site further downstream is reached probably by ribosomal scanning. Although the HCV IRES contains oligopyrimidine tracts also, these are not arranged in a fixed distance shortly upstream of the initiator AUG.

An important property of the IRES structures evaluated by experimental and pyhlogenetic data is that they appear to

be conserved mainly on the secondary structure level, whereas only some patches of primary sequence conservation are obvious (26). These small stretches of conserved primary sequence reside mostly in unpaired loops and bulges, indicating that they are involved either in tertiary interactions within the IRES element (42), or they may serve primarily as the actual binding sites for proteins.

TYPE I IRES ELEMENTS: THE ENTERO-/RHINOVIRUS GROUP

The first computer-generated models of the possible secondary structure of the IRES of poliovirus, the paradigm of the entero-/rhinovirus group, were based on comparative sequence analyses (43,44). These were refined by chemical and nuclease structure mapping of domains II and III (42), domain III (45), and domains IV and V (42,46,47), and by data obtained from genetic analysis of a variety of wildtype poliovirus strains that provided information about secondary structure restoring mutations in the proposed stem-loop structures (48). The 5´-border of the poliovirus IRES was mapped between positions (pos.) 79 and 139 (15,16,49) and thus between the stem-loops I and II. In contrast, the proximal domain I of the poliovirus 5´-UTR, the so-called "cloverleaf", is involved in replication (50,51). The 3´-border of the IRES was not identical with the actual startcodon of the polyprotein ORF at pos. 743, but was mapped to a region upstream of the authentic AUG startcodon, between pos. 564 and 600 within the stem-loop VI (15,52,53). The sequence of about 160 nts between the 3´-border of the IRES and the actual initiation codon at pos. 743 appears not to be important, given the case it contains no AUGs and no sequences capable of hairpin formation.

Accordingly, the current model of the poliovirus IRES (Fig. 1) consists of four conserved stem-loop structures (26,54), the domains II to V, plus the proximal part of the stem-loop VI. Domain II is crucial for poliovirus translation (55,56), whereas the small domain III is not essential for IRES function (56,57). Although the stem-loop IV is required for translation in vivo (56), it appears not to be essential for translation, since in some in vitro-experiments the translational activity of the IRES was not abolished

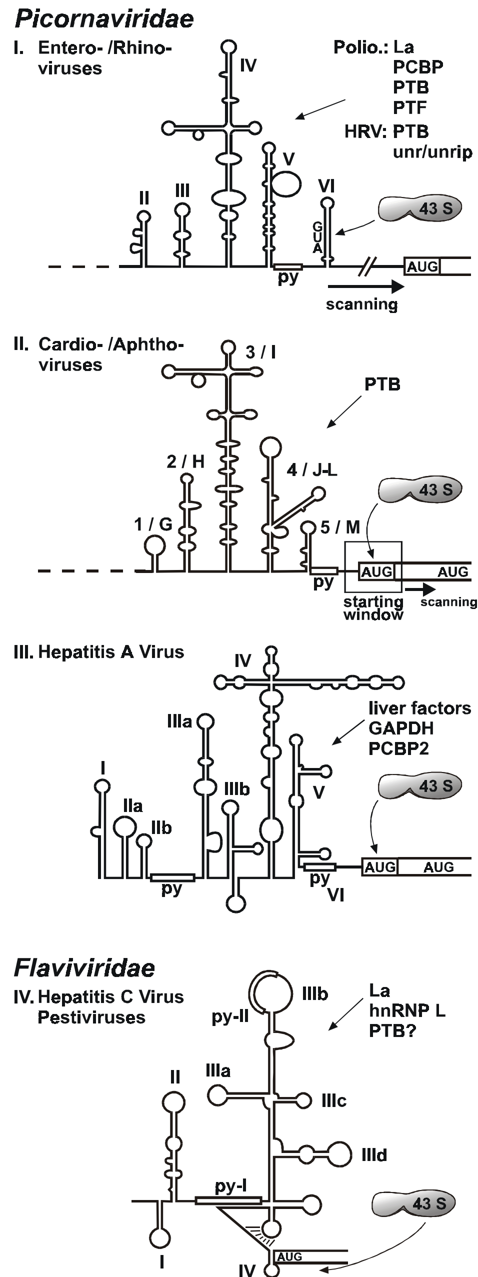


Fig. 1. Organisation of viral IRES elements. The IRES domains are numbered as mentioned in the text, and interacting non-standard protein factors are listed.

upon deletions in this structure (17,58). The domain V appears to be the most important structure of the poliovirus IRES, since nearly any mutation affecting its structure is deleterious for IRES activity (15,18,52,57-60).

The oligopyrimidine tract ("py" in Fig. 1) located upstream of the stem-loop VI is essential for IRES function (52,55,61-63). Sequences in this oligopyrimidine tract were proposed to hybridize to complementary sequences in the ribosomal 18S rRNA (53,64,65), and are possibly involved in a mechanism of guiding the eukaryotic ribosome to the translational start site in a way similar to that of prokaryotes.

The last stem-loop of the proposed poliovirus IRES structure appears not to be absolutely required as a folded entity, since the 3´-border of the actual IRES resides within this stem-loop (15,52,53). Nevertheless, primary sequences within this domain V are essential for IRES function, namely the AUG triplet at pos. 586. Although this conserved AUG is part of the tandem of cis-elements (an oligopyrimidine tract followed by an AUG) which is characteristic for picornaviruses, this "cryptic" AUG 586 is not used for translation initiation. Nevertheless, its actual primary sequence is important, but not the ability of this sequence to participate in base-pairs forming the stem-loop VII (62). However, when the context of the silent AUG 586 is changed to a favorable consensus sequence (6), this AUG can be used as an initiation codon (66). Thus, ribosomes appear to enter the viral RNA near this AUG and most probably then reach their authentic polyprotein initiator codon (AUG 743) by scanning. This view is supported by experiments in which an additional AUG was inserted into the variable linker between the conserved AUG 586 and the polyprotein start site at AUG 743. In large plaque revertants, this extra AUG was always deleted or mutated (67).

In human rhinoviruses (HRV), the distance between the conserved, silent AUG (pos. 576) and the authentic start site for polyprotein synthesis is shorter than in poliovirus, since HRV uses the AUG at pos. 611 as initiation codon. The actual site of ribosome entry appears to be within the sequence between pos. 554 and 568 (68), leaving the distance between the ribosome entry site and the polyprotein start site of about 50 nts significantly shorter than the one in poliovirus. AUG 611, which serves as the actual start site for polyprotein synthesis, is also located within the proposed structure of the stem-loop that corresponds to stem-loop VI in poliovirus (68).

Recently, a slightly altered structure for the poliovirus IRES was proposed, based on phylogenetic analyses in which the larger domains IV and V of the "classical" model are folded into several smaller domains, thereby obtaining a structure that should be more compact on the tertiary structure level (69). However, this proposed model appears to be at least in part not consistent with chemical and nuclease structural probing data (46) as well as with genetic data (48).

IRES determinants related to the neurovirulence of poliovirus were correlated mainly with a single site in the stem-loop V, a C residue at position 472, pointing to the interaction of tissue-specific cellular factors with this site (70-76). Recently, additional determinants for poliovirus neurovirulence were identified in an elegant approach using chimeric poliovirus/rhinovirus IRES elements (77). They reside in the apical parts of the stem-loops V and VI (not including pos. 472) and act synergistically, since both stem-loops have to be of the poliovirus type to mediate neurovirulence. A second site correlated with neurovirulence was identified in mice transgenic for the poliovirus receptor. The critical mutations reside within the stem-loop II and appear to require nucleotide pos. 107 (78,79).

TYPE II IRES ELEMENTS: THE CARDIO-/APHTHOVIRUS GROUP

The secondary structure model of the IRES elements of the cardio-/aphthovirus group is different from that of the type I IRES (Fig. 1). After identification of a functional IRES region in encephalomyocarditis virus (EMCV) (14,80), foot-and-mouth disease virus (FMDV) (81,82) and Theiler´s Murine Encephalomyelitis Virus (TMEV) (83,84), its 5´ border was first mapped between EMCV pos. 315 and 485 (85), and more exactly between pos. 406 and 416 (86). Thereby, cardio-/aphthovirus IRESs include the stem-loops 2 (H) to 5 (M), whereas the stem-loop 1 (G) contributes only very slightly to IRES activity (81,86).

The mechanism by which the ribosome enters the site that

contains the actual initiator AUG used for polyprotein synthesis in cardio-/aphthoviruses is different from the mechanism used in entero-/rhinoviruses. Whereas in entero-/rhinoviruses the AUG of the oligopyrimidine/AUG tandem is silent and the actual initiator AUG located more downstream is reached by scanning, in EMCV and TMEV the ribosome appears to be placed directly to a limited region at the 3´-border of the IRES usually containing the initiator AUG (87,88). This small area to which the ribosome is targeted was called the "starting window" (88). The determinants responsible for this targeting partly reside within the oligopyrimidine tract, but are mainly dependent on the entire structure of the IRES (87). When the AUG is moved to a more upstream position, closer to the IRES structure and the oligopyrimidine tract, initiation occurs at the next downstream AUG. This indicates that the entry of the small ribosomal subunit is independent of the presence of an AUG triplet at the actual target site (88). A possible hybridization between the oligopyrimidine tract and the ribosomal 18S rRNA has been proposed (65,89), but not yet proven.

The FMDV IRES appears to use a combination of both mechanisms, direct placing of the ribosome to the initiation AUG in the starting window directly at the 3´-border of the IRES, as well as the scanning mechanism to reach a second authentic start site located 84 nts farther downstream (90-92). These two AUGs lead to the synthesis of two different forms of the leader (L) protease of FMDV, which are both able to cleave eIF4G. However, the first AUG is not essential for virus growth, whereas mutation of the second AUG abolishes virus viability (93).

As for the structure of the poliovirus IRES, Palmenberg and Sgro proposed a new model also for the cardio-/aphthovirus group IRES elements (69) in which the largest domain of the "classical" Pilipenko model (83) of encephalomyocarditis virus (EMCV) (stem-loop 3 in FMDV, compare Fig. 1) is folded into three smaller domains. On one hand, this model appears to be compatible with experiments in which insertions or deletions introduced into the first third of this large stem-loop I of EMCV caused only mild effects on translation efficiency (86,94). Also, some of the data obtained with chemical modification and nuclease mapping data obtained with the EMCV IRES (83,85) are consistent not only with the classical model (83), but also with the newly proposed structure (69).

On the other hand, however, some cleavages with the double-strand specific nuclease CV at EMCV pos. 661-663 are consistent with the classical model regarding a stem in the base of domain 3 (I) , but not with a bulge in the Palmenberg model (83,85). Moreover, the base of the large stem-loop I in EMCV was reported to be base-paired (95). The proposed structure of the closely related FMDV IRES was developed mainly by sequence comparisons and the similarity of the computational folding data obtained with the EMCV IRES (83). There are only few structural probing data available for the FMDV IRES (96), and these support the classical Pilipenko model but not the Palmenberg model. Several nucleotides in the lower stem of stem-loop 3 (I) (corresponding to EMCV pos. 455, 456, and 461-463; compare (85)) are sensitive to digestion with the double-strand specific RNase V1, while they are postulated to be unpaired by the Palmenberg model (69). Some other nuclease CV cleavages in a bulge at EMCV pos. 663-666 are not consistent with both models (83,85). Thus, most of the chemical and nuclease structural mapping data obtained with the IRESs of the cardio-/aphthovirus group are consistent with both models, some support the classical but not the new model, and very few do not fit with both models.

However, the most important support for the classical model comes from a detailed mutational analysis of the basis of the predicted stem-loop 3 in FMDV. Mutations destabilizing the predicted base-paired structure were detrimental to IRES function, while subsequent restoration of the predicted RNA structure gave rise to a fully competent IRES (97).

The neurovirulence of TMEV in mice has not been depicted to a single, clear-cut determinant, but neurovirulence appears to be dependent on multiple determinants in the TMEV 5´-UTR. First, a large sequence region preceding the IRES element was found to affect neurovirulence (98). These authors found that also sequences within a large portion of the coding region appear to be involved in neurovirulence. Second, a three nucleotide insertion in the H loop of TMEV abolished neurovirulence in mice, although it did not largely affect the in vitro-translation

efficiency (99). Third, a single base deletion in the large central domain of the TMEV IRES, the stem-loop I, was reported to attenuate neurovirulence (100). Fourth, the oligopyrimidine tract/AUG tandem is important for neurovirulence. Alterations in the oligopyrimidine tract caused attenuation of the respective mutants in mice, and pseudorevertants that regained neurovirulence had a restored oligopyrimidine tract/AUG tandem (101). Fifth, deletion of the sequence coding for the leader peptide of TMEV attenuated neurovirulence, although having little effect on growth of TMEV in BHK-21 cells (102). However, until now none of these determinants could be linked to the interaction with a cellular protein and its probably tissue-specific function at the IRES.

TYPE III IRES ELEMENTS: HEPATITIS A VIRUS

A model for the structure of the hepatitis A virus (HAV) IRES (Fig. 1) was proposed according to secondary structure probing of the HAV 5´-UTR (103). The 5´-border of the HAV IRES was first mapped to be located 3´ to pos. 355 (103) or pos. 347 (104), respectively, within the large domain IV in the 5´-UTR of HAV (see Fig. 1). However, a later study mapped the 5´-border between pos. 150 and 257 (105), thus located directly preceding the stem-loop IIIb or within this stem-loop. The 3´-boundary was found to be located between pos. 628 and the authentic AUG at pos. 736 (105).

In addition to this "core" IRES located between domains IIIa and the AUG, some other elements, either enhancing or inhibiting, may influence the HAV IRES activity on top of a basic level. Outside the previously mapped 5´-border of the HAV IRES, the proximal oligopyrimidine tract, which is located between the domains IIb and IIIa, was reported to be important for HAV translation (106). In contrast, deletion of the first 138 nts of the HAV 5´-UTR, removing the stem-loops I, IIa, IIb and the 5´ oligopyrimidine tract, was reported to result in a loss of inhibition of translation (107), leaving a yet unresolved puzzle of enhancing and inhibiting effects of sequences from the very 5´-end of the HAV 5´-UTR. Moreover, sequences located within the first 114 nucleotides of the HAV core protein coding region, downstream of the previously mapped 3´-border of the IRES, appear to slightly enhance translation from the IRES (107).

TYPE IV IRES ELEMENTS: HEPATITIS C VIRUS AND PESTIVIRUSES

The current secondary structure model of the HCV 5´-UTR (108) includes four domains (see Fig. 1). The domains I and II are small, whereas domain III represents a large stem-loop with several branches and an oligopyrimidine tract (py-II) included in its apical loop (109). In pestiviruses, two small hairpin structures are present at the very 5´-end of the 5´-UTR instead of one in HCV (the domain I depicted in Fig. 1) (23,110-112). Another oligopyrimidine tract (py-I) is located between domains II and III. The distal part of this first oligopyrimidine tract interacts with a downstream region of the IRES directly preceding the initiator AUG (113). Domain IV consists of a small stem-loop that contains the authentic initiator AUG and is involved in a pseudoknot interaction with a small distal stem-loop of domain III. The actual starting window in HCV to which the ribosome is placed upon internal entry is limited to a very small area around the initiator AUG (114,115).

The data delimiting the actual 5´-border of the HCV IRES are controversial. Its location was first mapped between pos. 101 and 147 (20) and by that would not include the domains I and II. In fact, domain I was repeatedly reported not to be required for HCV IRES function (116,117). Also in pestiviruses, the 5´-border of the IRES elements was mapped to pos. 70 for BVDV (bovine viral diarrhea virus) (118) or to pos. 67 for CSFV (classical swine fever virus) (112), suggesting that the first two small hairpins of pestiviruses are not essential for translation. However, in later studies only the deletion of the first some 70 nts already caused a serious decrease in translation efficiency (21,116). In another study, even the deletion of only 21 nts of the HCV 5´-UTR caused a decrease in translation efficiency (119), indicating that all domains, including the stem-loops I and II, are necessary for HCV IRES activity.

In contrast to the results obtained with the three groups of picornaviruses, the 3´-border of the HCV IRES definitely extends into the coding region. The actual 3´-border of a

complete and fully active HCV IRES is not yet known. Although an HCV IRES ending exactly at the initiator AUG (pos. 342) has been shown to be active (21,120), the current understanding is that sequences of the N-terminal coding region are required for a fully active HCV IRES. Nucleotide sequences within the first 42 nt of the HCV core protein coding sequence were reported to be required for full IRES activity (117). A detailed in vitro study (121) has shown that an HCV IRES only including pos. 355 is essentially inactive. The 3´-border of an active IRES was mapped between pos. 355 and 374, and full in vitro-translation was only achieved when nucleotide positions 375 to 395, i.e., 54 nts of the core coding sequence, were also included. Also in another study, sequences up to pos. 371 were required for full IRES activity (122). These results were supported by infection experiments with poliovirus/HCV IRES chimera. When the HCV IRES did not include coding sequences, the chimera were not viable. A chimera only containing 14 nt of HCV core sequences (up to pos. 356) was viable, but exhibited a small plaque phenotype and produced 100-fold reduced amounts of infectious virus compared to a longer variant (123,124). In contrast to these results obtained with HCV, deletion of coding sequences downstream of the initiator AUG in the IRES of the pestivirus BVDV reduced translation efficiency only to 79 % (118).

A COMMON "CORE" STRUCTURE IN THE 3´-REGION OF IRES ELEMENTS?

A conserved common "core" structure in the most 3´-region of the IRES elements has been postulated for all three groups of picornaviruses (125) as well as for HCV and pestiviruses (126,127), and was extended further to cellular IRES elements (128). This predicted core structure of viral and cellular IRES elements appears to be similar to a proposed secondary structure of group I introns (127,128). The main prediction made by these compilations is a conserved secondary/tertiary core structure that involves one of the most distal domains of the IRES and an RNA pseudoknot. This pseudoknot is predicted to form between sequences in a bulge in the last IRES domain that precedes the oligopyrimidine tract on one hand, and sequences in the region between this last domain and the AUG on the other hand. In picornaviruses, this second sequence partner involves sequences within the oligopyrimidine tract or immediately downstream of it.

For the entero-/rhinovirus group, unfortunately no conclusions can be drawn to support or disprove this modelfor the region in question from the experimental nuclease and chemical mapping data available (46,47). For the cardio-/aphthovirus group, only very few data can be depicted from (83). In a bulge in domain 4 (Fig. 1, II), a UUAAA sequence was predicted to be involved in pseudoknot formation (127). The U-A bond (underlined in the sequence UUAAA) was found to be slightly sensitive to nuclease CV1 digestion and by that supposed to be double stranded in accordance with the pseudoknot model. In contrast, the second and the third A (UUAAA) were susceptible to DMS treatment and so supposed to be single-stranded (83), thereby not supporting the model.

Furthermore, the experimental data available for the HAV IRES do not allow a decision between the "classical" secondary structure model proposed (103) and the predicted "core structure" (127). For the upstream bulge sequence proposed to be involved (CUUAUG) no experimental data are available, whereas part of the involved downstream sequence was actually shown to be sensitive to double-strand specific probes (underlined in CAUUUAGG) (103). However, this downstream sequence is supposed to be involved in double-stranded interactions in both different secondary structure predictions. Information interfering with the "core structure" model comes from testing a sequence (CUGGA) some 15 nucleotides upstream of the first pseudoknot "partner" sequence. This 15 nt sequence should be located in a double-stranded stem according to the "core structure" model. However, this sequence was originally predicted to reside in a loop and is clearly sensitive to single-strand specific probing (103), leaving some doubts about the relevance of the proposed core structure.

In contrast, clear support for the presence of the predicted pseudoknot emerges from experiments with HCV and the pestiviruses. In the HCV IRES, mutations in both sequences participating in formation of the predicted pseudoknot structure reduced the efficiency of translation. When both mutations were combined, thereby restoring the predicted pseudoknot, translation efficiency was recovered to 30%

of the original level in vitro and to 10% in vivo. In addition, chemical and enzymatic probing revealed that these sequences are mainly single-stranded (120). A confirmation for the existence of the pseudoknot in the type IV IRES group comes from a similar experiment with the CSFV IRES. The translation efficiency of single site mutants interfering with the predicted pseudoknot structure reduced the efficiency of translation to background levels, while a double mutant restoring the predicted pseudoknot interaction attained nearly 100% of the original level (112).

Thus, convincing experimental evidence supporting the proposed secondary/tertiary structure model was provided until now only for HCV and a pestivirus. Nevertheless, it represents a model for a possible tertiary structure that is probably common to all IRES elements, and by that points to a common mechanism for ribosome binding used by all these IRESs.

INTERACTION OF STANDARD INITIATION FACTORS WITH VIRAL IRES ELEMENTS

During the process of translation initiation on normal eukaryotic cellular mRNAs, a 48S complex is formed including the mRNA and the 43S preinitiation complex, which includes the small ribosomal 40S subunit loaded with the ternary complex of eIF2·GTP·tRNAi and the initiation factors eIF1A and eIF3 (4). The 46 kDa RNA-helicase eIF4A and its stimulating cofactor eIF4B, which has a apparent molecular weight of 80 kDa, are assumed to melt secondary structures in the 5´-untranslated region of the mRNA (129) to allow processive scanning of the ribosomal preinitiation complex from the RNA 5´-end to the AUG startcodon. eIF1 and eIF1A act synergistically in enabling the scanning of the preinitiation complex to locate the initiation codon (7). When the preinitiation complex arrests on the initiator AUG, initiation factor eIF5 mediates the dissociation of initiation factors, and the large ribosomal subunit joins the 48S initiation complex (4), most likely supported by another factor (130).

At picornaviral IRES elements, almost the complete set of initiation factors was found to be required for internal initiation, except the cap-binding protein eIF4E (131-135). Both the RNA-helicase eIF4A (133) and its cofactor eIF4B were found to stimulate translation initiation from the EMCV IRES (136,137).

Substantial progress in understanding of the actual requirements of factors used for the assembly of ribosomal initiation complexes on picornaviral and HCV/pestivirus IRES RNAs came from recent in vitro studies. The requirements for formation of ribosomal initiation complexes with picornaviral IRES elements (demonstrated with the type II IRES of EMCV) are clearly distinct from those observed for HCV and the pestiviruses. For the binding of ribosomal 40S subunits to the EMCV IRES, the presence of eIF2, eIF3 and ATP is absolutely essential, while the additional presence of eIF4A, eIF4B and eIF4F serves to largely improve this basic binding (138). eIF3 is a large factor with eleven subunits of 170, 116, 110, 66, 48, 47, 44, 40, 36, 35 and 28 kDa that can provide many protein-protein and protein-RNA interactions with other components of the translational apparatus (139-145). It is even capable of binding to mRNA and ribosomal 18S rRNA (146). The factors eIF1 and eIF1A are not essential, but slightly support AUG selection in EMCV (7).

A key role in this process is attributed to eIF4G, the large subunit of eIF4F. It appears to take on the role of a "multipurpose adapter" that connects the mRNA with the ribosome (see Fig. 2) (147). eIF4G (p220, eIF4g) is a large protein of 154 kDa (1396 amino acids) (148), which is primarily cleaved by the FMDV leader (L) protease (at amino acid 479) or the rhinovirus 2A protease (at amino acid 486) into two fragments representing functional domains (149-154). These two domains of eIF4G are capable of making several contacts to other initiation factors. The N-terminal fragment interacts with eIF4E (155,156). In fact, the cleavage site for the FMDV L protease within the intact eIF4G appears to become exposed upon binding of eIF4E to eIF4G (157). The second, C-terminal fragment ("4Gc" in Fig. 2) interacts with two other factors, on one hand the ribosome-bound eIF3, and on the other hand the RNA-helicase eIF4A. This interaction of eIF4A with the C-terminal domain is mediated by two separate binding sites in eIF4Gc (158).

While the intact eIF4G molecule with the eIF4E protein bound to its N-terminal domain is necessary for cap-dependent translation, the C-terminal domain of eIF4G is

sufficient to confer internal translation initiation of several picornaviruses in the absence of eIF4E (134,159). The different types of IRES elements are differentially dependent of the cleavage of eIF4G. Entero-/rhinoviruses are highly dependent on action of a picornaviral proteinase, while type II IRESs are less dependent on such a cleavage of eIF4G (160,161). The only general exception of this rule appears to be the IRES of HAV, which requires intact eIF4G for translation and is therefore inhibited by the activity of picornaviral proteases (160,162,163).

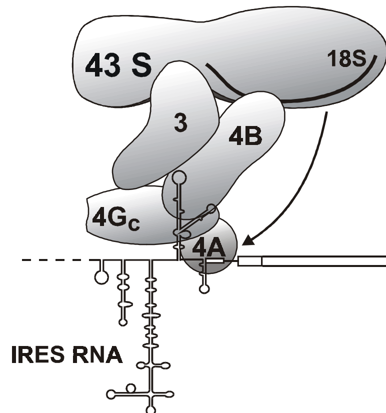
Moreover, eIF4G appears to be able to contact RNA directly. It has been found to bind to the Y-shaped stem-loop J-K-L of the type II IRES (EMCV) (138,164), thereby providing another bridge between the IRES element and the translational apparatus. Nevertheless, the order of binding events that involve eIF4G and lead to association of the IRES RNA with the ribosomal 40S subunit is not yet clear. eIF4G has been found to be present not only in ribosomal 48S complexes with normal mRNAs, but also in 43S complexes (165). This indicates that the association of eIF4G with the small ribosomal subunit is independent of the presence of (m)RNA. However, eIF4G was not found in complete 80S ribosomes, suggesting that it dissociates from the RNA upon joining of the 60S subunit (165). This supports a model in which eIF4G confers a functional bridge between the IRES and the ribosome, probably by interacting first with the ribosome-bound eIF3 and after that with the IRES RNA.

Also eIF4B may contribute essentially to the guiding of the small ribosomal subunit to the 3´-border of the IRES. eIF4B binds directly to several subdomains in the FMDV stem-loop 4 (166), and deletion of these subdomains affects binding of eIF4B and translation efficiency in parallel (167). Such an interaction of eIF4B with the IRES may introduce a second multivalent adapter, thereby providing two additional links between IRES and ribosome. One link provided by eIF4B may be the "bridge" IRES-eIF4B-eIF3-40S, facilitated by protein/protein interactions between IRES-bound eIF4B and the 170 kDa subunit of the ribosome-bound eIF3 (168).

A second link may be an IRES-eIF4B-18S rRNA interaction. eIF4B has two separate RNA-binding domains (169) and can therefore be suspected to connect two RNA molecules. This may be even enhanced by eIF4B dimer formation (168). Moreover, some observations point to a possible enzymatic function of eIF4B in positioning the starting window of the IRES to the appropriate site on the ribosome. One observation is that binding of eIF4B to the FMDV IRES is strictly ATP-dependent (166), suggesting that the ATP-dependent RNA-helicase eIF4A is involved as an interacting cofactor. Indeed, a direct binding of both eIF4A and eIF4B to the IRES has been demonstrated for EMCV (164). A second observation is that eIF4B has an RNA annealing activity (170). Such an eIF4A/eIF4B complex may help to hybridize sequences in the IRES oligopyrimidine tract to complementary sequences in the ribosomal 18S rRNA (53,65) by alternating annealing and melting events and by that adjust the IRES initiator AUG to the correct position on the small ribosomal subunit. Moreover, the energy-dependent binding of eIF4B and other initiation factors to the FMDV IRES is a prerequisite for binding of the small ribosomal subunit, and eIF4B is associated with the IRES not only in ribosomal 48S complexes, but also in complete 80S ribosomes (170a). Thus, in contrast to the weak association of eIF4B with normal mRNAs and the dissociation of all initiation factors upon entry of the large ribosomal subunit, eIF4B remains bound to the IRES even after association of the 60S subunit.

The HCV and CSFV IRES elements are functionally different from the picornavirus IRESs regarding the obviously not needed assistance of the "adapter" eIF4G. These IRESs fit to the ribosome by themselves and do not need a connecting factor. Small ribosomal 40S subunits from rabbit reticulocyte lysate (but not from wheat germ extract) bind to the HCV and CSFV IRES elements in the absence of any standard initiation factor, even of eIF2 and eIF3 (130). The binding site for the 40S subunits appears to be located at or close to the pseudoknot structure of these IRESs (130). The binding of the "naked" HCV/pestivirus IRES RNA to the ribosome may be facilitated by a possible base-pairing between a sequence in the 3´-region of the IRES and a complementary sequence in the ribosomal 18S RNA (126).

Nevertheless, the following step in assembly of translation-competent ribosomes with the CSFV IRES, the subsequent joining of the 60S subunit to form complete 80S ribosomes,



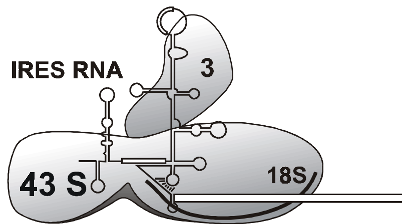


Fig. 2. A network of interactions guides the small ribosomal subunit to the IRES elements.

is absolutely dependent on the presence of eIF2 and eIF3. The finding that eIF3 is not required for formation of ribosomal 48S complexes, but for formation of 80S initiation complexes with the CSFV IRES (130), is supported by a direct interaction of eIF3 with the HCV and CSFV RNA. eIF3 binds to two parts of the domain III of HCV and CSFV, the stem-loops IIIb and IIIc (109,171). However, even for the joining of the 60S subunit, eIF4A, eIF4B and eIF4F are not required in the case of CSFV, while an additional, yet uncharacterized factor appears to be essential for subunit joining (130). Not unexpectedly, also eIF1 and eIF1A, which play an essential role in the process of initiation codon location during the scanning of ribosomes on normal mRNAs, are not required (7).

Taken together, the binding of viral IRES elements to the small ribosomal subunit appears to be facilitated by a network of interactions (Fig. 2). Picornavirus IRESs (so far demonstrated only for the type II IRES) use the IRES-eIF4G-eIF3-40S connection, probably supported by an IRES-eIF4B-eIF3-40S bridge and perhaps even a third link via IRES-eIF4B-18S rRNA. HCV and pestivirus IRESs are able to substitute these links by directly binding to the 40S subunit, although an IRES-eIF3-40S link may stabilize this interaction.

NON-CANONICAL CELLULAR PROTEINS

A simple way of looking at the so-called "unconventional" factors that support translation initiation at the viral IRES elements would be to assume that they are used to confer tissue specificity for the virus in question. While this assumption may hold probably for some viruses, like HAV, it is definitely not true for all of them. Many of the non-canonical factors involved in IRES-dependent translation initiation are also expressed in several tissues which are not permissive for propagation of the virus in question. Another observation contradicts the possible view that the unconventional translation factors should generally confer tissue specificity. Hepatitis C virus shows a strict specificity for liver tissue, although it carries an IRES elements that is able to form ribosomal 48S complexes in the absence of almost all initiation factors. Moreover, some of these cellular proteins have pleiotropic effects regarding their action on different viruses, like polypyrimidine tract-binding protein (PTB).

A feature in common to most of these "non-standard initiation factors" is that they are recruited from the large group of RNA-binding proteins involved in mRNA metabolism, mRNA transport and splicing, like PTB and hnRNP L. The only requirement for proteins that become recruited by an IRES element appears to be their ability to bind RNA. Moreover, many of the proteins have not only multiple RNA binding domains and are able to dimerize, but they bind indeed to multiple determinants on IRES

elements, suggesting that these RNA binding proteins serve as multi-contact adapters that stabilize a certain tertiary structure of the complex RNA.

The different groups of IRES elements differently depend on non-canonical cellular factors. The entero-/rhinovirus group IRES elements are the most demanding, followed by HAV and the cardio-/aphthovirus group, while the HCV/ pestivirus group appears to be the most simple at the first glance. However, at a closer look, also in this type IV IRES group some regulatory interactions seem to act on top of the basic ability to initiate translation.

FACTORS INTERACTING WITH THE TYPE I IRES ELEMENTS

Up to now, four cellular factors have been characterized that appear to be involved in the regulation of poliovirus translation: La, PCBP2, PTB and PTF. Probably due to these obviously complex factor requirements in addition to the standard eukaryotic initiation factors, no in vitro-system has been presented so far in which the formation of initiation complexes with the poliovirus IRES was demonstrated. None of these factors has been correlated with poliovirus neurovirulence. In contrast, three proteins were reported to be involved in stimulation of rhinovirus translation.

A) POLIOVIRUS

LA PROTEIN

The first cellular protein that is recruited by the poliovirus IRES, the autoantigen La, is a 52 kDa RNA-binding protein that appears to be involved in different aspects of regulation of the nucleic acid metabolism and translation. On one hand, it facilitates the release of cellular RNA-polymerase III transcripts and enhances transcription reinitiation (172). On the other hand, it regulates translation of the mRNAs coding for ribosomal proteins by binding to their 5´-terminal oligopyrimidine tracts (173). La protein has an RNA recognition motif (RRM) domain (174) and a C-terminal dimerization domain (175), and exhibits both a general RNA binding activity (176) and an RNA unwinding activity (177). It is present abundantly in extracts from HeLa cells, but not in rabbit reticulocyte lysate (RRL) or wheat germ extract, a fact that contributes to the poor efficiency of translation of poliovirus RNA in reticulocyte extracts.

La binds specifically to the stem-loop VI of the poliovirus IRES (178,179) that contains the silent AUG required for internal ribosome entry. It facilitates increased translation initiation at the authentic poliovirus polyprotein start site while decreasing "aberrant" translation initiation at downstream poliovirus AUG codons located within the polyprotein ORF. In this context, La was termed a "translation initiation correction" factor (180). Dimerization of La (175) is not necessary for the mere binding to the poliovirus stem-loop VI, but is indeed necessary for its activity enhancing and correcting poliovirus translation initiation (181). It may be possible that La protein is directly involved in recognition of the conserved but silent AUG which is part of the actual site of ribosome entry, since La protein was reported to bind to oligonucleotides that contain AUG codons and inhibit translation (182).

However, the actual mode of action of La on poliovirus translation is not yet clear, and some doubts are left about the physiological significance of La action on the poliovirus IRES. The concentrations of La reported for the stimulatory action on the poliovirus IRES are extremely high, in the range of 1.5 µM. This is in clear contrast to the much lower dissociation constant of 5 nM reported for La-IRES binding (181), and thus raises the question if the actual mechanism of action of La on poliovirus translation is really related to its binding to the stem-loop VI. In contrast, La could perhaps account for this effect on poliovirus translation by stoichiometrically covering the RNA and by that preventing aberrant initiation at downstream AUGs as reported for other reporter mRNAs (176).

PCBP

The second non-canonical factor which has been demonstrated to be actively involved in poliovirus translation is a protein of about 38 or 39 kDa that binds to the stem-loop IV of the poliovirus IRES (179). This protein has been identified as poly(rC)-binding protein (PCBP) (183,184). PCBP is a protein with three "KH" RNA-binding domains (for hnRNP K Homology) (185,186) and exists in two isoforms, PCBP1 and PCBP2. It was found

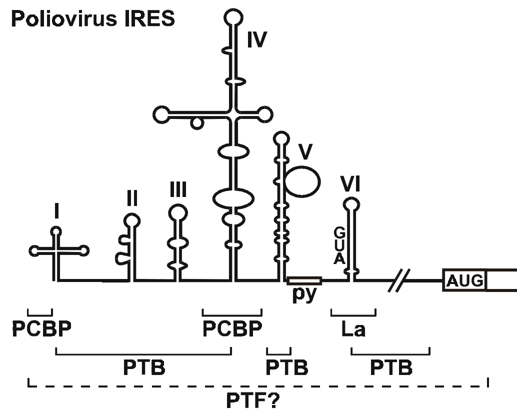


Fig. 3. Multiple determinants for the binding of cellular proteins to the poliovirus IRES.

in most mammalian tissues, including tissue culture cells of neuronal origin (183), although it has not been correlated with poliovirus expression in neuronal tissues. Both isoforms of PCBP form a heteroprotein complex which binds to the stem-loop IV of the poliovirus IRES (187). However, although antisera specific for each of the two PCBP isoforms inhibited poliovirus translation (187), only PCBP2, but not PCBP1 was shown to enhance poliovirus translation in a PCBP-depleted extract (187,188). In this function, PCBP1 cannot substitute for PCBP2 (184).

Moreover, PCBP1 and PCBP2 appear to be involved in the regulation of replication. Since they also bind to the 5´-terminal cloverleaf structure of the poliovirus RNA (187), they are suspected to play a role in the switch from translation to replication of the poliovirus RNA (see below). The positive effect of PCBP2 on IRES activity is then superseded by the activity of a complex of PCBP and viral 3CD protein forming at the cloverleaf (187,189).

PTB

The third cellular protein involved in poliovirus translation is the polypyrimidine tract-binding protein, PTB. It is one of the most intensively studied non-canonical "translation" factors; however, most studies have been performed with type II IRES elements (see below). PTB is a protein of about 57 kDa that exists in three different splice variants that appear often as a doublet or triplet of bands (190-192). In the cell, PTB recognizes pyrimidine tracts in the introns of various mRNAs and acts (probably tissue-specific) as a negative regulator of differential splicing and as a positive regulator of polyadenylation {reviewed in (193)}. For example, PTB functions as a coordinate repressor of three neuron-specific splicing events that are subject to developmental splicing changes in the rat cerebellum (194). PTB has four RRM or RNP domains (174,191,192). It is highly conserved between human, mouse, rat and swine, particularly within its RRM domain II, with levels of 91% to 97% overall amino acid sequence identity (195). RRM domain I interacts with hnRNP L, but it can also bind to RNA (196,197). RRM domain II does not bind to RNA but is responsible for the dimerization or oligomerization of PTB in solution (196,198). This domain II appears to be extremely important for the function of PTB, since not a single amino acid exchange in domain II was found between PTB sequences obtained from four different species, while various exchanges were found in domains I, III and IV (195). RRM III and IV are obviously those domains that contribute mainly to RNA-binding (196,198).

In the poliovirus IRES, three regions were found to act synergistically in binding PTB (199). The first determinant appears to be localized between pos. 70 and 286 (including stem-loops II, III and part of IV), and the second between pos. 443 and 539 (including most of stem-loop V). A third region between poliovirus pos. 630 and 750 (i.e., downstream of stem-loop VI) contributes slightly to PTB binding. Competition experiments using the major PTB-binding site of EMCV suggested a contribution of PTB to poliovirus translation (63), and immune-depletion of PTB from HeLa extracts using anti-PTB antiserum inhibited poliovirus translation, whereas preimmune-serum did not cause this inhibition. However, reconstitution of the translational activity by adding recombinant PTB failed (200), suggesting that other factors may have been co-depleted together with PTB in this assay. Although these studies may have caused widespread assumptions about the involvement of PTB in poliovirus translation, only very recently a functional depletion and add-back assay was used to definitely prove a modest stimulation of poliovirus

translation by PTB (201). Moreover, reduced binding of PTB to a poliovirus IRES carrying a mutation at pos. 472 in the stem-loop V was observed in extracts from neuroblastoma cells, but not from HeLa cells, and has been correlated with attenuation (202). However, only a second mutation at pos. 482 caused a more severe attenuation and extended the failure of PTB to bind this IRES also to HeLa extracts as a source for PTB, indicating that PTB may not be the only factor involved in this attenuation effect.

PTF

The fourth protein or protein complex possibly involved in poliovirus translation was called poliovirus translation factor (PTF). It was reported to be a large protein or protein complex with a molecular weight of some 300 kDa from HeLa cells that is involved in stimulation of poliovirus translation when tested in Xenopus oocytes (203). The activity of this factor was discovered by an elegant experiment in which the preinjection of HeLa mRNA into oocytes was shown to be an absolute prerequisite for poliovirus translation in these oocytes. PTF is either one protein or a extremely stable complex of proteins, since the PTF activity migrates on gel-filtration columns at the same position of about 300 kDa in the presence of 50 mM KCl, 1 M KCl or even 6 M urea. Unfortunately, no follow-up study investigating this potentially interesting factor, its binding sites in the poliovirus IRES and its possible interactions with other factors has been published to date.

B) RHINOVIRUS

In parallel with the identification of PTB interacting with the EMCV IRES (see below), PTB was identified to bind to the IRES of HRV-2 (204). In that study, already a functional assay for measuring a stimulatory activity was used to identify components that are involved in rhinovirus translation. These experiments have been extended by recent work (201). Half-maximal stimulation of translation in these assays (in the absence of another test system for the activity of these proteins, this value may be regarded as a functional equivalent of the KM value of enzymes) was observed to occur at PTB-concentrations of about 20 nM, and maximal stimulation was achieved using 100 nM PTB. These values are well in the range of the dissociation constants expected for typical RNA-binding proteins, which are in the range of 1-10 nM (201). However, until now, the binding site(s) for PTB in the HRV IRES have not been mapped.

The second activity that was originally characterized (204) and chromatographically separated from the stimulating activity of PTB is a complex including a 97 kDa protein (201,204). This was now identified as a complex of the "unr" protein and another protein of 38 kDa (205). unr is an RNA-binding protein with five cold-shock domains (CSDs). It stimulates the translation from the rhinovirus IRES (but not from the poliovirus IRES), and it acts synergistically together with PTB. This synergism was hypothesized to account for the decrease in activity of poliovirus or rhinovirus RNA in some translation assays (e.g., as in (181), Fig. 1B), when the RNA concentration was raised above certain values in extracts limited in the concentrations of PTB and/or unr, like rabbit reticulocyte lysate (205). According to this hypothesis, the efficiency of translation is proportional to the RNA concentration at low RNA concentrations, when both factors can bind to the same RNA molecules and act synergistically in stimulating translation. When the RNA concentration is increased further, these factors become limiting and become distributed separately to different RNA molecules. By that, the absolute amount of RNA molecules that have both factors bound decreases, and the overall efficiency of translation drops compared to that at lower RNA concentrations. By adding HeLa extract, in which these factors are present in higher concentrations, these limitations can be overcome, and translation is stimulated further also at higher RNA concentrations (205).

The 38 kDa protein that interacts with unr in stimulation of rhinovirus translation is a novel member of the GH-WD repeat protein family and has no intrinsic RNA-binding activity. This p38 was named "unrip" (for unr-interacting protein), since co-immunoprecipitation with antibodies against either protein showed that the two proteins interact with each other.

For rhinovirus-14, the same factors can be suspected to act on the IRES, since PTB and a 97 kDa protein had also been shown to bind the HRV-14 IRES (206). In addition,

these authors found that La protein and a 68 kDa protein bind to the HRV-14 IRES.

TYPE II IRES ELEMENTS

Shortly after characterization of the IRES of EMCV, a protein of about 57 kDa from different sources, like reticulocyte lysate, HeLa cells and Krebs cells, was found to bind to the EMCV IRES (207-209). In addition, also proteins of 52, 70 and 100 kDa were found to bind this RNA (210). The 57 kDa protein was identified as PTB (200). The major binding site for PTB in the EMCV IRES is the stem-loop H (formerly also named stem-loop «E» or «II»). This stem-loop H was shown to compete with the poliovirus IRES for p57 and by that decrease the level of poliovirus translation (63), indicating a functional role of p57 in EMCV translation. A second, separate binding site for PTB in the EMCV IRES is located in the IRES 3´-region and includes the stem-loops K, and L as well as the oligopyrimidine tract. Even a third area upstream from the 5´-border of the EMCV IRES, including the stem-loops D, E and F, binds PTB (211). The dissociation constant (KD) for the binding of PTB to the EMCV IRES is about 40 nM (211) and is by that in the range expected for RNA-binding proteins. The contacts of PTB to the EMCV IRES have been mapped also by nuclease and chemical protection experiments, and were found to be located in the apical loop of stem-loop H, at its basis and surrounding it, and also in the second binding region including the apical part of stem-loop K and the oligopyrimidine tract (95).

First evidence for the functional role of PTB in EMCV translation was provided by immune-depletion experiments (200). Using anti-PTB antibodies, the authors could inhibit EMCV translation, whereas a preimmune-serum did not show this effect. However, restoration of the original activity by adding back PTB failed, indicating that other factors may have been co-depleted together with PTB. In another approach, RNA-segments containing the PTB-binding site were used to sequester PTB, resulting in decreased efficiency of translation, and the original activity was restored after addition of exogenous PTB (212). In this study, also the effect of competition on the formation of 48S complexes with the EMCV IRES was analyzed, showing results correlating with those obtained for the translation activity. Later, this effect of PTB on 48S complex formation was also shown in an in-vitro system using purified components (138). In both studies, PTB enhanced the formation of ribosomal 48S complexes and the translation efficiency, respectively, but there was no absolute requirement for PTB.

A direct proof for the functional role of PTB in EMCV translation was provided using a depletion and add-back system. Reticulocyte lysate was physically depleted of endogenous PTB by passing the lysate over a column containing an RNA representing the major PTB binding site of EMCV. The efficiency of translation directed by the EMCV IRES assayed by a heterologous reporter cistron was poor in this PTB-depleted lysate, but addition of recombinant PTB resulted in full restoration of the IRES activity (213). However, these results were obtained with a mutant of the EMCV IRES that is not viable. This mutant contains an UA7 sequence instead of the wildtype UA6 sequence in the A-rich bulge following the K-loop in the IRES 3´-part. In contrast, the wildtype IRES with the UA6 sequence in the A-rich bulge renders EMCV translation independent of PTB when authentic viral sequences were used as a reporter. Intermediate levels of PTB-dependency were observed when either the mutant sequence UA7 was combined with the authentic viral coding sequences, or when the wildtype sequence UA6 was combined with the heterologous reporter sequence, while none of these parameters influenced the binding of PTB to the high-affinity site in the IRES (214). These results point to the idea that PTB is not an essential factor for EMCV translation, but its binding to the IRES may help to maintain the appropriate higher-order structure of the IRES when this is distorted, e.g., by an enlarged A-rich bulge, or influenced by downstream sequences (214). In contrast, the translation directed by the IRES of the related TMEV is not stimulated by PTB (213).

Like the EMCV IRES, also the FMDV IRES contains two separate binding regions for PTB, the stem-loop 2 in the 5´-part, and a second PTB-binding site including the stem-loop 4 in the 3´-part and the oligopyrimidine tract (215,216) (compare Fig. 1). The large stem-loop 3, which is located between these two binding sites and separates them, can

be entirely removed without any effect on the binding of PTB (166). Also in FMDV, the second hairpin in the Y-shaped stem-loop 4 contributes to the 3´-binding site for PTB (166,167), as well as the oligopyrimidine tract (216). The translation efficiency of mutants carrying changes in the oligopyrimidine tract correlated with the efficiency of binding of PTB to this 3´-binding site (216), suggesting that PTB may play an important role in FMDV translation.

Using an depletion and add-back system, the functional role of PTB was demonstrated also for FMDV (195,217). When the endogenous PTB contained in reticulocyte lysate was removed using poly(U)-sepharose, the amount of endogenous PTB was decreased from about 300 nM to less than 18 nM. The capacity of this depleted lysate to direct FMDV IRES-dependent translation dropped to about 30 % of the untreated lysate. After depletion, FMDV IRES-directed translation was dependent on PTB, since the translation was restored essentially to the original level by adding very small amounts of recombinant PTB. A concentration of about 20 nM PTB was required for half-maximal translation efficiency, comparable to the KM-value of 40 nM for in vitro-binding of PTB to the EMCV IRES (211) and to the KM-values reported for standard translation initiation factors like eIF-3 and eIF-4F, which are in the range of 10 nM (218). However, this KM is considerably lower than the concentrations reported for the stimulatory action of p52 La on the poliovirus IRES. It is not known if the residual level of expression of ~30 % in the lysate treated with poly(U)-Sepharose was caused solely by the residual endogenous PTB (about 9 nM in the translation reaction), or if additional factors contributed to this basic translation level. However, experiments in which the formation of ribosomal 48S complexes with the EMCV IRES was investigated suggest that PTB does indeed not account for the entire translational activity but only for enhancing it two- or three-fold (138).

Mutations in the major PTB-binding site of the FMDV IRES interfere simultaneously with the formation of initiation complexes, translation efficiency and PTB-cross-linking, suggesting a functional role of PTB in enhancing the formation of ribosomal complexes with the IRES (217). Surprisingly, PTB was identified to directly contact the FMDV IRES not only in ribosomal 48S complexes, but also in complete 80S ribosomes (217). Thus, PTB remains bound to the FMDV IRES even after association of the large ribosomal subunit. The binding of PTB to the IRES is temperature-independent and occurs prior to the binding of the ribosomes to the IRES.

The actual function of PTB in translation initiation is not yet clear. On one hand, by bridging its separate 5´- and 3´-binding sites in the IRES, PTB may simply stabilize a particular tertiary IRES structure required for the binding of other initiation factors. This "RNA chaperone" function of PTB may be helpful for some IRES elements like that of FMDV, while it may not be necessary for others, like that of TMEV (213), because these are able to assume the required tertiary structure by themselves. On the other hand, the PTB protein itself could also provide surface determinants for the binding of other factors.

TYPE III IRES ELEMENTS

The first evidence that HAV may require cell-type specific proteins for its translation came from experiments in which mouse liver extracts were shown to support HAV translation in reticulocyte lysate (219). Independently of these findings, proteins of 30, 39, 57 and 110 kDa were described to interact with pyrimidine-rich sequences in the HAV IRES (220). Of these, p30 and p39 were present only in cells permissive for HAV, e.g., FRhK-4 cells. One of these proteins, p39, was identified as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (221). GAPDH is a liver-specific enzyme and has been shown to have numerous non-glycolytic activities, e.g., in DNA repair by excision of uracil from DNA. It is a protein with RNA binding activity and binds to tRNA and AU-rich RNA (222,223). GAPDH interacts specifically with three regions in the HAV IRES, first the sequence between nucleotides 1 and 148 including the stem-loops I, IIa, IIb and the first oligopyrimidine tract (compare Fig. 1), second the sequence between nucleotides 155 and 235 representing stem-loop IIIa, and third nucleotides 596 to 746 including the stem-loop V and the initiator AUG. Binding of GAPDH to the stem-loop IIIa was competed efficiently by PTB. However, no functional role for GAPDH in HAV translation has been shown so far by the use of a depletion and add-back system.

Another protein of 39 kDa, PCBP2, is also present in FRhK-4 cells that are permissive for HAV. It binds to nucleotides 1-157 of the HAV 5´-nontranslated region, which includes a pyrimidine-rich sequence. The efficiency of translation directed by the HAV IRES was low in a HeLa cell lysate depleted of PCBP2 by passage over a column with the poliovirus stem-loop IV, and back-addition of PCBP2 to this depleted extract restored HAV translation efficiency (224). However, the deletion of the sequence to which PCBP2 binds eliminated the dependency on PCBP2 and lead to higher levels of expression. This indicates that also in HAV a regulatory mechanism could work similar to that involved in the regulation of poliovirus translation and replication (see below).

TYPE IV IRES ELEMENTS

As well as the type II IRES elements, also the type IV IRESs of HCV and the pestiviruses are basically able to attract the small ribosomal subunit without the help of non-standard initiation factors. In contrast to the type II IRESs, HCV and pestivirus IRES elements can bind to 40S subunits even in the absence of all standard initiation factors (see above), while subsequent binding of 60 subunits requires eIF2, eIF3 and other yet uncharacterized factors (130).

Nevertheless, some interactions of non-standard proteins have been found, mainly with the HCV IRES. The fact that PTB acts on the IRES elements of many picornaviruses suggested that perhaps PTB may also support initiation from the HCV and pestivirus IRESs. Indeed, PTB was shown to bind to the HCV IRES (225). However, these authors could not unambiguously demonstrate that the mere binding of PTB has functional consequences for the efficiency of HCV translation. Immune-depletion of PTB from reticulocyte lysate resulted in reduced HCV IRES activity. However, the attempt to reconstitute the original activity by adding PTB failed, suggesting that other factors required for translation had been affected by the depletion procedure. In contrast, another report demonstrated that the HCV IRES is translationally active in a reticulocyte lysate from which PTB has been physically removed (213). Similarly, PTB can also bind weakly to the IRES of the pestivirus BVDV, but the translation of BVDV is not stimulated by PTB added to a translation system devoid of endogenous PTB (226). Thus, the binding of PTB to the HCV and pestivirus IRES elements appears to have no functional consequences in the test systems used so far.

In addition to the weak binding of PTB to the HCV IRES, two other regions in the HCV RNA have been found to bind PTB even stronger. One is located in the 3´-part of the coding sequence of the core protein and includes an oligopyrimidine tract (227), and the other is located in the 3´-untranslated region of the HCV RNA that is involved in replication (228). The PTB-binding region in the 3´-part of the IRES core strongly inhibits translation (227), whereas the 3´-UTR appears to relieve this inhibiting effect and enhances HCV translation in cis (229). Possibly these two areas binding PTB interact with each other by means of the PTB protein. If so, this may represent a new type of regulation, possibly similar to the coordinate regulation of poliovirus translation and replication by PCBP2 (see below). Even a further protein, the 35 kDa hnRNP C which also binds to the HCV 3´-UTR (230), could be involved in this process.

The second protein that appears to be functionally involved in HCV translation is La. It binds to a region in the 3´-part of the noncoding region, between pos. 291 and 347. Consistent with the finding that La appears to recognize AUGs (182), this sequence includes the core AUG at pos. 342-344 (231). In a functional assay, very small amounts of recombinant La were able to stimulate translation directed by the HCV IRES in reticulocyte lysate by at least 20-fold. A concentration of 18 nM La was sufficient for full stimulation of HCV translation, which is well in the range expected for a specific interaction (see above for the discussion of the KD-values for PTB and La). These low concentrations of La needed for HCV translation stimulation are in sharp contrast to the high concentrations of La required for stimulation of poliovirus translation and point to a specific effect of La in the case of HCV.

Moreover, two other cellular proteins have been described to interact with the HCV IRES. One is hnRNP L, a protein of 68 kDa (232). It binds to a region of about 60 nucleotides between the authentic HCV AUG at pos. 342 and pos. 402 in the HCV core protein coding sequence, i.e., directly

downstream of the binding site for La mentioned above. This sequence represents the 3´-part of the IRES and contributes to IRES function. Binding of hnRNP L to this sequence is correlated with IRES activity, suggesting that hnRNP L is also involved in the regulation of HCV IRES activity. The close vicinity of the binding sites for La and hnRNP L points to a possible interaction between these two proteins in HCV translation, and even hnRNP L and PTB may interact since the N-terminal RRM domain of PTB was shown to bind to hnRNP L (197). The second is a yet uncharacterized protein of 25 kDa that binds to a sequence in the domain II of the HCV IRES, and mutations affecting the binding of this 25 kDa protein also affect translation (233).

In conclusion, on top of the basic ability of HCV RNA to bind to ribosomes in the absence of any additional initiation factors, a complex network of regulatory interactions appears to modulate the translational activity of the HCV IRES, perhaps also including cross-talk between the IRES and the 3´-end.

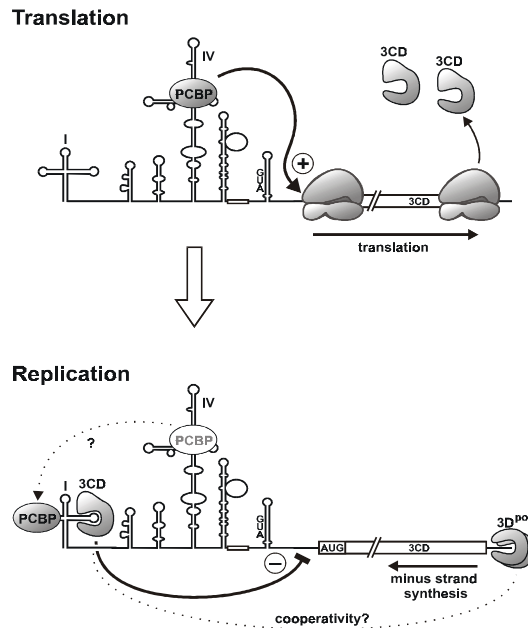


Fig. 4. The switch from translation to replication of poliovirus RNA is mediated by the interaction of PCBP and the polymerase precursor 3CD with the 5´-cloverleaf.

A SWITCH FROM TRANSLATION TO REPLICATION IN POLIOVIRUS

A coordinated regulation of translation and replication in poliovirus was suggested by experiments with PCBP and the precursor of the poliovirus 3D polymerase, the 3CD protein. 3CD binds to the D-loop of the 5´-terminal cloverleaf structure of the viral plus-strand (189,234). By this binding, it inhibits translation and so facilitates minus-strand synthesis which is initiated by the 3D polymerase at the 3´-end of the plus-strand (189,235). This binding of 3CD to the 5´-terminal cloverleaf is greatly enhanced by binding of the cellular RNA-binding protein PCBP2 to the B-loop of the cloverleaf. Thereby a ternary complex including the cloverleaf, PCBP2 and 3CD is formed (189). Moreover, the binding of the active 3D polymerase to the 3´-end of the viral plus-strand, the start-point for minus-strand synthesis, occurs in a highly cooperative manner (236).

From these data, a picture emerges that provides a possible mechanism for the switch from translation to minus-strand synthesis in the poliovirus replication cycle (Fig. 3). When the poliovirus RNA is liberated in the cell, PCBP2 binds to the stem-loop IV of the IRES and enhances translation (183,184,188), whereas simultaneous binding of PCBP2 to the B-loop of the 5´-terminal cloverleaf may have no effect at that time. When viral gene products have been translated and partially processed from the polyprotein by the viral 2A protease, 3CD binds to the cloverleaf D-loop. This binding of 3CD is cooperatively enhanced by PCBP2. When 3CD binds to the cloverleaf, translation is inhibited by a mechanism not yet known, perhaps by bringing PCBP2 to the B-loop of the cloverleaf, drawing it away from the stem-loop IV of the IRES. After the inhibition of translation, no ribosomes translate the plus-strand, and the way is free for the 3D polymerase to bind to the very 3´-end and by that start minus-strand synthesis. This binding of 3D to the 3´-end is perhaps cooperatively enhanced by the 3CD bound to the cloverleaf.

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