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REVIEW

Translation initiation of viral mRNAs

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SUMMARY

Viruses depend on cells for their replication but have evolved mechanisms to achieve this in an efficient and, in some instances, a cell-type-specific manner. The expression of viral proteins is frequently subject to translational control. The dominant target of such control is the initiation step of protein synthesis. Indeed, during the early stages of infection, viral mRNAs must compete with their host counterparts for the protein synthetic machinery, especially for the limited pool of eukaryotic translation initiation factors (eIFs) that mediate the recruitment of ribosomes to both viral and cellular mRNAs. To circumvent this competition viruses use diverse strategies so that ribosomes can be recruited selectively to viral mRNAs. In this review we focus on the initiation of protein synthesis and outline some of the strategies used by viruses to ensure efficient translation initiation of their mRNAs. Copyright © 2010 John Wiley & Sons, Ltd.

INTRODUCTION

Translation initiation of eukaryotic mRNAs

The expression of viral proteins is frequently subject to regulation at the level of the initiation of mRNA translation, a process considered to be

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Abbreviations used:
CaMV, cauliflower mosaic virus; CrPV, cricket paralysis virus; CV, coxsackievirus; dsRNA, double stranded RNA; DENV, dengue virus; eIFs, eukaryotic initiation factors; EMCV, encephalomyocarditis virus; FMDV, foot and mouth disease virus; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; HPV, human papillomavirus; HRV, human rhinovirus; Met-tRNAi, initiator tRNA; IRES, internal ribosome entry sites; ITAFs, IRES trans-acting factors; L, leader; mTOR kinase, mammalian target of rapamycin; N, nucleocapsid; NS1, nonstructural protein 1; PABP, poly(A)-binding protein; PCBP2, poly(rC) binding protein-2; PTB, polypyrimidine tract binding protein; PV, poliovirus; RRL, rabbit reticulocyte lysate; RRM, RNA recognition motif; SARS-CoV, severe acute respiratory syndrome coronavirus; sOFR, short ORF; TL, tripartite leader; TURBS, termination upstream ribosomal binding site; Unr, Upstream of N-ras; uORF, upstream ORF; UTRs, untranslated regions.
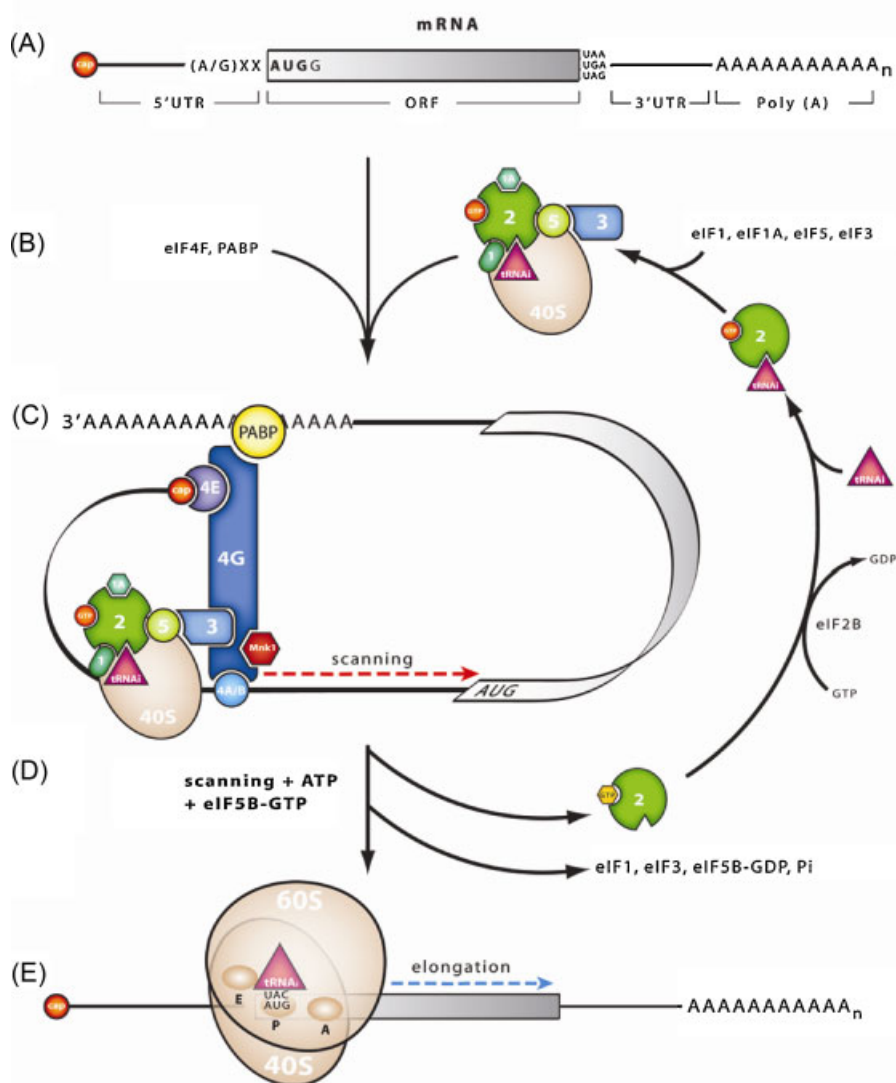
rate limiting for overall protein synthesis in eukaryotes and which has been previously reviewed [1]. Translation initiation is a stepwise process by which the 40S ribosomal subunit is recruited to the mRNA, scans in a 5′-3′direction until the first translated codon is encountered, at which point the 80S ribosome is assembled (Figure 1). A number of host proteins and *cis*-acting RNA elements participate in this process. Most eukaryotic mRNAs present a 5′terminal nuclear modification, known as the cap structure (^{7}mGpppN, where N is any nucleotide) and feature a poly(A) tail (50–300 nt in length) at their 3′ end [2,3]. The 5′ cap structure plays a key role in several important cellular processes, including RNA splicing, transport, stabilisation and translation. In translation the 5′cap serves as a 'molecular tag' that recruits the 40S ribosomal subunit in order to initiate the process of protein synthesis. Important in this recruitment process is the three subunit complex named eIF4F, composed of eIF4E, eIF4A and eIF4G (Figure 1B). EIF4E directly binds the 5′ cap structure and is obligatory for the start of cap-dependent translation initiation. EIF4A is a member of the DEA(D/H)-box RNA helicase family, a diverse group of proteins that couples

Figure 1. Schematic diagram of the canonical translation initiation pathway in eukaryotes. Relevant features of the eukaryotic mRNA are shown in (A). The 5′cap and 3′poly(A) structures, the 5′ and 3′ untranslated regions (UTRs), the initiation codon (AUG) in the optimal context, the ORF and the stop codons (UAA, UGA and UAG), are highlighted. Translation of mRNA begins after assembly of initiator tRNA, mRNA and both ribosomal subunits. The complex initiation process that leads to 80S ribosome formation depicted in (B–E) consists of several linked stages that are mediated by eukaryotic initiation factors (eIFs). See text for details. The 40S ribosomal subunit is recruited for initiation via a complex array of protein-RNA and protein-protein interactions (B). The pre-initiation complex binds to the mRNA at the 5′ terminal cap structure with help of the eIF4F protein complex. Initiation requires a protein mediated interaction between the 5′ and 3′ ends of the mRNA (C). In the closed-loop model of translation initiation the eIF4F complex interacts with both the 5′end of the mRNA (via eIF4E) and the poly(A) tail (via PABP) and recruits the 40S ribosomal subunit via its interaction with eIF3. Upon recruitment, the initiation complex migrates along the mRNA until it encounters the initiation codon (C), at which point the eIFs are released (D) and the 80S ribosome is reconstituted (E). Upon release the eIFs are recycled (B–D). This simplified model has been adapted from Reference [1]. This figure is available in colour online at www.interscience.wiley.com/journal/rmv

ATP hydrolysis with RNA binding and duplex separation. EIF4A participates in the initiation of translation by unwinding secondary structure in the 5′-untranslated region of mRNAs and facilitating scanning by the 40S ribosomal subunit towards the initiation codon. EIF4A alone possesses relatively

weak ATPase and helicase activities, but these are stimulated by eIF4G, as part of the eIF4F complex, by eIF4B and by eIF4H [4]. EIF4B is a dimeric protein (Mr 70 000 subunit) that has no independent catalytic activity and functions to stimulate the RNA binding, ATPase and helicase activities of

eIF4A and eIF4F, while eIF4H, a small protein (Mr 25 000), also enhances ATPase and the RNA helicase activity of eIF4A [5]. EIF4GI and eIF4GII (here generically referred to as eIF4G), serve as a scaffold for the coordinated assembly of the translation initiation complex, leading to the attachment of the template mRNA to the translation machinery [6]. EIF4G acts as a cornerstone for the multi-subunit eIF4F complex, as it possesses two binding sites for eIF4A and one binding site for eIF4E. Crucially, it thereby links the mRNA cap (via eIF4E) and the 40S ribosomal subunit (via eIF3). EIF4G also mediates the interaction of the 5′cap structure and the 3′poly(A) tail of the mRNA by binding the poly(A)-binding protein, PABP (Figure 1C). The poly(A) tail of most transcripts is coated with multiple copies of PABP, a protein of Mr 70 000 featuring four highly conserved RNA recognition motifs. PABP is a ubiquitous, essential factor with well-characterised roles in translational initiation and mRNA turnover. PABP interacts with eIF4G, causing circularisation of the mRNA by bridging its 5′ and 3′ termini (eIF4E/eIF4G/PABP) [7]. Interestingly, binding of PABP to eIF4G increases the binding of eIF4E to the cap [8]. It is in this context that the poly(A) tail interacts synergistically with the 5′cap in stimulating translation.

EIF4F is recognised as the key factor in selecting mRNA for translation, in that the binding of eIF4F to a 5′cap structure commits the translational apparatus to the translation of that mRNA [1]. The 40S ribosomal subunit is recruited to the mRNA as part of the 43S initiation complex, composed of the subunit bound to eIF2-GTP/Met-tRNAi, eIF1A, eIF1 and eIF3 (Figure 1B). EIF1A and eIF1, are required for binding to the mRNA and migration of the 43S complex in a 5′ to 3′ direction towards the initiation codon (ribosome scanning), a process that consumes energy in the form of ATP. EIF1A enhances eIF4F-mediated binding of the 43S complexes to mRNA, while eIF1 promotes formation of the 48S complex, in which the initiator codon is base paired to the anticodon of the initiator tRNA [9]. These proteins act synergistically to mediate assembly of ribosomal initiation complexes at the initiation codon and dissociate aberrant complexes from the mRNA. EIF1 also plays a role in the fidelity of initiation by acting as an inhibitor of eIF5-induced GTP hydrolysis. The 40S ribosomal subunit stops when it binds stably at the initiation codon to form the 48S initiation complex, primarily through RNA–RNA interaction of the AUG (mRNA) and the CAU anticodon of the bound Met-tRNAi (bound via eIF2). The initiation codon is usually the first AUG triplet in an optimal sequence context (G/AXXAUGG, where X is any nucleotide), downstream of the 5′cap [10]. Once positioned on the initiation codon the eIFs bound to the 40S ribosomal subunit are displaced. The first step in 60S ribosomal subunit joining is hydrolysis of eIF2-bound GTP and release of eIF2-GDP from 48S complexes. EIF5 induces hydrolysis of eIF2-bound GTP, leading to displacement of eIF2-GDP; the inactive eIF2-GDP is subsequently recycled to the activated eIF2-GTP by eIF2B, a guanine nucleotide exchange factor (Figure 1D). In the absence of eIF1, eIF5 induces rapid hydrolysis of eIF2-bound GTP in 43S complexes, while its presence in 43S complexes inhibits eIF5-induced GTP hydrolysis [11]. Interestingly, the establishment of codon-anticodon base-pairing in 48S complexes alleviates eIF1-mediated inhibition of GTP hydrolysis by eIF5. Thus, eIF1 plays the role of a negative regulator, inhibiting premature hydrolysis of eIF2-bound GTP until codon-anticodon base-pairing has taken place [11]. Hydrolysis of eIF2-bound GTP and release of eIF2-GDP leads to the release of eIF3 from 48S complexes assembled on the AUG triplet. Finally, eIF5B mediates joining of a 60S subunit, resulting in the formation of a protein synthesis-competent 80S ribosome in which initiator Met-tRNA is positioned in the ribosomal peptidyl (P) site (Figure 1E).

Leaky scanning, shunting and termination-reinitiation: non-canonical cap-dependent mechanisms of translation initiation

The scanning model depicted in Figure 1 predicts that ribosomes should initiate at the first AUG codon encountered by a scanning 40S subunit. For the vast majority of mRNAs initiation indeed usually occurs at the 5′ proximal AUG codon. However, the first encountered AUG codon can be by-passed if it is present in a non-optimal context. In this case the 40S subunit will initiate at a downstream AUG, in a process known as leaky scanning (Figure 2A). Leaky scanning is widely encountered in viral RNA, where it presumably helps economise on coding space. In HIV-1, for

(A) Leaky scanning

(B) Shunting

(C) Rotavirus NSP3- 3'UTR

(D) Internal Ribosome Entry Site
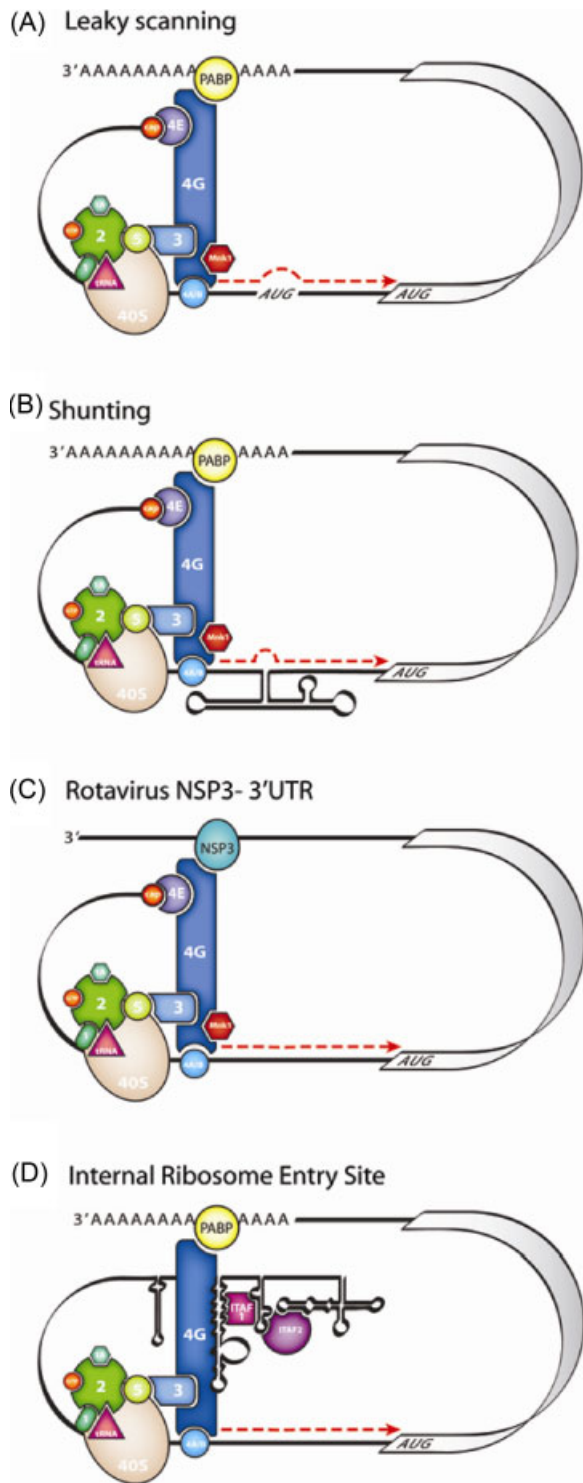
example, the envelope glycoprotein protein (ENV) is translated from different alternatively spliced mRNAs, all of which contain the upstream ORF (uORF) for Vpu, and several mRNAs also contain the Rev uORF in a different reading frame [12]. ENV is translated by leaky scanning and relies on suboptimal translation initiation codons at the upstream *rev* or *vpu* AUGs [12]. Leaky scanning is also the predominant mechanism for translation initiation of human papillomavirus (HPV) type 16 E7 oncoprotein from E6/E7 bicistronic mRNA, of the downstream ORF of the mRNA encoding rabies virus phosphoprotein [13,14], and certain bicistronic subgenomic mRNAs of the severe acute respiratory syndrome coronavirus, SARS-CoV [15–17].

The scanning model also postulates that when a scanning 40S ribosomal subunit encounters a hairpin loop in the 5'untranslated region (5'UTR), it does not skip over the loop but unwinds it. Nevertheless, there are some cases when a scanning 40S ribosomal subunit encounters the structures present in the 5'UTR and skips or shunts over a large segment, bypassing intervening segments including AUG codons and strong secondary structures that would normally block the scanning process (Figure 2B). First characterised in cauliflower mosaic virus (CaMV) 35S RNA [18], shunting has also been observed in Sendai Y mRNAs [19], in the duck hepatitis B virus bicistronic pregenomic RNA [20], papillomaviruses E1 mRNA [21], adenovirus Ad late mRNAs [22,23], and most recently in the prototype foamy virus (PFV), a nonpathogenic retrovirus [24]. In ribosome shunt-

Figure 2. Non-canonical mechanisms used by viruses to initiate viral mRNA translation. Viruses utilise unorthodox mechanisms to avoid restrictions naturally imposed by the classical scanning model used by most cellular mRNAs. (A) In the mechanism known as 'leaky scanning' the initiation complex initially recruited to the 5' cap structure may scan past a putative initiation codon if this is in a non-optimal context and translation may instead initiate at a downstream AUG. (B) The scanning complex might skip secondary structure or upstream initiation codons in a process termed 'ribosomal shunting'. (C) In viral mRNAs that lack a 3'poly(A) tail viral proteins may substitute for PABP by binding directly to conserved 3' RNA motifs in order to circularise the viral mRNA and ensure efficient translation initiation. (D) Recruitment of the initiation complex can be mediated by RNA structures known as internal ribosome entry sites (IRES) favouring a cap-independent mode of translation initiation. This figure is available in colour online at www.interscience.wiley.com/journal/rmv

ing, ribosomes start scanning upon recruitment at the cap but large portions of the 5′UTR are skipped. Thereby the secondary structure of the shunted region is preserved (Figure 2B). In cells infected with human Ad, viral mRNAs are selectively translated while the translation of cellular mRNAs is simultaneously suppressed during the late phase of infection. Late Ad mRNAs are capped but their translation is assisted by *cis*-acting RNA shunting elements in the 5′UTR known as the tripartite leader (TL). The TL is essential for viral translation as it confers on mRNAs the ability to be selectively translated during late Ad infection by ribosome shunting. The TL contains an extensive unstructured 5′ end, followed by a group of stable hairpin structures that form large single-stranded loops. Several of the hairpin structures possess complementarities to 18S ribosomal RNA [22,23]. However, it remains unclear whether these sequences function to bind 18S rRNA or whether they act structurally to promote ribosome shunting. In late Ad-infected cells, the TL directs translation solely by ribosome shunting in a manner that is stimulated by late viral gene products. The nonstructural viral Mr 100 000 protein, first of the late viral proteins, is encoded by the late region 4 (L4) mRNA and expressed in large quantities. The Mr 100 000 protein contains an RNA-binding element that interacts strongly with the TL and is essential for selectively promoting ribosome shunting on late viral mRNAs [25]. Additionally the Mr 100 000 protein binds the carboxyl terminus of eIF4G at the site normally occupied by the kinase Mnk1, which is responsible for phosphorylating the cap binding protein eIF4E [26]. The viral protein competitively displaces Mnk1 from cap initiation complexes, which prevents eIF4E phosphorylation, in turn affecting cellular cap-dependent mRNA translation [27]. The Mr 1 000 000 protein also binds mRNAs in the cytoplasm with a clear preference for late Ad TL-containing mRNAs, so forming complexes which enhance association between eIF4G and PABP and thereby increasing translation of late viral mRNAs by ribosome shunting [28]. The Mr 100 000 protein-eIF4G interaction thus provides concomitant inhibition of host-cell protein synthesis, while promoting ribosome shunting to the benefit of viral mRNA translation.

In the termination-reinitiation mechanism a second ORF located in the same mRNA can be translated without the 40S subunit becoming disengaged from the mRNA after reaching the stop codon of the first ORF. If the 5′-proximal AUG triplet in a mammalian mRNA is followed by a short ORF (sOFR), a significant fraction of ribosomes resume scanning after termination of the sORF and reinitiate at a downstream AUG. However, efficient reinitiation only occurs if the eIF4 family of factors participated in the initiation event for the sORF [29]. Termination–reinitiation in viral mRNAs was first described in the synthesis of the proton channel protein M2 of influenza B [30,31]. The process was shown to require the close proximity of termination and reinitiation codons and a defined region of mRNA upstream of the stop–start site that includes a functionally essential stretch of bases with complementarity to helix 26 of 18S rRNA. One suggestion postulates that base-pairing between the viral mRNA and the 18S rRNA could serve to tether the 40S subunit to the mRNA post-termination. Thus, the mechanism of termination–reinitiation is dependent on sequences upstream of the closely spaced termination and initiation codons [31]. Furthermore, it is the primary sequence of the RNA and not the protein encoded by the first ORF that is important in the process. Since the first description of termination–reinitiation in viral mRNAs the mechanism has also been documented in the expression of the M2-2 protein of RSV [32], and members of the calicivirus family [33]. The molecular mechanisms underlying translation termination–reinitiation are not common to all viruses. For example, in RSV no region with obvious complementarity to the 18S rRNA is present. In the caliciviruses, besides hybridisation between the viral mRNA and the 18S rRNA [34], a second essential primary sequence motif is required, termed the termination upstream ribosomal binding site (TURBS; ∼70 nt), located some 20–30 bases upstream of the stop–start overlap [35]. TURBS are thought to bind the post-termination ribosome, increasing the rate of reinitiation. It should be noted that tethering of the 40S subunit to the viral RNA is not the only function that has been proposed for the mRNA in this region. Cross-linking analysis has shown that the calicivirus mRNA is capable of binding eIF3 [36,37]. Interestingly, eIF3 plays a role in dissociating the 60S and 40S ribosomal subunits following termination, a process that is enhanced further by eIF1A and eIF1. eIF3 may therefore bind upstream of the stop–start

overlap and promote dissociation of the 60S ribosomal subunit following termination, accelerating the recycling of ribosomes and allowing reinitiation on the downstream ORF [37]. The bound eIF3 may then play a further role in recruiting initiation factors for reinitiation on the downstream ORF.

The closed-loop model of translation initiation: when the cap structure and/or the poly(A) tail are missing

As described, experimental evidence suggests that mRNA circularisation is required for efficient protein synthesis [7,38,39]. This is accomplished in the case of cellular mRNAs by RNA-protein and protein-protein interactions, generating the following complex [5'cap-eIF4E-eIF4G-PABP-3'poly(A) tail] [7]. Yet some viruses generate mRNAs that lack a 3'poly(A) tail (Figure 2C). Rotavirus, a member of the *Reoviridae*, contains 11 double-stranded RNA segments. All segments are transcribed into mRNAs that possess a 5'cap structure but lack 3'poly(A) tails. Instead, the 3' end sequences contain a tetranucleotide motif which is recognised by the virus encoded protein NSP3 [40,41]. NSP3 is composed of three functionally distinct domains. The N-terminal RNA-binding domain is responsible for binding to the viral mRNA, the C-terminal domain is required for its association with eIF4G (Figure 2C), and the central portion participates in NSP3-NSP3 interactions [42]. Thus, NSP3 binds specifically both to the conserved viral 3' end sequences and to eIF4G. And because eIF4G shows a higher affinity for NSP3 than for PABP, the interaction between PABP and eIF4G is disrupted in rotavirus-infected cells [43]. The two consequences of NSP3 expression, then, are reduced efficiency of host mRNA translation and circularisation-mediated translational enhancement of rotavirus mRNAs. The mRNA of Dengue virus (DENV), a mosquito-borne member of the family Flaviviridae, also harbours a 5'cap yet lacks a poly(A) tail and in this particular case PABP interacts with the 3'UTR of the viral mRNA to bring about its circularisation [44].

Translation initiation mediated by internal ribosome entry sites

The mRNAs of positive-strand RNA viruses in the Picornaviridae family lack a 5'cap structure. 1988 heralded the discovery that translation of uncapped picornaviral mRNA is mediated by an RNA structure which allows assembly of the translational machinery at a position close to or directly at the initiation codon, the Internal Ribosome Entry Site (IRES) [45,46]. This finding broke one of the cardinal rules of translation initiation; that is, that eukaryotic ribosomes can bind to mRNA only at the 5' end (Figure 2D). Not surprisingly IRES-mediated translation initiation has been extensively reviewed [47–55]. Functionally, the IRES was identified by inserting the poliovirus (PV) 5' UTR into the intercistronic spacer of a bicistronic construct encoding two proteins. Expression of the second cistron documented the ability of the inserted sequence to promote internal ribosome binding and translation independent of the first cistron. In general, IRES-mediated translation is independent of the nature of the extreme 5' end of the RNA as it does not require a cap structure. In the artificial bicistronic mRNA model, translation of the downstream cistron occurs even when translation of the upstream cistron is abolished, for instance by leaving the 5' end uncapped. In an alternative to bicistronic constructs, circular mRNA can be used to identify IRESes [56]. The rationale behind this approach lies in the observation that in cell free systems eukaryotic ribosomes are unable to bind small circular RNAs 25-110 nucleotides in length, and thus that eukaryotic ribosomes can only bind mRNAs via a free 5'end. In describing *in vitro* translation of circular mRNAs, Chen and Sarnow [56] showed that even the spatial constraints imposed by circularising IRES-containing mRNAs do not interfere with IRES function, confirming that IRESes allow recruitment of the 40S ribosomal subunit entirely independently from the 5' and 3' ends of the mRNA.

Since the initial characterisation of picornavirus IRESes, other RNA and DNA viruses have been shown to initiate translation internally. These include members of the *Flaviviridae* [48,57–59], the *Retroviridae* [51,60], and the *Herpesviridae* [61–63]. IRESes have also been found in insect [64–66] and in plant viruses (some members of the *Comoviridae and Luteoviridae*) [67], and been described in insect and rodent retrotransposons [68–70]. As a general rule and perhaps surprisingly, there are no significant structural or mechanistic similarities between individual IRESes (unless they are from

related viruses) [50,51,53]. Viral IRESes have been divided into four groups [47,53]. The proposed division is based on IRES secondary structure, the requirement for eIF, the location of the start codon relative to the site of recruitment of the 40S ribosomal subunit, and the ability of the IRES to operate in rabbit reticulocyte lysate (RRL) with or without supplementation [53]. The mechanism of internal initiation is not restricted to viruses; IRESes have been increasingly recognised in cellular mRNAs also [49,71,72]. In most cases IRES-mediated translation initiation is dependent on the structural integrity of the IRES [50,57,73]. Small deletions or insertions, and even substitution of single nucleotides within the IRES elements can severely reduce or enhance their activity [74,75]. The tertiary structure of IRESes is supported by both RNA-protein and long-range RNA–RNA interactions between functional domains. The latter interactions are strand specific and dependent on RNA concentration, ionic conditions and temperature *in vitro*, suggesting that IRES folding is a dynamic process. It is likely that the structural dynamism shown by IRESes plays an important role in their biological function, that is, IRESes may adopt alternative structures featuring distinct translational activities depending upon the specific environmental conditions.

In vitro reconstitution of the translation initiation complex using the encephalomyocarditis virus (EMCV) IRES showed that formation of 48S complexes is ATP-dependent and requires almost the same factors as the cap–dependent initiation mechanism, with the exception of eIF4E [76,77]. Specifically, the cap-binding protein complex eIF4F can be replaced by a complex of eIF4A and the central domain of eIF4G (lacking the eIF4E binding domain, see below). Both eIF4A and the function of the central domain of eIF4G are essential for 48S complex formation, exemplified by the profound inhibition of EMCV IRES-mediated translation by dominant negative mutant forms of eIF4A, which sequester the eIF4A/4G complex in an inactive form [78]. However, the requirement by IRESes for eIFs is not a general feature. Biochemical reconstitution of the initiation process on the HCV and cricket paralysis virus (CrPV) IRESes revealed that in these particular cases, formation of the 40S/IRES complex is eIF-independent [79–81]. In the case of HCV, the 40S ribosomal subunit binds specifically to the IRES without any requirement for initiation

factors and in such a way that the ribosomal P site is placed in the immediate proximity of the initiation codon. Subsequent addition of the eIF2-GTP/Met-tRNAi complex is necessary and sufficient for formation of 48S complexes. Although eIF3 is not required for 48S complex formation, it binds specifically to the HCV IRES and is likely to be a constituent of 40S/IRES complexes *in vivo*. Significantly, initiation on the HCV IRES does not require ATP or any factor associated with ATP hydrolysis and, as would be expected, is resistant to inhibition by dominant negative eIF4A mutants [82]. The case of the CrPV IRES is even more unanticipated as this element does not require any initiation factors or even initiator tRNA in order to assemble elongation-competent 80S ribosomes [65,83]. In fact the first encoded amino acid is not methionine at all and thus initiation does not occur at a cognate AUG codon, or even a weak cognate codon such as CUG or GUG. Instead the N-terminal residue of the CrPV capsid protein precursor is either alanine (encoded by GCU or GCA) or glutamine (encoded by CAA) [84]. Studies aimed at understanding the molecular basis of this mechanism have revealed that the CrPV IRES mimics the base-paired initiation codon and anticodon of Met-tRNA$_i$, thus, in the ribosome-IRES binding complex the P-site is occupied not by Met-tRNA$_i$ but by the viral IRES and the first triplet to be decoded is located in the A-site of the ribosome [81,85].

Studies conducted to examine the mechanism by which picornaviruses inhibit translation of capped cellular mRNAs revealed the advantage conveyed to viral replication by the IRES [86]. Infection of cells by PV, rhinoviruses and aphthoviruses, results in rapid inhibition of host cell protein synthesis. During infection eIF4G is the target of viral proteases 2A in the case of PV, coxsackievirus (CV) and human rhinovirus (HRV) or of the leader (L) protease in the case of foot and mouth disease virus (FMDV). Cleavage results in an amino-terminal fragment, which contains the eIF4E-binding site and a carboxy-terminal fragment (p100), which contains the binding sites for eIF3 and eIF4A [6,86]. Consequently, cleavage of eIF4G following viral infection results in inactivation of the eIF4F complex in terms of its ability to recognise capped mRNAs and hence in severe inhibition of cap-dependent translation initiation (Figure 3). Yet, while p100 supports cap-independent translation initiation, its interaction with the IRES requires host
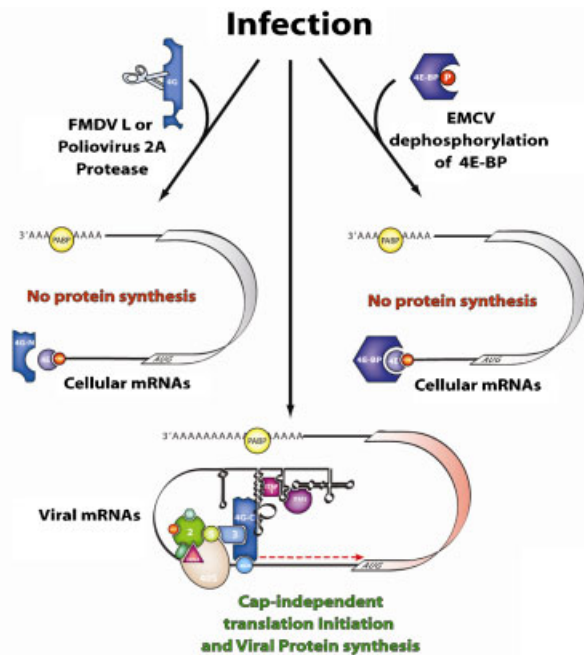
Figure 3. Hijacking the cellular translational machinery by specific inhibition of cap-dependent translation initiation. The animal picornavirus poliovirus (PV) and the foot-and-mouth disease virus (FMDV) both cause rapid cleavage of eIF4G during infection. Cleavage of eIF4G is accomplished by the PV 2A or the FMDV leader (L) proteinases. Cleavage of eIF4G inhibits cap-dependent translation initiation yet stimulates viral IRES mediated translation initiation. Some picornaviruses such as the encephalomyocarditis virus (EMCV) inhibit host cell protein synthesis by inducing dephosphorylation of the 4E-binding protein (4E-BPs). 4E-BPs strongly interact with eIF4E when in their hypophosphorylated state, displacing eIF4G. This figure is available in colour online at www.interscience.wiley.com/journal/rmv

factors [87]. Therefore, upon infection with these viruses, host protein synthesis is blocked and the viral mRNA is translated without competition from cellular mRNAs for the required host components. Inhibition of cap-dependent translation initiation through cleavage of eIF4G by virus encoded proteases is a strategy recently extended to other IRES containing viruses. In fact, the retrovirus encoded protease, a protein responsible for virus maturation, also cleaves eIF4G, with a similar impact on cap-dependent translation initiation [6,88]. However, not all *Picornaviridae* use this strategy to inhibit cap-dependent translation; the cardioviruses inhibit host cell protein synthesis by inducing dephosphorylation of the 4E-binding protein (4E-BPs) [89]. The eIF4E-eIF4G interaction is of central importance for cap-dependent initiation, and can be blocked by small regulatory proteins that bind to eIF4E, known as 4E binding

proteins (4E-BPs) [90]. EIF4G and the 4E-BPs occupy mutually exclusive binding sites on eIF4E. The interaction of 4E-BPs with eIF4E is modulated by the extent of 4E-BP phosphorylation, normally via the mammalian target of rapamycin (mTOR kinase). The 4E-BPs strongly interact with eIF4E when in their hypophosphorylated state and dissociate from eIF4E upon hyperphosphorylation (Figure 3).

Despite being independent of the presence of cap-binding complexes, translation of some IRES-containing mRNAs is stimulated by the presence of a poly(A) tail. Translation driven by the IRESes of PV, EMCV and HAV is significantly enhanced in the presence of a poly(A) tail [91,92]. However, for picornaviruses, the mechanism by which the poly(A) enhances IRES-mediated translation is far from clear as poly(A) stimulation of translation is reported to be PABP independent in the context of the viral RNA [93]. These observations are consistent with data showing that both eIF4G and PABP are cleaved during poliovirus infection, under which circumstances poly(A) enhancement of IRES activity must be PABP independent since the eIF4G-PABP interaction and eIF4G-IRES recognition domains are separated. Potentially of course interactions between the IRES and 3′RNA regions may involve as yet undetermined mechanisms in which PABP does not play a role. This suggestion is borne out by the RNAs of barley yellow dwarf virus, satellite tobacco necrosis virus and HCV which lack both a 5′cap and a poly(A) tail, yet which contain sequences in the 3′ UTR that are required for efficient IRES-dependent translation initiation [94–97]. Such observations suggest that an RNA–RNA or an RNA–protein bridge is established between sequences or factors interacting with specific elements within the 3′UTR and the IRES. There are certainly a number of proteins, unrelated to PABP, capable of interacting with viral 3′ UTRs that are also implicated in IRES interaction, providing potential links between 3′ and IRES sequences. Favouring this notion a recent report describes that the cellular protein insulin-like growth factor-II mRNA-binding protein 1 (IGF2BP1) stimulates HCV IRES-mediated initiation by bridging the viral 5′ and 3′UTRs [98]. Long range RNA–RNA interactions between 5′ and 3′ UTRs, that may be implicated in the modulation of viral mRNA translation, have also recently been described for HCV [99]. Furthermore, there is an interesting report

demonstrating that RNA–RNA interaction between the 5′ and 3′ UTRs of an uncapped and non-polyadenylated plant viral mRNA confers stimulation of translation initiation [100].

IRES trans-acting factors: proteins that regulate IRES activity

The precise molecular mechanism by which the host translation apparatus recognises IRESes is unknown, but accumulating data strongly suggest that canonical initiation factors, as well as specific cellular proteins not normally involved in cap-mediated initiation known as IRES *trans-acting* factors (ITAFs), are important in this recognition process [48,49,72,101,102]. Initial support for the notion that some IRESes might require additional factors to enable their activity came from evidence that the IRESes of the EMCV, FMDV and Theiler's murine encephalomyelitis virus (TMEV) were all active in RRL, whereas translation mediated by PV and HRV IRESes was inefficient unless RRL was supplemented with HeLa cell extracts [103–105]. Cell extracts contributed with host factors required for viral IRES activity. This phenomenon is not restricted to PV and HRV as similar evidence was reported for HIV-1 IRES activity [106,107].

The list of known ITAFs is continually growing. Among the most intensively studied, the human La autoantigen (La) and the polypyrimidine tract binding protein (PTB) are important for IRES activity of some picornaviruses [101], La is required by the HCV IRES [108], the Upstream of N-ras (Unr) protein specifically activates the IRES of HRV and PV [109,110], while the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) stimulates activity from the HRV and HIV-1 IRES [49,107]. Functional *in vitro* assays revealed that some IRES elements require not just one, but a specific combination of several ITAFs for efficient translational activity: PTB plus ITAF$_{45}$ for the IRES of FMDV [111]; PTB, PCBP2, La, Unr, SRp20 for the PV IRES [48,49]; PTB, La, hRNPA1, Unr plus the poly(rC) binding protein-2 (PCBP2) for the HRV IRES [49,112]; PTB, PCBP2, La, hnRNP D and hnRNP L for the HCV IRES [48,49]. Interestingly, cellular proteins cannot only stimulate IRES mediated translation initiation but they can also, in some instances, inhibit IRES activity [113–115].

The mechanism by which ITAFs facilitate the recruitment of ribosomal subunits remains unknown [72,102]. One hypothesis posits that ITAFs possess RNA chaperone activity and help to fold the IRES into the conformation required for translational activity [48,116,117]. This hypothesis is based mainly on the structural properties of these RNA-binding proteins [48,116,117]. All possess multiple-RNA-binding domains, such as cold shock domains in the case of Unr, RNA recognition motif (RRM) domains for La, and PTB and KH domains for PCBP2 [48]. Furthermore, most of these proteins dimerise in solution. Accordingly, these proteins may make multiple contacts with the IRES and modulate IRES conformation by a concerted interaction between several RNA binding sites.

IRES-activity when cap-dependent translation is compromised

A distinguishing hallmark of IRES-mediated translation is that it allows for enhanced or continued protein expression under conditions where normal, cap-dependent translation is shut off or compromised [72,118]. This general notion suggests that IRES-mediated translation represents a regulatory mechanism that helps the cell cope with transient stress. Viral mRNAs that posses IRES elements might thus always be expected to demonstrate efficient translation under all conditions where cellular cap-dependent translation initiation is blocked. However, this view is likely to be somewhat simplistic since there have already been reports to the contrary [119,120]. This suggests that the switch from cap- to IRES-mediated translation initiation is not mediated exclusively through the increased availability of canonical translation initiation factors due to inhibition of cap-dependent translation. Instead, IRES-mediated translation may also be sensitive to the particular mix and availability of ITAFs under any given physiological stress condition [52,72,102], a conclusion that would seem consistent with the notion of stress-induced translational control of IRES activity. Heat shock, a major such cellular stress, disrupts cap-dependent translation initiation while IRES-dependent translation of HCV and EMCV is enhanced [119]. In contrast, IRES-dependent translation of PV remains unaffected under continuous heat shock stress [119], supporting the notion that heat shock differentially affects the activities of IRES elements. The IRESes present in the

HCV [121], some members of the *Picornaviridae* [122], and HIV-1 [106] encoding mRNAs are active during the G2/M phase of the cell cycle, and yet, while cap-dependent translation is prevalent in the G1/S phase, it is inhibited in the G2/M phase [123]. Our current understanding states that this translation blockage is the result of multiple events that lead to disruption of the eIF4F complex [123]. Two such events are the dephosphorylation of eIF4E and the hypophosphorylation of 4E-BPs at mitosis, which prevent eIF4F function and assembly, respectively. In contrast to cap-dependent translation, IRES-mediated translation initiation is independent of the cap, and is therefore independent of eIF4F integrity. Nonetheless, even though it is tempting to propose that all IRESes are active during G2/M, again this does not turn out to be the case [120].

Cap-independent translation initiation: when viral proteins act as cap-analogues

It is noteworthy that the presence of an IRES is not an absolute requirement for cap-independent translation initiation on viral mRNAs. Alike Picornaviridae, the positive-strand RNA viruses in the *Caliciviridae* family lack a 5′cap structure. The *Caliciviridae* utilise a mode of initiation of protein synthesis that is independent both of a 5′cap and of the presence of an IRES [36,124]. This alternative mechanism involves the interaction of translation initiation factors with a viral protein covalently linked to the viral RNA. Calicivirus generates naturally bicistronic mRNAs that lack a 5′cap structure and feature a 3′poly(A) tail. The 5′ ends of calicivirus mRNAs are covalently linked to a viral protein of Mr 12 000–15 000, known as VPg. Translation initiation from the 5′ORF involves protein–protein interactions between VPg and eIF4E and/or eIF3 allowing the recruitment of the translation initiation complex [36,124]. While VPg thus acts as a cap-analogue, allowing translation initiation from the first ORF, translation from the second ORF is achieved by a termination/reinitiation mechanism as discussed above.

Translation initiation of viral mRNAs mediated by viral cap-binding proteins

The influenza A viruses are the causative agents of yearly epidemics of respiratory disease and occasionally more severe pandemics. The influenza

A viruses belong to the family *Orthomyxoviridae* and posses a single-stranded, negative-polarity RNA genome made up of 8 RNA segments. Virus RNA transcription initiation takes place by a cap-snatching process whereby the viral polymerase recognises the 5′cap structure of cellular pre-mRNAs in the nucleus, cleaves these some 15 nt downstream of the cap and utilises the resulting capped oligonucleotides as primers to copy the virus template RNA. Transcription terminates by reiterative copying of the virus polyadenylation signal, an oligo-U sequence located close to the 5′-end of the template. This process generates a 5′capped and 3′polyadenylated viral mRNA. Although distinct pathways synthesise cellular and viral mRNAs, both types of mRNA are structurally similar. During infection influenza virus efficiently shuts off host cell protein synthesis [125,126], accompanied by selective translation of viral mRNAs, with sequences within the 5′UTRs playing a critical role. As described above, the function of the 5′cap is mediated by cap-binding proteins that play a key role in translational control. Interestingly, viruses such as influenza have evolved cap-binding proteins capable of preferentially recognising the 5′cap structures present on viral mRNAs, in spite of the fact that these 5′ cap structures are of 'snatched' cellular origin [127,128]. The observation that viral cap-binding proteins exist in complexes comprising other viral proteins, cellular proteins and the viral mRNA 5′UTR suggests that these act as essential cofactors for the influenza virus cap-binding protein. Among viral proteins that participate in translation initiation complexes such as these, the influenza non-structural protein 1 (NS1) appears to play a key role [125,126]. NS1, which shows binding-specificity towards viral RNAs can bind to eIF4G in an RNA-independent fashion and to PABP independently of eIF4G [128–130]. Thus, NS1 binds to viral mRNA 5′UTRs and recruits key translation initiation factors to stimulate the assembly of translation initiation complexes on influenza viral mRNAs. Additionally, through its preference for viral RNAs and its affinity for eIF4G, NS1 may be partially responsible for the shut-off of host-cell protein synthesis observed during viral infection, via the sequestration of eIF4G from host-cell mRNAs.

The strategy of using cap-binding proteins of viral origin to favour translation of viral mRNA

over cellular mRNA during infection is not restricted to influenza virus [127]. In fact, members of the poxviruses, coronaviruses, flaviviruses, reoviruses, and the bunyavirus families have evolved similar cap-binding proteins capable of preferentially binding 5′cap structures when in the context of a viral mRNA to favour translation initiation from the viral mRNA during infection. Among this group of viral cap-binding proteins the hantavirus nucleocapsid (N) exhibits some unique abilities. Replication of hantavirus, a member of the *Bunyaviridae* family, is exclusively cytoplasmic where generation of viral mRNA uses a cap-stealing mechanism, akin to orthomyxovirus, yielding mRNAs with 5′m7G-cap structures derived from cellular mRNAs [131]. The hantavirus N protein is capable of substituting for the activities of eIF4F to mediate mRNA translation [132]. N binds with high affinity to the capped 5′ end of viral mRNAs, an activity that mimics that of eIF4E, and acts as substitute for eIF4G by directly recruiting the 43S pre-initiation complex to the 5′ mRNA cap, as well as replacing the helicase, eIF4A, in the cap-binding complex. N in fact augments translation of both viral and non-viral mRNA; however, viral mRNA is recognised preferentially by N, and translation of viral mRNA is more robust in competitive translation reactions with non-viral mRNA [132].

Translational control and viral pathogenesis

Factors related to both the host and the infectious agents determine pathogenesis of virus-induced disease. In this sense there is significant genetic evidence that regulation of translation initiation plays a role in virus tropism and pathogenesis [48,133]. In the case of PV, the efficiency of viral mRNA translation is a major determinant of neurovirulence and disease pathogenesis [134,135]. In humans the expression of the neurovirulent phenotype of PV results in paralytic poliomyelitis. Repeated passages of PV strains in animals and cultured cells generated the corresponding attenuated vaccine strains (Sabin types 1-3). Thus, the improved ability of these PV variants to replicate in non-nervous tissue compromised their ability to spread within the nervous system, as demonstrated by decreased neurovirulence in monkeys. The live,

attenuated Sabin vaccine strains of PV contain point mutations within the IRES resulting in compromised translation efficiency, specifically in neuronal cells. This effect is mediated by impaired binding of eIF4G, eIF4B and the ITAF PTB to the IRES leading to an impaired association of ribosomes with the viral RNA [136]. In support of these findings, the reversion of Sabin strains towards a pathogenic phenotype, a major cause of vaccine-associated paralytic poliomyelitis, is associated with compensatory mutations within the IRES sequence leading to a concomitant recovery in secondary structure and translational activity.

Another example is the HAV, sole member of the hepatovirus genus of the *Picornaviridae*. HAV is characterised by its lack of sequence relatedness with other members of the picornavirus family and by several unique biological characteristics, including slow non-cytopathic growth in cell culture and an inability to shut down host cell protein synthesis. HAV possesses an IRES in its 5′ UTR and translation is the rate-limiting step for virus replication. However, in sharp contrast to the major types of picornavirus IRESes, the activity of the HAV IRES requires intact eIF4F [137,138]. Highly replicating HAV was recovered following successive passage in cells that normally allowed poor virus replication only. In common to PV, HAV RNA was shown to have acquired mutations in its IRES element that enhance replication by facilitating cap-independent translation in a cell-type-specific fashion. Interestingly, passage of HAV in different cell types engendered different sets of mutations; however, all adaptive events were clustered within the IRES [139–141].

The activity of the HCV IRES also varies according to cell type and furthermore studies comparing the efficiencies of IRES elements from different HCV genotypes have established differential translation initiation capabilities [142,143]. A correlation was also described between *in vivo* tropism of HCV and the ability of the viral IRES to support translation initiation [144]. This translation specific tropism might be due to the availability or otherwise of ITAFs required for adequate IRES mediated translation initiation in different cell types [48,49]. Alternatively, HCV IRES tropism may be regulated by microRNA-122 (miR-122), a highly abundant liver-expressed microRNA that binds the HCV 5′UTR [145]. MicroRNAs (miRNAs)

are post-transcriptional regulators of gene expression that have emerged as important factors for the control of a multitude of processes in mammalian cells [146–148]. Increasing evidence suggests that miRNAs are also an important component of the host's antiviral response mechanism [149–151]. Remarkably, some viruses have evolved approaches to make use of miRNAs to increase the efficiency of their own replication within infected cell [149–151], notably several members of the herpesvirus family express miRNAs to regulate their own replication and modulate cellular gene expression [149–151]. In the case of HCV, binding of miR-122 to its 5′UTR results in an up-regulation of viral RNA levels and in an enhancement of IRES-mediated translation initiation [145,152,153]. In agreement with its role in HCV replication a marked suppression of viremia is observed in chronically HCV infected chimpanzees when the function of miR-122 is antagonised [154]. Taken together these observations point towards the participation of mRNA translation initiation in virus tropism and underline the role of IRESes in virus pathogenesis.

During infection many viruses regulate host protein synthesis favouring translation from their own mRNAs [55,86,133,155–157]. As discussed, host cells respond to viral infection by activating several defence mechanisms aimed at targeting viral replication at multiple stages including translation initiation [158,159]. One such host antiviral response is the activation of the RNA-dependent protein kinase (PKR) [158]. When activated, PKR phosphorylates the alpha subunit of eIF2 leading to the general inhibition of protein synthesis. eIF2 is inactive when associated with GDP (Figure 1D). To regain its capacity to interact with the Met-tRNAi, an essential step for the recruitment of the initiator tRNA to the initiation complex, GDP must be exchanged for GTP (Figure 1D-B). When the alpha subunit of eIF2 is phosphorylated the exchange of eIF2-GDP for eIF2-GTP is reduced. Therefore, the phosphorylation of the alpha subunit of eIF2 affects general translation initiation. Regulation of the canonical mechanisms of protein synthesis by the infected host cell thus represents an important antiviral response strategy. Viruses such as adenovirus, Epstein-Barr virus, HSV-1, influenza virus, HCV, among others, have however evolved escape mechanisms to overcome the cellular response mediated by PKR [125,126,156,157,160].

Therefore, the development of strategies by viruses to overcome cell-imposed translational regulation in combination with the use of alternative mechanisms of translation initiation render some viral mRNA immune to host mechanisms for controlling protein synthesis.

CONCLUDING REMARKS

Much effort has been directed at understanding the mechanisms by which viral mRNA initiate protein synthesis. A better knowledge of the mechanisms by which for example viral IRES activity is regulated may lead to the design and validation of drug candidates that specifically inhibit virus replication by targeting translation initiation from the viral mRNAs. In the case of HCV this notion has already received attention. Protein synthesis inhibitors are well known in antibacterial therapy; however, to date no antiviral agents that target viral protein synthesis are used despite the fact that several viruses of extreme medical significance (e.g. HCV and HIV) possess unique *cis*-acting RNA elements such as IRESes that are essential for viral mRNA translation. Antisense aptamers and peptide nucleic acids [161–163], both targeting specific domains of the HCV IRES, and chimeric RNA molecules composed of a hammerhead ribozyme targeting the HCV genome and an aptamer directed towards the viral IRES [164], have been reported to strongly inhibit viral protein synthesis *in vitro* and in cell culture. Morpholino phosphoramidate antisense oligonucleotides that specifically target the HCV IRES inhibited translation by more than 95% for at least 6 days in living mice [165]. Together these studies support the notion that IRES functionality in pathogenic viruses is a viable target for novel antiviral strategies. Indeed benzimidazole 5-carboxamide derivatives, initially identified as inhibitors of the RNA-dependent-RNA polymerase of HCV [166], have been shown to bind to and alter the conformation of the HCV IRES, ultimately leading to inhibition of IRES-driven translation in HCV-infected cells [167]. Knowledge of the mechanisms specific to ribosome recruitment by viral mRNAs should also provide novel indirect targets for putative drugs designed to inhibit viral protein synthesis. In this sense the targeting of ITAFs or miRNAs required for viral protein synthesis has emerged as a feasible antiviral approach [154]. To conclude, understanding the molecular mechan-

isms underlying the alternative and often unorthodox strategies employed by viruses to selectively enhance protein synthesis initiation on their own mRNAs will likely prove instrumental in the development of novel antiviral strategies that specifically target viral protein synthesis.

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