A cell cycle-dependent protein serves as a template-specific translation initiation factor

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Cap-independent translation initiation on picornavirus mRNAs is mediated by an internal ribosomal entry site (IRES) in the 5' untranslated region (5' UTR) and requires both eukaryotic initiation factors (eIFs) and IRES-specific cellular *trans*-acting factors (ITAFs). We show here that the requirements for *trans*-acting factors differ between related picornavirus IRESs and can account for cell type-specific differences in IRES function. The neurovirulence of Theiler's murine encephalomyelitis virus (TMEV; GDVII strain) was completely attenuated by substituting its IRES by that of foot-and-mouth disease virus (FMDV). Reconstitution of initiation using fully fractionated translation components indicated that 48S complex formation on both IRESs requires eIF2, eIF3, eIF4A, eIF4B, eIF4F, and the pyrimidine tract-binding protein (PTB) but that the FMDV IRES additionally requires ITAF₄₅, also known as murine proliferation-associated protein (*Mpp1*), a proliferation-dependent protein that is not expressed in murine brain cells. ITAF₄₅ did not influence assembly of 48S complexes on the TMEV IRES. Specific binding sites for ITAF₄₅, PTB, and a complex of the eIF4G and eIF4A subunits of eIF4F were mapped onto the FMDV IRES, and the cooperative function of PTB and ITAF₄₅ in promoting stable binding of eIF4G/4A to the IRES was characterized by chemical and enzymatic footprinting. Our data indicate that PTB and ITAF₄₅ act as RNA chaperones that control the functional state of a particular IRES and that their cell-specific distribution may constitute a basis for cell-specific translational control of certain mRNAs.

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The control of mRNA translation is an important component of the regulation of gene expression, and various cellular processes are regulated by enhancement or repression of translation initiation. Message-specific translational control relies on structural elements in either 5' or 3' nontranslated regions of mRNAs that may bind regulatory proteins, determine the affinity of binding of canonical translation initiation factors, or enable mRNAs to utilize variants of the canonical initiation mechanism (Standart and Jackson 1994).

Most examples of mRNA-specific translational control involve repression by RNA-binding proteins. Repression of initiation is exemplified by ferritin mRNA, one of a group of mRNAs that contain a cap-proximal 5' UTR iron-responsive element (IRE; Hentze and Kühn 1996).

Binding of the iron regulatory protein to the IRE sterically prevents recruitment by eIF4F of the 43S ribosomal complex to the cap-proximal region of the 5' UTR before scanning to the initiation codon and thus represses translation (Muckenthaler et al. 1998). RNA protein interactions can also direct patterns of expression that are spatially restricted within cells or organisms, for example, during development or differentiation, and commonly involve repression of translation of specific mRNAs by regulatory 3' UTR-binding proteins (Gray and Wickens 1998). Translational enhancement of specific mRNAs is less common. All mRNAs that are translated following conventional cap-mediated initiation require the full set of canonical eIFs, and variations in initiation factor levels have not been associated with translational enhancement of specific mRNAs that use this mechanism. Cell type-specific translational regulation is more likely to affect those mRNAs that require noncanonical protein factors for initiation in addition to the canonical set of eIFs.

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Picornavirus 5' NTRs contain a ~400-nt-long internal ribosomal entry site (IRES) that mediates cap-independent translation initiation (Jackson and Kaminski 1995). These IRESs are divided into major groups on the basis of structural properties. One group includes poliovirus and rhinovirus and the other contains encephalomyocarditis virus (EMCV), foot-and-mouth disease virus (FMDV), and Theiler's murine encephalomyelitis virus (TMEV). There is significant genetic evidence that IRESs contain determinants of cell specificity. A mutant poliovirus in which the IRES had been substituted by the rhinovirus IRES replicated as well as wild-type poliovirus in HeLa cells, but replication of the mutant (but not wild-type) viruses was completely restricted in neuronal cells (Gromeier et al. 1996). Single substitutions in the IRESs of poliovirus vaccine strains are important determinants of their attenuated neurovirulence and impaired function in translation initiation, particularly in neural cells (Svitkin et al. 1985, 1988). Mutations in the IRES also attenuate TMEV neurovirulence in mice while having only minor effects on viral growth in cell culture (Pritchard et al. 1992; Pilipenko et al. 1995, 1999). Although such noncoding determinants of IRES function have been characterized in detail, the molecular basis for their cell-specific influence is not known.

IRES-mediated initiation may require both canonical initiation factors and message-specific cellular IRES *trans*-acting factors (ITAFs) that are not involved in cap-mediated initiation. EMCV, TMEV, and FMDV IRESs are all active in rabbit reticulocyte lysate (RRL), where translation mediated by poliovirus and rhinovirus IRESs is inefficient unless the lysate is supplemented by HeLa cell extracts (Brown and Ehrenfeld 1979; Dorner et al. 1984; Borman et al. 1993). Biochemical analyses led to the identification of the ITAFs present in these extracts as the pyrimidine tract-binding protein (PTB; Borman et al. 1993; Hellen et al. 1993), the La autoantigen (Meerovitch et al. 1993; Svitkin et al. 1994), the poly(rC)-binding protein 2 (PCBP2; Blyn et al. 1996, 1997), and unr and a unr-interacting protein (Hunt et al. 1999). All of these proteins except for the unr-interacting protein are RNA-binding proteins that bind specifically to structural elements in different IRESs and are thought to maintain these RNAs in an active conformation (Kaminski and Jackson 1998; Kolupaeva et al. 1998; Gamarnik and Andino 2000). A reasonable hypothesis is therefore that tissue-specific differences in the expression of an essential ITAF could determine those cells in which the IRES is active.

We report here the identification of a novel cellular mRNA-specific ITAF. We found that the activities of TMEV and FMDV IRESs were similar in RRL and in transfected BHK-21 cells but that replacement of the IRES of the neurotropic TMEV by the IRES of the epitheliotropic FMDV yielded viable but completely attenuated viruses that fail to replicate in mouse neurons. To identify the factor that is required for FMDV IRES function, we compared the factor requirements for initiation on TMEV and FMDV IRESs by biochemical reconstitution to the stage of 48S complex formation using fully

fractionated components. Using this approach, we have previously found that initiation on the EMCV IRES up to the stage of 48S complex formation requires the canonical factors eIF2, eIF3, eIF4A, eIF4B, and eIF4F, and is enhanced less than twofold by PTB (Pestova et al. 1996a, 1996b). We also report that 48S complex formation on FMDV and TMEV IRESs requires the same set of canonical factors and that initiation on all three IRESs involves specific binding of a complex made up of the eIF4A and eIF4G components of eIF4F to the IRES. However, initiation on the TMEV IRES depended strongly on PTB, where initiation on the FMDV IRES required both PTB and a second protein, ITAF₄₅, that is not required for TMEV initiation. ITAF₄₅ bound specifically to a central domain of the FMDV IRES and acted synergistically with PTB to enhance binding of eIF4F to an adjacent domain. ITAF₄₅ is a proliferation-dependent protein that is not detectable in murine brain cells and may thus function as a tissue-specific factor that controls translation of particular mRNAs.

Results

In vitro properties of TMEV GDVII/FMDV chimeras

TMEV GDVII causes rapid fatal encephalitis in mice following intracerebral inoculation due to lytic infection of neurons (Theiler 1937). FMDV replicates in epithelial cells, causing an acute systemic infection, but does not replicate in the central nervous system (CNS; Bachrach 1968). We used FMDV and TMEV IRESs to investigate the molecular basis for cell-specific differences in IRES function. Chimeric GD68 and GD80 variants of TMEV GDVII were made by substituting its IRES by the FMDV IRES. IRES-mediated translation was assessed using constructs in which the luciferase coding region had been fused to TMEV nt 1–1124 in frame and downstream of the initiation codon AUG_{1068–70} (Fig. 1A). In the pLGF-68 and pLGF-80 chimeras, TMEV sequences from nt 628 (upstream of domain H) to either nt 1047 (the pyrimidine tract) or nt 1124 (past AUG_{1068–70}) were replaced by equivalent FMDV sequences (nt 280–693 or nt 280–740, respectively; Forss et al. 1984). These substitutions had little effect on the efficiency of translation in RRL, and both caused a small reduction in IRES-mediated translation in BHK-21 cells (Fig. 1B). TMEV containing these substitutions was viable and genetically stable in BHK-21 cells, yielding plaques that were slightly reduced in diameter relative to wt TMEV GDVII (Fig. 1C).

Attenuation of neurovirulence of TMEV GDVII/FMDV chimeric viruses

Several picornavirus IRESs have restricted activity in some cells and therefore contain determinants of the pattern and severity of host viral infection. We assayed the neurovirulence of the chimeric GD68 and GD80 viruses in mice. No clinical signs were observed 6 wk after intracerebral inoculation with up to 10⁸ TCD₅₀ of GD68

Figure 1. Structures of chimeric Theiler's murine encephalomyelitis virus (TMEV)/foot-and-mouth disease virus (FMDV) RNAs, template activities, and viral phenotypes. (A) Schematic representations of the TMEV IRES showing the positions of the oligopyrimidine tract (Yn) and the initiation codon AUG₁₀₆₈₋₇₀ and of constructs derived from the recombinant cDNA for TMEV GDVII (white) and FMDV O₁K (black) fused to a luciferase reporter gene (grey). The sizes of cDNA fragments used in these constructs are as indicated; TMEV nucleotide positions (Law and Brown 1990) are shown above pLG, and FMDV nucleotide positions are shown below pLGF-68 and pLGF-80. (B) Template activities of pLG, pLGF-68, and pLGF-80 mRNA transcripts translated in RRL and in BHK-21 cells. (C) In vitro growth characteristics and neurovirulence of wt and chimeric TMEV GDVII viruses. Standard plaque assays were done using virus stocks produced by three accumulating passages post-transfection in BHK-21 cells, and plaques were measured 3 days postinfection. Neurovirulence was assayed by intracerebral inoculation of BALB/c mice with tenfold dilutions of virus. The paralytic activity of viruses was expressed as the TCD₅₀ dose causing paralysis in 50% of animals (PD₅₀).

and up to $10^{7.5}$ TCD₅₀ of GD80 (Fig. 1C). For comparison, the PD₅₀ of wt TMEV GDVII was $10^{2.5}$ TCD₅₀. Thus, GD68 and GD80 were completely attenuated as a consequence of replacing the TMEV IRES by that of FMDV. The sequences of these IRESs are ~40% homologous (Kühn et al. 1990). Virus-specific PCR fragments were easily detected 1 day postinfection (p.i.) in all CNS samples from three mice inoculated with GD68 and from four mice inoculated with GD80. This result confirms that all mice were properly inoculated. Significantly, no virus-specific fragments were PCR-amplified

from CNS samples prepared from the same number of mice 7 and 14 day p.i. Previously, we readily detected moderately attenuated TMEV mutant genomes up to 33 day p.i. (Pilipenko et al. 1995). TMEV GDVII replicates almost exclusively in neurons (Aubert and Brahic 1995). The results reported here imply that GD68 and GD80 did not replicate in the CNS and, more specifically, may be unable to interact functionally with the translational apparatus in neurons. These results indicated that cell-specific differences in the factors required for translation initiation could be analyzed using FMDV and TMEV IRESs.

Factor requirements for initiation on the TMEV GDVII IRES

IRES-mediated initiation involves recruitment of the 40S ribosomal subunit, Met-tRNA^{Met}_i, and eIFs to an internal site on an mRNA, leading to 48S complex formation at the initiation codon. We identified the factors required for 48S complex formation on the TMEV GDVII IRES by reconstituting this process in vitro, using purified translation components. The position of 48S complexes on this mRNA was determined by toeprinting, which involves cDNA synthesis by reverse transcriptase on a template RNA to which a ribosome or protein is bound. cDNA synthesis is arrested by the bound complex, yielding toeprints at its leading edge. Eukaryotic 48S complexes inhibit primer extension at positions that can vary from 14–21 nt 3' to the A of the initiation codon depending on the mRNA on which the 48S complexes have assembled (Pestova et al. 1996a, 1998a,b). The positions of toeprints described here are shown on a structural model of an appropriate segment of the TMEV IRES (Fig. 2A).

48S complexes accumulate when GMPPNP (a nonhydrolyzable GTP analog) is included with mRNA in RRL. Primer extension done on 48S complexes assembled on wt TMEV GDVII mRNA in RRL in the presence of 1mM GMPPNP yielded prominent toeprints 17 and 18 nt and weaker toeprints 19 and 20 nt 3' to the A of AUG₁₀₆₈₋₇₀ (Fig. 3, lane 3).

We next assayed formation of 48S complexes in vitro on this mRNA, using purified translation components. Incubation of TMEV GDVII mRNA with eIF2, eIF3, eIF4A, eIF4B, and eIF4F, PTB, 40S subunits, and Met-tRNA^{Met}_i resulted in formation of 48S complexes that yielded toeprints similar to those detected in RRL (Fig. 4A, lanes 5,9). Each translation component was then individually omitted. PTB enhanced 48S complex formation fivefold (average of six assays) (Fig. 4A, lanes 8,9). 48S complex formation was absolutely dependent on 40S subunits, Met-tRNA^{Met}_i, eIF2, eIF3, and eIF4F (Fig. 4A, lanes 3,4; data not shown). eIF4A was present as a subunit of eIF4F, and these experiments therefore did not enable us to determine the requirement for eIF4A. Neither eIF1 nor eIF1A were required for 48S complex formation (data not shown). This observation is consistent with reports that initiation on the TMEV IRES does not

Figure 2. Secondary structures of segments of (A) Theiler's murine encephalomyelitis virus (TMEV) GDVII and (B) foot-and-mouth disease virus (FMDV) 0, K internal ribosomal entry sites (IRESs; Pilipenko et al. 1989). The FMDV IRES was linked differently to downstream TMEV sequences in GD68 and GD80 as shown. FMDV and TMEV initiation codons are underlined. Prominent sites of RT arrest caused by eIF4F, PTB, and 48S complexes are indicated by symbols as described in the key at bottom right.

involve scanning (Pilipenko et al. 1994) for which these two factors are absolutely required (Pestova et al. 1998a). Taken together, these results indicate that eIF2, eIF3, eIF4F, and possibly eIF4A and eIF4B constitute a minimum set of factors that are sufficient for 48S complex formation on the TMEV IRES and that this process is strongly enhanced by PTB. We have previously found that eIF2, eIF3, eIF4A, eIF4B, and eIF4F constitute the minimum set of factors required for 48S complex formation on the EMCV IRES and that this process is enhanced less than twofold by PTB (Pestova et al. 1996a).

Assembly reactions done using the TMEV IRES and all translation components also yielded prominent new toeprints at UUUCC₁₀₁₉₋₂₃ and weaker C₁₀₇₂ and G₁₀₇₅ toeprints (Fig. 4A, lanes 4,5,7-11). Their relative prominence increased on omission of 40S subunits (Fig. 4A, lanes 4,5), suggesting that they result from binding of factors to the IRES. Stable complexes were formed by the binding of eIF4F to the IRES and yielded the same pattern of toeprints at UUUCC₁₀₁₉₋₂₃ at the base of the J-K domain (Fig. 4B, lane 2). Mutations in the J-K domain that

impair binding of eIF4F also strongly impair the ability of the IRES to support internal initiation and abrogate virus viability (T.V. Pestova and E.V. Pilipenko, unpubl.). The toeprints at C₁₀₇₂ and G₁₀₇₅ were seen only in the presence of eIF4F (Fig. 4A, cf. lanes 2 and 4), and they appear to be caused by the binding of a complex of its eIF4A and eIF4G subunits to the IRES because they were not observed in the presence of either eIF4G or eIF4A alone (E.V. Pilipenko and T.V. Pestova, unpubl.). These toeprints were strongly enhanced in reactions that contained PTB (Fig. 4A, cf. lanes 7,9,11 with lanes 8 and 10). This observation suggests that PTB binding facilitates formation of a specific ribonucleoprotein (RNP) complex on the GDVII IRES. Taken together, these results and previous reports (Pestova 1996a,b; Kolupaeva et al. 1998) suggest that the initiation processes on the related EMCV and TMEV IRESs differ in their requirement for PTB but that they otherwise occur by similar mechanisms that involve direct binding of eIF4F to the IRES upstream of the initiation codon and recruitment of a 43S complex.

Figure 5. Purification of internal ribosomal entry site (IRES) *trans*-acting factors from rabbit reticulocyte lysate required for internal ribosomal entry on the foot-and-mouth disease virus (FMDV) 0₁K IRES, using toeprinting to assay 48S complex assembly. GD68 mRNA was incubated under standard reaction conditions for assembly of 48S complexes with translation components as indicated and (A) with the 70%–95% AS fraction (lane 8) or with the 50%–70% AS fraction (lane 9) and with the 100 mM KCl DEAE flow-through (lanes 4, 7), the 250-mM KCl DEAE elution fraction (lanes 5, 7), or the 400-mM DEAE elution fractions (lanes 6, 7) of the RSW 70%–95% AS fraction; (B) with the 400-mM KCl phosphocellulose elution fraction (lane 3) or with 185-mM KCl (lane 4) or 210-mM KCl (lane 5) MonoQ elution fractions derived from this phosphocellulose elution fraction; and (C) with recombinant PTB (lanes 2, 4, 6) and recombinant ITAF₄₅ (lanes 2, 5, 6). A primer was annealed to Theiler's murine encephalomyelitis virus (TMEV) nt 1195–1214 in the coding region of GD68 mRNA and was extended with AMV-RT. The full-length cDNA extension product is marked E. cDNA products labeled +17–+20 nt terminated at these positions 3' to the A of TMEV initiation codon. Other cDNA products terminated at the sites indicated on the right. Reference lanes T, A, C, and G depict the negative-strand sequence derived using the same primer and GD68 plasmid DNA. The junction between TMEV GDVII and FMDV 0₁K nucleotides and the position of the initiation codon are indicated.

on GD68 mRNA when included with 40S subunits and eIF2, eIF3, eIF4A, eIF4B, and eIF4F. However, a combination of 0%–40% and 70%–95% AS fractions together provided the additional activities necessary for efficient 48S complex formation on the FMDV IRES (data not shown).

The active subfractions of the 0%–40% AS fraction were identical to those that contain PTB (Hellen et al. 1994). Recombinant PTB replaced the 0%–40% AS fraction without loss of activity in reactions that also contained the 70%–95% AS fraction (Fig. 5A, lane 8; data not shown). PTB is therefore essential for initiation on the FMDV IRES and was included in all subsequent reactions. The active component of the 70%–95% AS fraction was initially purified by DEAE-cellulose chromatog-

raphy (Fig. 7A). The active 100 mM KCl flow-through fraction (Fig. 5A, lane 4) was applied to a phosphocellulose column, and proteins in it were separated by step-elution. The IRES *trans*-acting factor was in the 250–400-mM KCl fraction (Fig. 5B, lane 3), which was dialysed against 50 mM KCl buffer, applied to an FPLC MonoQ column and eluted with a 50–500 mM KCl gradient. Two well-resolved major peaks that eluted with 185 mM and 210 mM KCl both contained nearly homogenous 45-kD proteins (e.g., Fig. 7B). The former peak was half as large as the latter. The electrophoretic mobility and the activity of these proteins were identical (Fig. 5B, lanes 4, 5). We named this protein IRES *trans*-acting factor 45 (ITAF₄₅) on the basis of its function and apparent molecular weight. ITAF₄₅ and the minimum set of eIFs

Figure 6. Identical activities of native and recombinant ITAF₄₅ in foot-and-mouth disease virus internal-ribosomal-entry-site-mediated translation initiation. Toeprinting analysis of 48S and RNP complexes assembled on GD80 mRNA under standard conditions for assembly of 48S complexes as follows: with aminoacylated initiator tRNA and eIFs eIF2, eIF3, eIF4A, eIF4B, and eIF4F (lanes 2–6) with 40S subunits (lanes 3–6), recombinant PTB-1 (lanes 2,4,6) and (A) native ITAF₄₅ (lanes 2,5,6) or (B) recombinant His₆-ITAF₄₅ (lanes 2,5,6). A primer was annealed to Theiler's murine encephalomyelitis virus nt 1195–1214 of GD80 mRNA and was extended with AMV-RT. The full-length cDNA extension product is marked E. Other cDNA products terminated at sites indicated on the right of each panel. Reference lanes T, A, C, and G depict the negative-strand sequence derived using the same primer and GD80 plasmid DNA. The position of the initiation codon is indicated.

without PTB were not sufficient to mediate 48S complex formation on GD68 mRNA (Fig. 5C, lane 5). Initiation on the FMDV IRES therefore requires both PTB and ITAF₄₅. Separation of ITAF₄₅ into two populations suggests that it may be modified posttranslationally.

GD68 contains FMDV IRES sequences to the middle of the pyrimidine tract followed by TMEV nucleotides (Fig. 1). To confirm that initiation on the FMDV IRES requires both PTB and ITAF₄₅ in addition to canonical eIFs, 48S complexes were also assembled on GD80 mRNA, which contains FMDV IRES sequences up to and including the initiation codon AUG₇₁₄₋₆ and eight additional codons. PTB and ITAF₄₅ were both required, along with the minimum set of eIFs for 48S complex assembly on this mRNA, yielding prominent toeprints at positions 18–22 nt 3' of the A of AUG₇₁₄ (Fig. 6A, lanes 3–6). 48S complex formation was absolutely dependent on Met-tRNA^{Met}, eIF2, eIF3, eIF4F, and 40S subunits but did not require eIF1 or eIF1A (data not shown).

Inclusion of ITAF₄₅ in 48S complex assembly reactions on *wt* TMEV GDVII mRNA confirmed that ITAF₄₅ could not substitute for PTB in this process and did not alter the pattern or intensity of toeprints caused by bound 48S complexes or by bound eIF4F (cf. Fig. 4A, lanes 9–11). These results confirm that initiation on the TMEV IRES requires PTB and show that the additional requirement for ITAF₄₅ is specific for FMDV. This conclusion was supported by the observation that recombinant ITAF₄₅ did not influence initiation on either the EMCV IRES or on capped native globin mRNA (data not shown).

Molecular cloning of ITAF₄₅

The identity of ITAF₄₅ was determined by amino acid sequence analysis. The amino terminus of ITAF₄₅ was

blocked (data not shown). The sequences of two well-resolved tryptic peptides from ITAF₄₅ were LVKPGN-QNTQVTEAWNK and RRFDAMPFTLR. They correspond exactly to amino acids 156–172 and 271–281 of murine proliferation-associated protein (*Mpp1*; Nakagawa et al. 1997) and PAG24, its human homolog (Lamartine et al. 1997), as well as to sequences of p38–2G4, a possible smaller (37kD) murine *Mpp1* isoform (Radomski and Jost 1995).

To confirm that ITAF₄₅ and *Mpp1* are the same protein and that it plays a specific role in FMDV IRES-mediated initiation, cDNA for this protein was amplified by PCR, cloned, and sequenced. The single long ORF encodes a 394 amino acid protein with a calculated molecular mass of 43,669 Daltons. This value is in agreement with that estimated for ITAF₄₅ by SDS-PAGE. The ITAF₄₅ cDNA sequence differed from that of *Mpp1* at two nucleotides, both of which resulted in coding changes (A₂₇₉ and R₃₁₁ in ITAF₄₅ in place of T₂₇₉ and K₃₁₁ in *Mpp1*). As reported previously for *Mpp1* and p38–2G4, homologous proteins of a similar size occur in many eukaryotes. These range from the closely related *PAS1* protein from *Fugu ribripes* to a more distantly related DNA-binding protein from *Schizosaccharomyces pombe* (Yamada et al. 1994; Gellner and Brenner 1999). Significant sequence homologies also exist between these proteins, ITAF₄₅, and type II methionine aminopeptidases from archaea and eukaryotes (Fig. 7C,D). These similarities extend over the entire ITAF₄₅ coding sequence and include four of the five signature amino acid residues that coordinate two cobalt ions in these metallo-enzymes (Tahirov et al. 1998). This group of enzymes includes p67, a methionine aminopeptidase that regulates translation by interacting with the γ subunit of eIF2 and inhibiting phosphorylation of its α subunit (Ray et al. 1993; Arfin et al. 1995). ITAFs such as PTB, PCBP2, and unr are, respectively, RNP-motif, KH-

Figure 7. Purification and structure of ITAF₄₅. (A) Scheme for the purification of ITAF₄₅ from the 0.5 M KCl RSW from RRL. (B) Resolution by SDS-PAGE of native rabbit ITAF₄₅ (2 µg) after purification by MonoQ chromatography and elution with 210 mM KCl. The positions of molecular weight markers are indicated to the left and those of ITAF₄₅ to the right. (C) Schematic illustration of ITAF₄₅ with p67 and MAP to show regions of homology. (D) Deduced amino acid sequence of ITAF₄₅ and alignment of it with sequences of the PAS1 protein from *Fugu ribripes* (PAS1; Gellner and Brenner 1999), p42 protein from *Schizosaccharomyces pombe* (p42; Yamada et al. 1994), and with type II methionine aminopeptidases from human (p67; Arfin et al. 1995) and from the hyperthermophile *Pyrococcus furiosus* (MAP; Tahirov et al. 1998). The sequences of these proteins and ITAF₄₅ show 71%, 26%, 23%, and 35% amino acid identity and 80%, 44%, 43%, and 48% similarity, respectively. Residues in bold are present in the majority of sequences and have been introduced to facilitate visualization of alignments.

domain, and cold shock domain proteins, but ITAF₄₅ does not belong to any of these recognized families of RNA-binding proteins.

To confirm that the IRES-trans-activating activity of ITAF₄₅ is not due to a contaminant, recombinant His₆-ITAF₄₅ was purified to homogeneity and its activity assayed in assembly reactions done on GD80 mRNA. Inclusion of PTB and His₆-ITAF₄₅ or native ITAF₄₅ together with the minimum set of eIFs led to the formation

of prominent 48S complexes that yielded identical toe-print patterns (Fig. 6A,B, lane 6). Significant assembly of 48S complexes on this mRNA did not occur unless PTB and ITAF₄₅ were present together (Fig. 6A,B, lanes 3–6). Native and recombinant ITAF₄₅ therefore have identical activities.

Ribosomal complexes assembled in these reactions were also resolved from RNP complexes by sucrose density gradient centrifugation to verify that they corre-

Figure 8. Dependence of foot-and-mouth disease virus (FMDV) internal-ribosomal-entry-site (IRES)-mediated internal ribosomal entry on PTB and ITAF₄₅, assayed by sucrose-density gradient centrifugation. Assays were carried out under standard conditions using GD68 or GD80 nt 1–1334 mRNA as indicated. Assembly reactions contained eIFs eIF2, eIF3, eIF4A, eIF4B, and eIF4F, and included recombinant PTB-1 and/or His₆-ITAF₄₅, as indicated. Sedimentation was from right to left. Fractions from the upper part of the sucrose gradient were omitted for clarity.

spond to authentic 48S complexes and to confirm the factor requirements for their formation. 48S complexes were formed on GD68 mRNA in reactions that contained eIF2, eIF3, eIF4A, eIF4B, and eIF4F only if both PTB and His₆-ITAF₄₅ were present but not if either or both were omitted (Fig. 8). In parallel experiments done using GD80 mRNA, eIF2, eIF3, eIF4A, eIF4B, and eIF4F were not sufficient to mediate efficient 48S complex formation (Fig. 8). Inclusion of either ITAF₄₅ or PTB individually in reactions containing these eIFs yielded small amounts of 48S complex. Inclusion of ITAF₄₅ and PTB together in reactions had a very strong synergistic effect on 48S complex assembly (Fig. 8). These observations are exactly consistent with the results noted previously for assembly reactions on this mRNA assayed by toeprinting. Taken together, the results obtained using recombinant PTB and ITAF₄₅ confirm that these two *trans*-acting factors both are necessary and are together sufficient for assembly of 48S complexes on the FMDV IRES in the presence of eIF2, eIF3, eIF4A, eIF4B, and eIF4F.

mer GD80 nt 1–1334 RNAs was comparable and was equally resistant to challenge by tRNA/rRNA competitors (Fig. 9B). In parallel experiments, recombinant and native ITAF₄₅ also became strongly labeled after UV cross-linking to the EMCV IRES (data not shown). The interaction of ITAF₄₅ with these RNAs is therefore specific and not caused by contaminants in preparations of the native protein.

Interactions of initiation factors with the FMDV IRES assayed by toeprinting

IRES-mediated initiation involves specific interactions between the IRES and the components of the translation apparatus. We used toeprinting and footprinting to map the interactions of factors with the FMDV IRES. The presence of eIF2, eIF3, eIF4A, eIF4B, and eIF4F in assembly reactions on the FMDV IRES of GD68 mRNA with or without 40S subunits enhanced a toeprint at

UV cross-linking of ITAF₄₅

The role of ITAF₄₅ in promoting initiation on the FMDV IRES could involve specific binding to it. A UV cross-linking assay was used to investigate whether ITAF₄₅ is a specific RNA-binding protein (Fig. 9A). ITAF₄₅ was labeled significantly more strongly after UV cross-linking to [³²P]UTP-labeled nt 1–1334 GD80 mRNA (which contains the FMDV IRES) than to β-globin mRNA (Fig. 9A, lanes 1,5), both in the presence of a 50-fold molecular weight excess of unlabeled tRNA. Native ITAF₄₅ was as strongly labeled after UV cross-linking to [³²P]UTP-labeled wt TMEV nt 1–1334 RNA as to the equivalent chimeric GD80 RNA (Fig. 9A, lanes 1,3). The interaction of ITAF₄₅ with wt TMEV and chimeric GD80 RNAs was specific since it was not significantly affected by the additional inclusion in cross-linking assays of a 15-fold molecular weight excess of unlabeled tRNA, where UV cross-linking of ITAF₄₅ to β-globin RNA was slightly reduced by this competitor (Fig. 9A, cf. lanes 1,3,5 with lanes 2,4,6). Labeling of recombinant and native forms of ITAF₄₅ after UV cross-linking to [³²P]UTP-labeled wt TMEV and chi-

Figure 9. UV cross-linking of ITAF₄₅ to wt Theiler's murine encephalomyelitis virus (TMEV) GDVII, chimeric foot-and-mouth disease virus (FMDV)/TMEV GD80 and β-globin mRNAs. (A) Native and (B) recombinant ITAF₄₅ were UV cross-linked to [³²P]UTP-labeled wt TMEV GDVII nt 1–1334, chimeric FMDV/TMEV GD80 nt 1–1334 or β-globin RNA nt 1–600 RNAs in the presence of a 50× molecular weight excess of rRNA and in the presence or absence a 15× molecular weight excess of ribosomal RNA, as indicated.

U₆₆₄ and led to the appearance of prominent new toeprints at AA₆₄₈₋₉ and G₆₆₅ (e.g., Fig. 5C, lanes 1-3). Similar toeprints appeared on GD80 mRNA in the presence of these factors (Fig. 6A,B, lanes 1-3). UG₆₆₄₋₅ and AA₆₄₈₋₉ map to the base of the J₁ and K₁ helices of the J-K domain, respectively (Fig. 2B), and correspond to the binding site for the eIF4G subunit of eIF4F on EMCV and TMEV IRESs (Figs. 4B, 2A; Pestova et al. 1996a,b; Kolupaeva et al. 1998). The prominence of the AA₆₄₈₋₉ toeprints was reduced on GD68 and more strongly on GD80 mRNA on inclusion of PTB in assembly reactions, and more strongly on inclusion of ITAF₄₅ with PTB (Fig. 5C, lanes 3-6; Fig. 6A,B, lanes 3-6), implying some changes in the conformation of the IRES or in the character of its interaction with eIF4F as a result of binding PTB and ITAF₄₅. Binding of eIFs to GD68 mRNA yielded weak toeprints at C₁₀₇₂ and G₁₀₇₅ (e.g., Fig. 5C, lane 3) that were previously detected on GDVII RNA (Fig. 4A). These toeprints resulting from binding of the eIF4A/eIF4G complex to the IRES are markedly enhanced in the presence of PTB or ITAF₄₅ individually and are enhanced to an even greater extent on inclusion of PTB and ITAF₄₅ together (Fig. 5C, lanes 2,6). We do not know whether these toeprints are indicative of conformational changes in the IRES or whether they represent additional contacts of the IRES with the eIF4G/4A complex. In any case, enhancement of these toeprints in the presence of PTB and ITAF₄₅ indicates that these two proteins may facilitate binding of the eIF4G/4A complex to the IRES, which might in turn enhance 48S complex formation.

Toeprint analysis of RNP complexes formed with different combinations of factors indicated that eIF4F bound specifically to the FMDV IRES, yielding toeprints at UG₆₆₄₋₅ in the J-K domain (data not shown). More detailed analysis indicated that the central domain of eIF4G (residues 607-1076) bound specifically but weakly to the FMDV IRES, yielding a toeprint at G₆₆₅ (Fig. 10A, lanes 1,2). Inclusion of eIF4A with eIF4G₆₀₇₋₁₀₇₆ resulted in the appearance of an additional toeprint at U₆₆₄ (Fig. 10A, lanes 2,3). eIF4A alone did not lead to the appearance of toeprints anywhere on the IRES (data not shown). The intensity of the G₆₆₅ toeprint due to bound eIF4G₆₀₇₋₁₀₇₆ and of the UG₆₆₄₋₅ toeprints due to the bound eIF4A/eIF4G₆₀₇₋₁₀₇₆ complex were enhanced by PTB and ITAF₄₅ individually and together (Fig. 10A, lanes 1-9). Inclusion of PTB in these reactions led to the appearance of toeprints at GCCG₆₈₁₋₄ and UUU₆₉₀₋₂ that did not depend on the presence of other factors, including eIF4G, eIF4A, eIF4B, and ITAF₄₅ (Fig. 2; Fig. 10A, lanes 1,4,6,7,9,10; Fig. 10B, lanes 1,4). The toeprints at UUU₆₉₀₋₂ correspond to residues that are protected by PTB from chemical modification (Kolupaeva et al. 1996). Toeprints appeared at GU₆₇₁₋₂ on inclusion of PTB and ITAF₄₅ in binding reactions only in the presence of eIF4G₆₀₇₋₁₀₇₆ (cf. Fig. 10A, lane 6 and Fig. 10B, lane 4). Taken together, these observations indicate that the specific binding of eIF4G to the FMDV J-K domain is enhanced by eIF4A, PTB, and ITAF₄₅.

Figure 10. (A,B) Primer extension analysis of a ribonucleoprotein complex formed on the foot-and-mouth disease virus (FMDV) internal ribosomal entry site (IRES). GD80 mRNA was incubated under standard conditions with ITAF₄₅, PTB, eIF4A, eIF4B, and eIF4G₆₀₇₋₁₀₇₆ individually or in combination as indicated. An oligonucleotide was annealed within the coding sequence and extended with AMV-RT. The cDNA products labeled U₆₆₄, G₆₆₅, GU₆₇₁₋₂, GCCG₆₈₁₋₄, and UUU₆₉₀₋₂ terminated at these nucleotides. Reference lanes C, T, A, and G depict the negative strand FMDV sequence.

Localization of factor-binding sites on the FMDV IRES by footprinting

Footprinting was used to map binding sites for eIFs, PTB, and ITAF₄₅ on the FMDV IRES and to identify structural changes in it caused by their binding. In these experiments we used RNase T₁ (which cleaves RNA specifically after unpaired G residues) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulphate (CMCT), which reacts with N-3 of unpaired uracil and N-1 of unpaired guanine residues (Ehresmann et al. 1987). Chemical modification or strand scission both arrest reverse transcriptase and can thus be analyzed by

primer extension. CMCT modification causes primer extension arrest at the nucleotide immediately 3' to the modified position, and enzymatic cleavage results in arrest of primer extension at the nucleotide on the 3'-side of the cleaved bond. Some residues in the FMDV IRES at which cleavage or modification patterns appear to be altered in the presence of factors such as G₄₀₁ coincide with strong stops formed during primer extension on this highly structured RNA. Altered cleavage or modification of such residues is therefore equivocal and is not discussed below. Results of this analysis are summarized in Fig. 12 (see below).

Individually, neither eIF4A nor eIF4G_{607–1076} protected any part of the IRES from cleavage or modification (Fig. 11A, lanes 4,7; data not shown). However, together they specifically protected the IRES from cleavage at G₅₆₉, G₆₁₁, and G₆₅₃ in the J-K domain and at G_{670–1} and G₆₇₃ in domain L (Fig. 11A, lane 5). These results are consistent with the results of toeprinting (Fig. 10A) and with the identification of eIF4G binding sites at identical locations in EMCV and TMEV IRESs.

PTB alone protected the FMDV IRES from cleavage at G₃₁₇ in domain H (Fig. 11C, lane 3) and at G_{670–1} and G₆₇₃ in domain L (Fig. 11A, lane 3). The protected site in domain H is very close to residues in its apical loop that are protected by PTB from CMCT modification (Kolupaeva et al. 1996; Fig. 12). PTB alone also enhanced cleavage at G_{680–1} at the apex of domain L and, weakly, at G₅₆₉ at the junction of domains J and K (Fig. 11A, lane 3). Binding of ITAF₄₅ alone to the IRES protected it from cleavage at G₃₅₁ in the basal part of domain I (Fig. 11C, lanes 2,5; Fig. 12) and enhanced CMCT modification at G₄₉₁ in the apical half of domain I and at U₅₅₄, U₆₇₂, G₆₇₈, and G₆₈₅ near and within domain L (Fig. 11D, lane 2). Taken together, the data indicate that PTB and ITAF₄₅ interact functionally with the IRES at several specific sites and may therefore act as RNA chaperones that foster correct IRES folding. Importantly, the footprint pattern caused by binding of PTB and ITAF₄₅ together to the IRES differed somewhat from the summation of the footprints caused by binding of these two proteins individually. Thus, PTB alone enhanced RNase T₁ cleavage at G₃₅₅, near the base of domain I, and this enhanced cleavage was suppressed by ITAF₄₅ (Fig. 11C, lanes 3,4). A combination of PTB and ITAF₄₅ enhanced cleavage at G₄₉₁, G₅₅₃, and G₅₅₅ and exposed a cleavage site at G₆₆₅ (Fig. 11B, lane 2). These results are indicative of a cooperative chaperone-like action of PTB and ITAF₄₅ in RNA recognition and folding, and are consistent with and can account for the synergistic enhancement by these two proteins of 48S complex formation on the FMDV IRES.

The strong protection of the FMDV IRES from cleavage at nt 670–673 caused by the binding of eIF4G_{607–1076}, eIF4A, PTB, and ITAF₄₅ is consistent with the appearance of additional toeprints at GU_{671–2} on the IRES on inclusion of both PTB and ITAF₄₅ with either eIF4G_{607–1076} or, to a greater extent, with both eIF4G_{607–1076} and eIF4A (Fig. 10A, lanes 2,4–6,10).

The results of footprinting and toeprinting are thus

wholly consistent. They indicate that ITAF₄₅ binds at the base of domain I; that PTB makes multiple contacts with the IRES in domain H, domain K, and downstream of domain L; and that the eIF4G/eIF4A binary complex binds to the J-K domain. eIF4A does not bind specifically or stably to the IRES except in the presence of eIF4G. PTB and ITAF₄₅ also increase the susceptibility of the IRES to cleavage at several positions by inducing conformational changes in it and synergistically enhance binding of the eIF4G/eIF4A complex in a manner that may account for the dependence of FMDV IRES function on PTB and ITAF₄₅.

Discussion

We report here that we have identified a cellular RNA-binding protein, ITAF₄₅, that mediates mRNA-selective translation initiation. We identified ITAF₄₅ by reconstituting 48S complex formation in vitro on FMDV and TMEV IRESs, using fully fractionated translation components. Initiation on these IRESs and the related EMCV IRES required the same set of canonical eIFs, but they had strikingly different ITAF requirements. TMEV IRES function was enhanced fivefold by PTB, but initiation on the FMDV IRES was dependent on ITAF₄₅ as well as on PTB. EMCV IRES function did not depend on either of these ITAFs.

The role of canonical initiation factors in internal ribosomal entry

The canonical factors eIF2, eIF3, eIF4A, eIF4B, and eIF4F are required for 48S complex formation on TMEV and FMDV IRESs just as for initiation on the EMCV IRES (Pestova et al. 1996a). Significantly, we found that eIF4F binds specifically to an analogous site in the J-K domains of each of these IRESs (Fig. 4; Pestova et al. 1996a,b; Kolupaeva et al. 1998). The specificity of binding to each of these IRESs is due to its eIF4G subunit. We noted that inclusion of eIF4A with eIF4G in toeprinting assays strongly enhanced its binding to the FMDV IRES and led to the appearance of a new toeprint at U₆₆₄. Moreover, individually neither eIF4A nor eIF4G protected any part of the IRES from cleavage or chemical modification in footprinting assays. Taken together, these results indicate that the eIF4G/4A complex rather than eIF4G alone is responsible for recognition of the FMDV IRES. This conclusion is consistent with our finding that the affinity of eIF4G for the EMCV IRES is enhanced by up to two orders of magnitude by eIF4A (Lomakin et al. 2000). There is no eIF4E requirement for this interaction or for 48S complex formation. The IRES–eIF4G/4A interaction is essential for initiation; mutations in either the J-K domain of these IRESs or in the IRES-binding domain of eIF4G that abrogate this interaction also abrogate IRES function (Pestova et al. 1996a; T.V. Pestova and E.V. Pilipenko, unpubl.). The central domain of eIF4G contains binding sites for eIF4A and eIF3 (Morino et al. 2000) and is presumed to coordinate their activities. In the context

Figure 11. Chemical and enzymatic footprinting of the foot-and-mouth disease virus (FMDV) internal ribosomal entry site (IRES) in complexes formed with the IRES and ITAF₄₅, PTB, eIF4A, eIF4B, and eIF4G₆₀₇₋₁₀₇₆ individually or in combination as indicated. Polyacrylamide-urea gel fractionation of cDNA products obtained after primer extension showing the sensitivity of the internal ribosomal entry site to RNase T₁ cleavage (**A**, lanes 2–7; **B**, lanes 1–3; **C**, lanes 2–5) and to CMCT modification (**D**, lanes 1,2) either alone or complexed with factors as indicated. cDNA products derived from untreated RNA are shown in lane 1 of panels **A** and **C**, in lane 4 of panel **B**, and in lane 3 of panel **D**. A dideoxynucleotide sequence generated with the same primer was run in parallel on each gel and is shown in panels **A**, **B**, and **D**. The positions of protected residues or enhanced cleavage are indicated to the right of each panel. The positions of FMDV nucleotides at 50-nt intervals are indicated by black squares to the right of each panel. Individual subdomains are indicated to the left of each panel.

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Figure 12. Summary of changes in modification by CMCT and in cleavage by RNase T₁ of the foot-and-mouth disease virus (FMDV) internal ribosomal entry site (IRES) caused by binding of ITAF₄₅, PTB, eIF4A, and eIF4G_{607–1076} individually or in combination as indicated. These probes are indicated by symbols, as described in the key at top right. Results are displayed on a secondary structure model (Pilipenko et al. 1989). The initiation codon AUG₇₁₄ is underlined.

of these picornavirus IRESs, the role of the eIF4G/4A complex may be to prepare the IRES for attachment of a 43S complex in the vicinity of the initiation codon, for example, by interaction with associated factors such as eIF3 and eIF4B or by causing conformational changes in the IRES/factor RNP complex.

The role of IRES trans-acting factors in internal ribosomal entry

The requirement for PTB in FMDV IRES-mediated initiation is consistent with previous reports that it binds specifically to this IRES (Luz and Beck 1990; Kolupaeva

et al. 1996) and is involved in FMDV translation (Niepman 1995). However, PTB alone is not sufficient to enhance 48S complex formation above background levels on GD68 and GD80 mRNAs, and its activity is dependent on a second factor, ITAF₄₅. ITAF₄₅ and PTB bind specifically to nonoverlapping sites on the FMDV IRES and individually and together cause localized structural changes in adjacent regions of it (Figs. 10–12). These two ITAFs enhance binding of the eIF4G/4A complex to the IRES and likely promote their assembly into a stable RNP complex (Fig. 10). The binding sites for protein components of this RNP complex include all IRES domains except for the apical region of domain I and extend from the 5' border of the IRES to within 15 nt of the initiation codon. The latter distance is only slightly greater than the number of residues upstream of the initiation codon of an mRNA that are covered by the 40S ribosomal subunit (Legon 1976; Kozak 1977). Ribosomes are recruited to the FMDV IRES at or immediately upstream of the initiation codon at the 3' border of the IRES without scanning from an upstream position (Belsham 1992; Lopez de Quinto and Martinez-Salas 1999). The function of ITAFs on the FMDV IRES may therefore be to prepare an RNP complex that contains RNA and protein binding determinants for components of the 43S complex oriented in three dimensions in such a way that it can be recruited and loaded onto the mRNA precisely, placing the initiation codon at the ribosomal P site.

A robust model for IRES-mediated initiation should be able to account for the significant differences between closely related IRESs in their requirement for ITAFs. For example, ribosomal attachment to the initiation codon without scanning is also characteristic of EMCV and TMEV IRES (Kaminski et al. 1990; Pilipenko et al. 1994). There is significant conservation of sequence and structure between EMCV, TMEV, and FMDV IRESs (Pilipenko et al. 1989; Jackson and Kaminski 1995), and as described above, initiation on each of them requires the same set of canonical factors and involves the same interaction between the eIF4G/4A complex and the IRES J-K domain. EMCV, FMDV, and TMEV IRESs all bind PTB with high affinity (Jang and Wimmer 1990; Luz and Beck 1990; Witherell et al. 1993; Kaminski et al. 1995), and the location of PTB binding sites on them appears to be similar, although not identical (Kolupaeva et al. 1996). We have reported here that ITAF₄₅ can be strongly UV cross-linked to both TMEV and FMDV IRESs (Fig. 9). Despite these many similarities, initiation on the EMCV IRES is only weakly dependent on PTB, initiation on the TMEV IRES is strongly dependent on PTB, and initiation on the FMDV IRES is strongly dependent both on PTB and on a second protein, ITAF₄₅, that has no influence on EMCV or TMEV IRESs. Differences in the ITAF requirement for these three IRESs must therefore be related to the small sequence and structural differences between them.

One hypothesis is that ITAFs have a chaperone activity (cf. Herschlag 1995) that functions in IRES-mediated initiation to remodel aberrantly folded IRESs so that they achieve and maintain an active conformation. This

hypothesis is supported by the finding that EMCV IRES-mediated translation (which does not require PTB) became strongly PTB dependent following insertion of a single extra residue into the A-rich loop between J and K domains (Kaminski and Jackson 1998). The presence of an additional A residue in this same loop in the IRES of the TMEV GDVII strain relative to the equivalent loop of the TMEV DA strain IRES may account for the apparent difference in their PTB-dependence for activity (Kaminski et al. 1995; this paper). This hypothesis is also consistent with the conclusion that it is the requirement for, rather than the binding of per se, PTB and ITAF₄₅ to EMCV, FMDV, and TMEV IRESs that is altered as a consequence of nucleotide differences between them.

We suggest that the observation that FMDV IRES requires ITAF₄₅ in addition to PTB, where the TMEV IRES requires only PTB for activity may reflect sequence differences between these IRESs that lead to differences between them in the mode of binding PTB. For example, cardiovirus (EMCV, TMEV) IRESs contain pyrimidine residues in the bulge between the two helices of domain K that are specifically bound by PTB (Kolupaeva et al. 1996), whereas analogous residues are not present in the FMDV IRES. We hypothesize that PTB alone cannot fold the FMDV IRES into a functionally competent structure because of such differences in PTB binding sites and that this IRES only achieves an active conformation in the presence of both RNA chaperones, PTB and ITAF₄₅.

It is interesting to note that members of the second major group of picornavirus IRESs are also closely related yet also appear to have different ITAF requirements. Initiation on the poliovirus IRES is strongly dependent on PTB and PCBP2 and does not respond to *unr*, where in contrast to the poliovirus IRES, initiation on the rhinovirus IRES is strongly dependent on *unr* and is enhanced by PTB but is less responsive to PCBP2 (Blyn et al. 1997; Hunt and Jackson 1999; Hunt et al. 1999; Walter et al. 1999). Nevertheless, PTB, PCBP2, and *unr* all bind to both poliovirus and rhinovirus IRESs (Borman et al. 1993; Hellen et al. 1993, 1994; Hunt et al. 1999; Walter et al. 1999; T.V. Pestova, unpubl.).

ITAF₄₅ is a proliferation-dependent protein that is distributed throughout the cytoplasm from metaphase through to telophase (Radomski and Jost 1995; Lamarque et al. 1997; Nakagawa et al. 1997). Its expression is up-regulated in response to mitogen stimulation, and it is not detectable in nonproliferating differentiated cells such as murine brain cells (Radomski and Jost 1995). The strict difference in factor requirements for initiation of translation on TMEV and FMDV IRESs is sufficient to limit the activity of the latter, and it is thus tempting to speculate that the requirement for ITAF₄₅ may be a cell-specific determinant that limits replication of the chimeric GD68 and GD80 viruses as well as FMDV. An attractive hypothesis is that ITAF₄₅ may function as a tissue-specific and cell-cycle-dependent factor that specifically binds and controls translation on a particular set of mRNAs during cell proliferation and/or specific phases of the cell cycle. Indeed, IRES-mediated translation of a number of cellular proteins, including ornithine

decarboxylase, *c-myc*, the p58PITSLRE protein kinase, and platelet-derived growth factor B, is specifically activated in a cell-cycle or proliferation-dependent manner (Bernstein et al. 1997; Cornelis et al. 2000; Pyronnet et al. 2000).

Materials and methods

Plasmids

Plasmids pET(His₆-eIF4A), pET(His₆-eIF4B) (Pestova et al. 1996a), pBS-(β -globin), and pE15 (Hellen et al. 1993) have been described. Mouse embryo cDNA (Clontech) corresponding to the ITAF₄₅ coding region (Nakagawa et al. 1997) was amplified by PCR using the primers 5'-CGGGATCCGATGTCGGGC-GAAGACGAGC-3' and 5'-CTCCGCTCGAGTTATCAGTC-CCCAGCTCCATTCTC-3', digested with *Bam*H1 and *Xho*I and inserted into pET28b (Novagen) to yield pET(His₆-ITAF₄₅).

Chimeric TMDV/FMDV IRES-luciferase reporter constructs were derived from TMEV GDVII plasmids pUGD (Pilipenko et al. 1994), pGLV and pLG (Pilipenko et al. 1999), and the FMDV O₁K plasmid pSP449 (Luz and Beck 1990). pLG contains TMEV GDVII nt 1–1124 (corresponding to the entire 5' UTR, the initiation codon, and 18 codons) fused in-frame to the luciferase gene and downstream of a T₇ promoter. pLGF-68 and pLGF-80 were derived from pLG by replacing TMEV nt 628–1047 with FMDV O₁K nt 280–693 and TMEV nt 628–1124 with FMDV O₁K nt 280–740, respectively. FMDV nucleotides are numbered starting after the poly(C) tract (Forss et al. 1984). Chimeric full-length TMEV clones GD68 and GD80 were derived from an infectious full-length *wt* TMEV GDVII clone GD18 by replacing TMEV nt 628–1047 with FMDV O₁K nt 280–693 and TMEV nt 628–1124 with FMDV O₁K nt 280–740, respectively.

Translation of luciferase expressing plasmids

Plasmids pLG, pLGF-68, and pLGF-80 were linearized with *Sal*I, and mRNA was transcribed, purified, and translated in RRL at 30°C for 1 hr (Pilipenko et al. 1994). Template activity was quantified by measurement of [³⁵S]methionine incorporation into the luciferase band separated by SDS-PAGE. For *in vivo* translation, 1-day-old quadruplicate cultures of BHK-21 cells grown in 50-mm plastic dishes were each transfected with 2 μ g of mRNA using DEAE-dextran (Pilipenko et al. 1999). Cells were removed after 3 hr incubation at 37°C, and luciferase activity was determined using the Promega Luciferase Assay System according to the manufacturer's protocol. The signals were normalized to 10⁶ cells by determining protein concentration in the lysates using the Lowry method. The signal variation in quadruplicate cultures did not exceed 15% of the average value for a given template. The saturating amount of RNA in this assay corresponded to 1 μ g/dish.

Preparation and characterization of chimeric viruses

Viruses were recovered from transcript RNA-transfected BHK-21 cells, accumulated, and characterized (Pilipenko et al. 1994, 1995). A region of the viral RNA (between nt 579 and 1214 of the *wt* TMEV GDVII sequence) was amplified by RT-PCR and sequenced as described (Pilipenko et al. 1995). No additional sequence changes were detected.

Neurovirulence assay and detection of replicating virus in mouse CNS

BALB/C mice (10–12 g) were inoculated intracerebrally with tenfold virus dilutions and monitored for clinical signs for 6

week p.i. Thirty mice were inoculated with 10⁸ TCD₅₀ (50% tissue culture infective dose) of GD68 virus, and 10 mice were each infected with 10⁷ TCD₅₀–10³ TCD₅₀ doses. For GD80 virus, 25 mice were inoculated with 10^{7.5} TCD₅₀, and 12 mice each with 10^{6.5} TCD₅₀ and 10^{5.5} TCD₅₀ doses. The paralytogenic activity was expressed as the viral dose causing paralysis in 50% of animals (PD₅₀). The presence of the viral genome in the CNS was detected by RT-PCR amplification of the appropriate region of the viral RNA (nt 579–1214), as described above. Four mice for GD80 and three mice for GD68 were sacrificed at 1-, 7- and 14-day time-points p.i. Forty cycles of PCR amplification were performed with RNA samples extracted from CNS at days 7 and 14 p.i.

Purification of factors and 40S ribosomal subunits

Ribosomal subunits, eIF2, eIF3, and eIF4F were purified from RRL as described (Pestova et al. 1996a, 1998b). ITAF₄₅ was purified from the ribosomal salt wash (RSW) 70%–95% ammonium sulfate (AS) precipitation fraction from RRL. This fraction was applied in buffer A (20 mM Tris at pH 7.5, 1 mM DTT, 0.1 mM EDTA, 10% glycerol) containing 100 mM KCl to a DEAE cellulose column. The flow-through fraction was applied to a phosphocellulose column and fractionated by step-elution using buffer A with 100 mM and then 250 mM, 400 mM, 600 mM and finally 800 mM KCl. The active 250–400-mM KCl elution fraction was dialyzed against buffer A with 50 mM KCl and applied in this buffer to an FPLC MonoQ HR 5/5 column. Fractions were collected across a 50–500 mM KCl gradient. Peaks of active, apparently homogenous ITAF₄₅ eluted with 185 and 210 mM KCl. Recombinant PTB-1 and eIFs eIF1, eIF1A, eIF4A, and eIF4B were purified as described (Pestova et al. 1996a, 1998a). Recombinant ITAF₄₅ was expressed in *Escherichia coli* BL21(DE3) and purified by Ni²⁺-NTA (Qiagen) and MonoQ HR 5/5 column chromatography.

Sequencing of ITAF₄₅

Purified ITAF₄₅ was resolved by gel electrophoresis. Sequencing of two well-resolved ITAF₄₅ tryptic peptides was done as described (Pestova et al. 1998a,b).

Assembly and analysis of ribosomal and ribonucleoprotein complexes

For toeprinting analysis, ribosomal complexes were assembled essentially as described (Pestova et al. 1998a). For 5 min, 0.5 μ g of GD18, GD68, or GD80 mRNA were incubated at 30°C in a 40- μ l reaction volume that contained buffer B (2 mM DTT, 100 mM KAc, 20 mM Tris at pH 7.6, 2.5 mM MgAc, 1 mM ATP, 0.1 mM GMPPNP, 0.25 mM spermidine), eIF2 (3 μ g), eIF3 (6 μ g), eIF4A (2 μ g), eIF4B (0.3 μ g), eIF4F (1.5 μ g), 6 pmole of 40S subunits, and 6 pmole of Met-tRNA^{Met}_i and with or without PTB (0.5 μ g), ITAF₄₅ (1 μ g), eIF1 (0.3 μ g), eIF1A (0.3 μ g), or different RSW fractions (4–8 μ g protein), as indicated. Incubation was continued for 3 min at 30°C following addition of 4 pmole of primer 5'-CCAGAAGACGTCATCGTCCA-3' (complementary to TMEV nt 1195–1214). Ribosomal and RNP complexes were analyzed by primer extension (Pestova et al. 1996a) using avian myeloblastosis virus (AMV) reverse transcriptase (Promega) in the presence of [α -³²P]dATP (~6000 Ci/mmol; ICN Radiochemicals). cDNA products were compared with appropriate dideoxynucleotide sequence ladders.

For toeprinting analysis of 48S complexes assembled in RRL, 0.5 μ g of RNA was incubated in 15 μ l of RRL in the presence of 1 mM GMPPNP for 5 min at 30°C. The reaction mixture was

diluted with buffer B to 40 μ l final volume before the addition of 4 pmole of primer 5'-CCAGAAGACGTCATCGTCCA-3' complementary to TMEV nt 1195–1214. Toeprint analysis was done as described above.

Ribonucleoprotein (RNP) complexes were formed by incubating eIFs and FMDV IRES mRNA for 5 min at 30°C in buffer (100 mM KAc, 2 mM MgAc, 2 mM Tris-HCl at pH 7.5, 1 mM DTT). Reactions contained 1 μ g of RNA (4 pmole), 1 μ g of eIF4G_{607–1076} (10 pmole), 1 μ g of eIF4A (22 pmole), 1.5 μ g (21 pmole) of eIF4B, and 0.75 μ g (12 pmole) of PTB and ATP (1 mM), as indicated in the text, in a 20- μ l volume. Toeprint analysis of FMDV mRNA/factor complexes was done essentially as described (Pestova et al. 1996a,b), using the primer 5'-CCAGAAGACGTCATCGTCCA-3' (complementary to TMEV nt 1214–1195).

Ribosomal complexes were assembled for analysis by sucrose-density gradient centrifugation essentially as described above for toeprinting analysis, except that the reaction volume was increased to 100 μ l and mRNAs were labeled during transcription with [³²P]UTP (~3000 Ci/mmol; ICN Radiochemicals) to a specific activity of 200,000 cpm/ μ g. Reaction mixtures containing translation components as indicated in the text were incubated for 5 min at 30°C. 48S and RNP complexes were resolved by centrifugation through 10%–30% sucrose-density gradients as described (Pestova et al. 1996a).

UV cross-linking of ITAF₄₅ to RNA

The UV cross-linking reaction was done using a UV-Stratalinker (Stratagene) essentially as described (Pestova et al. 1996b). Purified *wt* TMEV RNA, chimeric GD80 RNA, or β -globin RNA (100 ng) that had been labeled with [³²P]UTP during transcription with T₇ polymerase were incubated for 10 min at 30°C in a 20 μ l reaction volume containing buffer B and ITAF₄₅ (3 μ g) either with or without competitor RNAs, as indicated in the text. Samples were digested with cobra venom nuclease and RNase A. Proteins were separated by 12% SDS-PAGE.

Chemical and enzymatic footprinting

RNP complexes were probed with RNase T₁ and CMCT as described (Kolupaeva et al. 1996, 1998). Cleaved or modified sites were identified by primer extension, using AMV RT and the primers 5'-CCAGAAGACGTCATCGTCCA-3' (complementary to TMEV nt 1214–1195), 5'-CAGTCAGTTGTATTCATG-3' (complementary to FMDV nt 730–713), or 5'-CCAGTATCAATGTCACC-3' (complementary to FMDV nt 539–523), as appropriate.

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