Sequence and secondary structure requirements in a highly conserved element for foot-and-mouth disease virus internal ribosome entry site activity and eIF4G binding

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Foot-and-mouth disease virus (FMDV) and other picornaviruses initiate translation of their positive-strand RNA genomes at the highly structured internal ribosome entry site (IRES), which mediates ribosome recruitment to an internal site of the virus RNA. This process is facilitated by eukaryotic translation initiation factors (eIFs), such as eIF4G and eIF4B. In the eIF4G-binding site, a characteristic, discontinuous sequence element is highly conserved within the cardio- and aphthovirus subgroup (including FMDV) of the picornaviruses. This conserved element was mutated in order to investigate its primary sequence and secondary structure requirements for IRES function. Both binding of eIF4G to the IRES and IRES-directed translation are seriously impaired by mutations in two unpaired dinucleotide stretches that are exposed from the double-stranded (ds)RNA. In the base-paired regions of the conserved element, maintenance of the double-stranded secondary structure is essential, whilst in some cases, the primary sequence within the dsRNA regions is also important for IRES function. Extra eIF4F added to the translation reaction does not restore full IRES activity or eIF4G binding, indicating that disturbances in the structure of this conserved element cannot be overcome by increased initiation factor concentrations.

INTRODUCTION

Foot-and-mouth disease virus (FMDV), a member of the family Picornaviridae, causes a highly contagious disease with considerable impact in cattle and other cloven-hoofed animals (Brown, 1999; Ferguson et al., 2001; Stone, 2002). Picornaviruses are small, non-enveloped, positive-strand RNA viruses that replicate in the cytoplasm (Paul, 2002). Of the nine genera of picornaviruses (Stanway et al., 2002), the majority can be divided into three groups: the enteroviruses (including poliovirus) and rhinoviruses, the cardio- and aphthoviruses [which include FMDV and encephalomyocarditis virus (EMCV)] and hepatoviruses (including hepatitis A virus), based on the sequence and predicted structure of the genome region that is implicated in the initiation of translation. After infection of a susceptible cell, these viruses bypass the general route of cellular cap-dependent translation. The internal ribosome entry site (IRES), a cis-acting RNA element with highly conserved secondary structures that is located in the 5′ untranslated region of the virus RNA genome (Fig. 1), guides the ribosome to an internal site of the virus RNA (Pilipenko et al., 1989; Jackson & Kaminski, 1995; Niepmann, 1999; Ehrenfeld & Teterina, 2002). At the 3′ end of the IRES, a conserved oligopyrimidine tract is followed by an AUG triplet (Beck et al., 1983). In cardio- and aphthoviruses, this AUG is usually the one that initiates translation. Alternatively, a second start site 84 nt further downstream can also be used after scanning in FMDV (Sangar et al., 1987; Belsham, 1992). Most picornaviruses utilize the obvious advantage of translating cap-independently and shut down cellular cap-dependent protein synthesis. The FMDV leader (L-) protease (Devaney et al., 1988) cleaves eukaryotic translation initiation factor (eIF) 4G (Lamphear et al., 1995), which is the major component of the cap-binding protein complex eIF4F. Also, changes in the intracellular ionic environment during picornavirus infection may contribute to the preferential translation of picornavirus RNA (Carrasco & Smith, 1976). In particular, optimum translation of FMDV occurs under high potassium and chloride conditions (Niepmann, 2003).

Binding of the ribosome to virus RNA is mediated by a number of cellular RNA-binding proteins (Jackson, 2002), which fall into two groups. On the one hand, the standard
eIFs that mediate translation of cellular mRNAs are also involved in picornavirus translation, except for the cap-binding protein eIF4E (Pestova et al., 1996a). On the other hand, picornavirus IRES elements additionally bind several other cellular RNA-binding proteins that are not usually involved in translation. Some of these proteins have been found to modulate IRES activity, such as the 57 kDa poly-pyrimidine tract-binding protein (PTB), which enhances translation driven by several IRES elements, such as that of FMDV (Niepmann, 1996; Niepmann et al., 1997) and EMCV (Kaminski et al., 1995). Other cellular proteins that stimulate picornavirus IRES activity are the La protein (Meerovitch et al., 1993), poly(rC)-binding protein 2 (PCBP2; Blyn et al., 1997), the protein encoded by the gene upstream of N-ras (unr; Hunt et al., 1999; Boussadia et al., 2003) and a 45 kDa IRES-interacting translation factor (ITAF45; Pilipenko et al., 2000).

A key role in translation initiation is attributed to eIF4G, which is a large, multifunctional adaptor protein that is a component of the cap-binding complex eIF4F and connects the RNA that is to be translated to the ribosome (Hentze, 1997; Prevot et al., 2003). In contrast to the indirect interaction of eIF4G with capped cellular mRNAs via the cap-binding protein eIF4E, eIF4G binds directly to the IRES 3' regions of FMDV (López de Quinto & Martínez-Salas, 2000; López de Quinto et al., 2001; Saleh et al., 2001), EMCV (Pestova et al., 1996b) and poliovirus (Ochs et al., 2003), probably synergistically with the associated ATP-dependent RNA helicase eIF4A and its stimulating cofactor eIF4B (Meyer et al., 1995; Kolupaeva et al., 1998; Ochs et al., 1999, 2002; Rust et al., 1999). In the FMDV strain O,K IRES, the binding site for eIF4G comprises stem–loops 4 and 5 (Fig. 1; Pilipenko et al., 2000; Saleh et al., 2001), whereas in a study with the FMDV C-S8c1 strain, only stem–loop 4 was required (López de Quinto & Martínez-Salas, 2000).

At the primary sequence level, only some short sequence stretches are conserved among the type II IRES elements of cardio- and aphthoviruses (Jackson & Kaminski, 1995). Two of these comprise a characteristic, discontinuous sequence element (Fig. 1b, boxed) that resides in the stem of the apical subdomain 4-1 (named J in the related EMCV IRES) within the Y-shaped domain 4 of the IRES. This sequence element is discontinuous at the primary sequence level, but forms a compact, continuous element at the secondary structure level, which consists of an apical 2 bp stack and two unpaired dinucleotide stretches flanking a 4 bp stack (Fig. 1). The absolute conservation of both its primary sequence and secondary structure among all cardio- and aphthoviruses (Jackson & Kaminski, 1995) points to an essential role of this element in the virus life cycle.

As IRES domain 4 carries the determinants for its interaction with eIF4G, we suspected that this element may be involved in the interaction with this initiation factor that is crucial for FMDV translation. Therefore, we have mutated this element in the context of the complete FMDV IRES and analysed the effects of these mutations on eIF4G binding and IRES activity.

**METHODS**

**Plasmids.** pSP449 (Luz & Beck, 1991) contains the FMDV O,K IRES and coding sequences from positions 363 to 831 (including 27 nt of coding sequence). Mutations in the IRES domain 4 sequence were derived by PCR mutagenesis. All mutant IRES elements were also placed in the monocistronic expression vector pM12 (Ochs et al., 1999), which contains the FMDV IRES (positions 363–805) with the 11th ATG of FMDV fused to the firefly luciferase gene. A set of selected mutants was also placed in the dicistronic expression vector pD128 (Niepmann et al., 1997), which contains the chloramphenicol acetyltransferase (CAT) gene and the FMDV IRES (positions 363–805) with the 11th ATG of FMDV fused to the firefly luciferase gene.
Preparation of RNAs and in vitro translation. The pSP449 series plasmids were linearized with Smal in the linker downstream of the FMDV sequence. Labelled RNAs were synthesized by using SP6 RNA polymerase in the presence of 2-5 μM [α-32P]UTP (400 Ci mmol⁻¹; Amersham Biosciences) plus 10 μM unlabelled UTP. For in vitro translations, the pM12 and pD128 series plasmids were linearized with Smal downstream of the luciferase gene and mRNA was synthesized in the presence of 500 μM unlabelled nucleotides. Then, 0-2 μg RNA was used in a 10 μl reaction that contained 4-4 μl rabbit reticulocyte lysate (RRL; Promega) and 0-2 μl [35S]methionine. In addition to the 50 mM endogenous potassium acetate that is added to RRL by the supplier, KCl was added to a final potassium concentration of 125 mM, unless otherwise indicated. Reactions were incubated at 30 °C for 60 min and 5 μl of the reaction was analysed by gel electrophoresis and autoradiography. FMDV L-protease was translated in RRL from plasmid pFMDV14 linearized with BamHI (Saleh et al., 2001). Purified eIF4F (Scheper et al., 1992) was kindly provided by Adri Thomas or prepared freshly as described by Grifo et al. (1983).

Transfections. BHK cells were split into 12-well plates and grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 100 μg penicillin/streptomycin ml⁻¹ to 90 % confluence. One day before transfection, the medium was altered to DMEM plus FCS, but without antibiotics. For transfection, 0-5 μg of each dicistronic plasmid and 1 μl Lipofectamine (Invitrogen) were each diluted separately in 25 μl DMEM (without FCS and antibiotics) and incubated at room temperature. Within 5 min, both dilutions were mixed together, incubated for another 20 min at room temperature and then added to the cells. After 24 h, cells were washed in PBS; the PBS was then aspirated, 250 μl passive lysis buffer (Promega) was added and the cells were lysed by gentle agitation. Lysates were collected, centrifuged for 30 s at 13 000 g and 15 μl supernatant was used for luciferase and CAT reporter enzyme assays (Niepmann et al., 1997).

UV cross-linking assays. UV cross-linking assays (Ochs et al., 1999) were performed with 4-4 μl RRL and 0-2 pmol [α-32P]UTP-labelled IRES RNA in a volume of 10 μl at 125 mM final potassium concentration, unless otherwise indicated. Competitor RNAs were added if indicated. Reactions were incubated at 30 °C for 30 min under irradiation with 254 nm UV light. Excess RNA was digested with 2 μg RNase A ml⁻¹ at 37 °C for 60 min. Proteins were separated on 8 % SDS-PAGE gels and analysed by autoradiography.

RESULTS

Effect of mutations in the unpaired dinucleotide stretches in the conserved element

In order to analyse the contribution of the two characteristic, unpaired dinucleotide stretches that are exposed from the double-stranded region of the conserved sequence element, we deleted them either separately (mutants up-del or down-del) or in combination (mutant up/down-del) (Fig. 2a). The activities of these mutant IRES elements were reduced almost to background levels, both in vivo after transfection of BHK cells (Fig. 2b) and in vitro in translation reactions with RRL (Fig. 2c, lanes 2–4), indicating that the presence of these unpaired dinucleotides is essential for IRES activity. In the UV cross-linking assay, binding of the large translation initiation factor eIF4G, which migrates at an apparent molecular mass of 200 kDa, was almost completely abolished by these mutations (Fig. 2d, lanes 2–4). The identity of eIF4G was confirmed by treatment with the L-protease of FMDV. Although binding of some proteins to the radiolabelled IRES competes to some extent with unlabelled RNA that encodes the L-protease, as its expression is also driven by FMDV IRES sequences (Fig. 2e, lane 2), expression of L-protease clearly results in cleavage of the 200 kDa full-length eIF4G and generates a 110 kDa C-terminal fragment that appears in the UV cross-linking assay (Fig. 2e, lanes 3 and 4), as well as in the immunoblot (Fig. 2f, lane 2) (Lamphear et al., 1995; Saleh et al., 2001).

In addition, binding of eIF4B (Ochs et al., 1999; Rust et al., 1999) was also reduced in parallel with eIF4F by these mutations, as eIF4G, eIF4A and eIF4B bind synergistically to the IRES (Kolupaeva et al., 1998). Also, binding of PTB (Niepmann et al., 1997) was affected. Some RNA determinants that are contacted by PTB are interspersed between the sites that are contacted by the standard factors (Kolupaeva et al., 1996). Accordingly, mutations in the eIF4F-binding site in the virus RNA that result in a complete loss of eIF4F binding may, in turn, also have a moderate effect on PTB binding, whereas mutations that still allow residual eIF4F binding do not result in detectable interference with PTB binding, as PTB binds to the IRES very efficiently (Fig. 4e). In contrast, binding of other proteins, such as that of about 47 kDa, appeared to be unaffected (Figs 2d and 3–5). The identity of this 47 kDa protein is not known. Its binding to the FMDV IRES is not specific. A protein of the same size binds unspecifically to the poliovirus IRES, but is probably not identical to the La protein (Ochs et al., 2002). We can only speculate that this 47 kDa protein may be identical to the ‘major mRNA-associated protein p50’ of Evdokimova & Ovchinnikov (1999). When these dinucleotide residues were not deleted, but mutated (from AC to GC in mutant up-CG or from GA to AU in mutant down-AU), only residual IRES activity and binding of eIF4F and eIF4B could be detected (Fig. 2d, lanes 5 and 6).

As expected from the absolute conservation of these dinucleotide residues among the cardio- and aphthoviruses in the primary sequence, as well as from their characteristic exposure from an otherwise dsRNA stem at the secondary structure level, these unpaired residues appear to be essential for binding eIF4G which, in turn, is crucial for IRES activity (López de Quinto & Martínez-Salas, 2000; Saleh et al., 2001).

Mutations in the 4 bp stack abolish binding of eIF4G and IRES activity

In the same way, we analysed the effect of mutations in the lower 4 bp stack of the discontinuous, conserved sequence element. This 4 bp stack is expected to contribute largely to the stability of the stem of the FMDV IRES subdomain 4-1. We introduced sequence changes separately in either (i) the left (upstream) part of the primary sequence (mutant up-4) (Fig. 3a), which disrupt the predicted structure of
the wild-type (wt) sequence (data not shown), even if they still allow formation of weak base pairs with the opposite strand, or (ii) in the right (downstream) part (mutant down-4), which also disrupts the secondary structure. Both changes completely abolish in vivo translation, both in BHK cells (Fig. 3b) and in RRL (Fig. 3c, lanes 2 and 3), as well as binding of eIF4G to the IRES (Fig. 3d, lanes 2 and 3). Surprisingly, even a compensatory mutant (up/down-4) in which the primary sequences of the two sides of the stack were altered to restore stable base-pairing in the dsRNA could rescue neither IRES activity (Fig. 3b, c, lane 4) nor binding of elf4G and elf4B (Fig. 3d, lane 4). Not only is base-pairing evidently important in this lower part of the conserved element, but both the integrity of the original dsRNA secondary structure and the primary sequence are crucial for eIF4G binding and IRES activity.

**Sequence and structure requirements in the upper 2 bp stack of the conserved element**

When primary sequence changes were introduced into the upper base stack by mutating either the left or the right part of the 2 bp (mutants up-2 or down-2, respectively) (Fig. 4a), thereby disrupting the RNA secondary structure, we found that IRES activity was reduced to levels of about 10% compared to the wild-type (Fig. 4b, c, lanes 2 and 3). Binding of elf4G was also consistently reduced to levels below the detection limit (Fig. 4e, lanes 2 and 3). However, when both primary sequence mutations were combined to yield a compensatory mutant (up/down-2) in which only the primary sequences were mutated, but the secondary structure was restored, we found, surprisingly, that IRES

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**Fig. 2.** Effect of mutations in the unpaired dinucleotide stretches of the conserved element. (a) In mutants up-del and down-del, either the unpaired dinucleotide sequence in the upstream (left) part or in the downstream (right) part of the conserved element, respectively, were deleted; in mutant up/down-del, both were deleted in combination. In mutants up-CG and down-AU, the unpaired sequences AC or GA were altered as indicated. (b) In vivo translation efficiencies of the wt and mutant FMDV IRES elements in BHK cells. Luciferase readings were standardized according to the activity of the wt IRES. (c) In vitro translation directed by the IRES elements in RRL in the presence of [35S]methionine. Samples were separated on 12% SDS-PAGE gels and exposed to X-ray film. Molecular masses of 14C-labelled marker proteins (M) are indicated on the left and the luciferase protein (Luc) on the right. Densitometric scan values of the luciferase bands are shown below the gel (as percentages of luciferase expression driven by the wt IRES). (d) UV cross-linking of [α-32P]UTP-labelled IRES elements in RRL. Samples were separated on 12% SDS-PAGE gels and exposed to X-ray film. (e, f) Identification of elf4G: (e) UV cross-linking assay with normal RRL (lane 1) or RRL pre-incubated with an RNA that expressed the FMDV L-protease for the times indicated (min L-protease, lanes 2–4). In lane 1, no L-protease RNA was added. This RRL was then used for UV cross-linking reactions with [α-32P]UTP-labelled FMDV wt IRES RNA. Half of each sample was applied to an 8% SDS-PAGE gel and analysed by autoradiography. elf4Gc is the C-terminal fragment of elf4G. (f) Western blot: 50% of the same samples that were loaded in lanes 1–4 in (e) were separated on an 8% SDS-PAGE gel, proteins were transferred to nitrocellulose and elf4G was immunostained by using an antiserum that was elicited against C-terminal sequences of elf4G (Saleh et al., 2001).
activity was partially restored both in BHK cells (Fig. 4b) and in vitro (Fig. 4c, lane 4). Accordingly, eIF4G binding was also partially restored (Fig. 4e, lane 4). Thus, maintenance of the secondary structure of the dsRNA is important, whereas the primary sequence in this part of the conserved element is not absolutely essential for eIF4G binding and IRES activity, but confers a considerable advantage to IRES activity.

To confirm that the loss of detection of eIF4G in the UV cross-linking assay with the above IRES mutants is indeed due to loss of binding, we performed competition reactions (Fig. 4f). The unlabelled, wt competitor IRES competed very well with binding of eIF4G to the radiolabelled IRES RNA (Fig. 4f, lanes 2–4). In contrast, even a 64-fold excess of the up-2 or down-2 mutant competitor RNAs (lanes 5–12) did not compete with binding of eIF4G to the wt IRES, whereas the compensatory up/down-2 mutant competed with intermediate efficiency (lanes 13–16), indicating that the loss of eIF4G detection is indeed caused by loss of binding to the mutant IRES, rather than by impaired label transfer from RNA to protein by UV cross-linking.

We then used another set of mutants to further analyse the influence of primary sequence and secondary structure alterations in the 2 bp stack on eIF4G binding and IRES activity. In mutant up-2AU, the upstream CC sequence was now altered to AU. Correspondingly, the downstream GG sequence was mutated to AU in mutant down-2AU, and both mutations were performed in combination in the compensatory mutant up/down-2AU (Fig. 4a). Mutant down-2AU showed greatly reduced IRES activity in vivo and in vitro (Fig. 4b, c, lane 6) and also showed markedly reduced eIF4G binding (Fig. 4e, lane 6). In contrast, to our surprise, mutant up-2AU, as well as the compensatory mutant up/down-2AU, was quite active in translation (Fig. 4b, c, lanes 5 and 7) and in eIF4G binding (Fig. 4e, lanes 5 and 7). However, taking the possible formation of standard cis-Watson–Crick/Watson–Crick A–G and U–G base pairs into account (Leontis et al., 2002), as illustrated in Fig. 4d, these results are in accordance with the idea that the intact secondary structure of the RNA double strand is important, whereas the primary sequence is not. In mutant up-2AU, an A–G and a U–G base pair (both of which involve two normal hydrogen bonds plus a bridging water; Leontis et al., 2002) may allow retention of the dsRNA secondary structure (Fig. 4d), whereas in mutant down-2AU, the weak A–C interaction (involving only two normal hydrogen bonds) in combination with the weak U–C interaction (involving only one normal hydrogen bond and a bridging water; Leontis et al., 2002) may not be sufficient to allow stable helix formation in the apical part of the IRES subdomain 4-1.

The relationship between the integrity of the RNA secondary structure in the upper part of the conserved element and IRES activity was also evident when eIF4G interaction and IRES activity were investigated with the up-2 and down-2 mutants at different potassium concentrations (Fig. 5). With the wt IRES, eIF4G binding decreased only slightly when the potassium concentration was increased from 50 to 150 mM (Fig. 5a, lanes 1–5), but wt IRES activity was
optimal at 100 and 125 mM (Fig. 5b, lanes 1–5). This enhanced activity is due to the stimulation of ribosome association by potassium ions, reflecting adaptation of the FMDV IRES activity to the high intracellular potassium concentration, whereas the decrease of translation activity at higher KCl concentrations is due to chloride anions (Weber et al., 1977; Niepmann, 2003).

However, with mutant up-2, very low expression was obtained at 75 mM K⁺, which decreased markedly at 100 mM K⁺ (Fig. 5b, lanes 6–8), whereas mutant down-2 showed slightly higher expression, with a maximum at 100 mM K⁺ (lanes 11–14). Also, binding of eIF4G was slightly better with mutant down-2 (Fig. 5a, lane 11) than with mutant up-2 (lane 6) at low potassium concentrations. These observations are consistent with the fact that the two stacks of opposing G residues in mutant up-2 are unable to form cis-Watson–Crick/Watson–Crick base pairs, while the two stacks of opposing C residues in mutant down-2 may form weak cis-Watson–Crick base pairs (Leontis et al., 2002).

To check the activities of the IRES mutants in relation to the expression of a control gene, we cloned selected IRES mutants into a dicistronic mRNA system with CAT as the first gene and firefly luciferase as the second gene, which is under IRES control (Niepmann et al., 1997). Expression results after transfection in BHK cells (Fig. 6a) and in vitro (Fig. 6b) with the mutations in the 4 bp stack and the 2 bp stack confirmed the results obtained above.

In conclusion, these results show clearly that the integrity of the dsRNA secondary structure in the upper part of the conserved sequence element is the main contributor to eIF4G binding and IRES activity, whereas the primary sequence in this part of the conserved element confers only a minor advantage to IRES function.

Functional IRES defects cannot be relieved by increased eIF4F concentrations

In order to analyse whether increased initiation factor concentrations could compensate for IRES defects, we performed translation reactions with the wt FMDV IRES and a selected set of IRES mutants that affected both the 4 bp and 2 bp stacks (up-4, up-2 and up/down-2). To these reactions, we added increasing amounts of purified eIF4F, the cap-binding protein complex that contains eIF4G as its major component, as present in the living cell (Fig. 7a). eIF4F was freshly purified before the experiments. On
addition of eIF4F, activity of the wt IRES increased by 40%, according to a shift in the chemical equilibrium caused by the increased eIF4F concentration. However, concentrations of up to 40 nM eIF4F (0-4 pmol eIF4F in a 10 µl reaction) could not relieve the disadvantage of the IRES mutations.

Consistently with the stimulation of wt IRES activity by the eIF4F preparation, the intensity of eIF4G binding to the wt FMDV IRES in the UV cross-linking assay was increased significantly (Fig. 7b, lanes 1–3), as well as the binding of eIF4B. In contrast, no binding of eIF4G to the largely inactive IRES mutants up-4 (lanes 4–6) and up-2 (lanes 7–9) could be detected, whilst a faint eIF4G band was detected with the compensatory mutant up/down-2 (lanes 10–12), corresponding to the intermediate activity of this mutant.

In conclusion, these functional assays demonstrate that disturbances in the structure of this conserved element in the IRES affect eIF4G binding in a way that cannot be overcome by increased eIF4G concentrations.

**DISCUSSION**

The IRES elements of picornaviruses are highly structured, cis-acting RNA regions that provide recognition sites for components of the translational machinery. A key component of the translational apparatus is the large, multidomain, translational adaptor protein eIF4G (Scheper et al., 1992; Pestova et al., 1996b; Hentze, 1997; Prevot et al., 2003). eIF4G directly contacts both the picornavirus RNA (Kolupaeva et al., 1998) and the ribosome-bound eIF3 (Lamphear et al., 1995; Korneeva et al., 2000), providing the bridge between the virus RNA on one side and the small ribosomal subunit where the RNA enters on the other. Binding of eIF4G to the viral IRES is supported by the synergistic binding of eIF4A and eIF4B (Meyer et al., 1995; Kolupaeva et al., 1998).

Among the type II IRES elements of members of the cardio-/aphthovirus group in the picornavirus family, the Y-shaped domain 4 (Fig. 1) in the IRES 3' region carries the determinants for recruitment of eIF4G to the virus RNA. In previous studies, either after optimization of the spurious detection of eIF4G in the UV cross-linking assay or by using purified initiation factors, eIF4G was found to contact the type II IRES elements (FMDV or EMCV, respectively) at many different sites. There are many contacts in the base of domain 4 (Kolupaeva et al., 1998, 2003; López de Quinto & Martínez-Salas, 2000; Pilipenko et al., 2000). Detailed mutagenesis of the lower stem of domain 4 revealed that the secondary structure, rather than the primary sequence, appears to be important for FMDV IRES activity, whereas in the first small, unpaired bulge at the base of domain 4, the primary sequence is important (López de Quinto & Martínez-Salas, 2000). Virtually all nucleotides of the unpaired, A-rich bulge (subdomain 4-3 in Fig. 1) are contacted by eIF4G (Pilipenko et al., 2000; Kolupaeva et al., 2003), an observation that is consistent with the extreme sensitivity of this bulge to changes in the number of A residues (Kaminski & Jackson, 1998). Other contact sites are in the base of subdomain 4-2 (named K in EMCV) (Kolupaeva et al., 2003). Also, the lower stem and apical loop of domain 5 appear to be involved in the contacts of eIF4G with the type II IRES (Pilipenko et al., 2000; Saleh et al., 2001; Kolupaeva et al., 2003), although another study found that eIF4G also bound well to the FMDV CS8 strain IRES in the absence of domain 5 (López de Quinto & Martínez-Salas, 2000).

Several contacts for eIF4G were also detected in the stem of subdomain 4-1 (named J in EMCV; Kolupaeva et al., 2003).
In this subdomain, a characteristic, conserved, discontinuous sequence element is present (Jackson & Kaminski, 1995) (Fig. 1). The absolute conservation of both secondary structure and primary sequence within this sequence element among all members of the cardio-/aphthovirus group argues strongly for an essential function of this element. We and others have identified subdomain 4-1 to be essential for binding of eIF4G (Saleh et al., 2001; Stassinopoulos & Belsham, 2001). Interestingly, the unpaired dinucleotides are contacted directly by the eIF4G protein. In structure-probing experiments (Pilipenko et al., 2000; Kolupaeva et al., 2003), eIF4G protects the unpaired A residues from dimethyl sulphate or RNase T1 treatment and eIF4G protects the 4 bp stack between the unpaired residues from hydroxyl radical treatment (Kolupaeva et al., 2003), indicating that this conserved element within subdomain 4-1 is an essential determinant for direct protein–RNA contacts between eIF4G and the IRES RNA.

Here, we show that this conserved sequence is essential for recruitment of eIF4G to the FMDV IRES, as well as for IRES activity. Several mutations in this element abrogate eIF4G binding and IRES activity. Consistently, additional eIF4F in the translation system cannot restore full IRES activity of the mutants, although additional eIF4F stimulates wt IRES activity. In particular, mutations in two unpaired dinucleotide stretches almost completely abolish eIF4G binding and IRES activity. Consistently, additional eIF4F in the translation system cannot restore full IRES activity of the mutants, although additional eIF4F stimulates wt IRES activity. In particular, mutations in two unpaired dinucleotide stretches almost completely abolish eIF4G binding and IRES activity.

**Fig. 6.** Translation of selected IRES mutants in a dicistronic mRNA context. mRNAs with the CAT gene as the first cistron, followed by the FMDV IRES and the firefly luciferase as the second cistron, were translated *in vivo* (a) and *in vitro* (b) as described for Fig. 2. Values show luciferase expression as a percentage of CAT expression.

**Fig. 7.** Effects of purified eIF4F on selected FMDV IRES mutants. (a) Translation directed by 0-25 pmol wt and mutant FMDV IRES elements in RRL, supplemented either with buffer only (containing no additional eIF4F) or in the presence of increasing amounts of purified eIF4F as indicated. (b) UV cross-linking assays with [α-32P]UTP-labelled FMDV IRES wt or mutant RNAs. Reactions were supplemented with eIF4F as indicated at the top.
stretches affects IRES activity, particularly mutations of those residues that are contacted by eIF4G; the A in the upstream unpaired dinucleotide stretch (Kolupaeva et al., 2003) and both nucleotides of the downstream dinucleotide stretch (Kolupaeva et al., 1998, 2003; Pilipenko et al., 2000). Together with these studies on the EMCV IRES, our results obtained with the FMDV IRES highlight the evolutionary importance of the conserved element for eIF4G binding and IRES function among the type II IRES elements of the cardio- and aphthovirus group of picornaviruses.

Mutations in a 4 bp stack almost completely abolish eIF4G binding and IRES activity, even if only the primary sequence is affected, but the dsRNA secondary structure is maintained in the compensatory mutant up/down-4 and, with weakened base-pairing, in mutant up-4. This result is consistent with the finding that eIF4G contacts the upstream AGGU sequence of the 4 bp stack in the conserved sequence element (Kolupaeva et al., 2003), pointing to an absolute primary sequence requirement that is probably conferred by the need for direct recognition of particular bases in this sequence by the eIF4G protein. In contrast, mutations in the 2 bp stack that affect the primary sequence, but not the secondary structure, impair, but do not completely abolish, IRES activity. Consistently, no direct contacts of eIF4G with specific bases of this 2 bp stack have been observed. In contrast to the lower 4 bp stack, in this 2 bp stack, IRES activity and eIF4G binding correlate preferentially with the stability of base-pairing (Leontis et al., 2002), supporting the hypothesis that exposure of the unpaired upstream AC dinucleotide stretch from the RNA double strand may be the main task of this dsRNA stem region. Nevertheless, the observation that changes in the primary sequence that keep the secondary structure in the 2 bp stack intact do not completely abolish eIF4G binding and IRES activity was a surprise, as the absolute primary sequence conservation of this sequence among type II IRES suggested that not only the RNA structure, but also the exact sequence of this region, was important. We note that, in this case, a certain preference for a distinct sequence has resulted in conservation of the primary sequence among this subgroup of the picornaviruses during virus evolution.

Taken together, the binding site for eIF4G in the picornavirus type II IRES is composed of several essential determinants that cover almost the entire domain 4 (named J–K in EMCV). Although the exact tertiary structure of the IRES domain 4 is not yet known, these determinants appear to act synergistically in the binding of eIF4G, as mutations in any of these determinants seriously affect both eIF4G binding and IRES activity. Moreover, the cellular proteins that assemble on the IRES also bind by synergistic interactions. First, several interaction sites within the large eIF4G protein are involved in contacting the virus IRES (Kolupaeva et al., 2003). Second, the initiation factors eIF4F, eIF4A and eIF4B form a multiprotein complex that requires synergistic involvement of all three components. Binding of eIF4B to the IRES is ATP-dependent (Meyer et al., 1995), although only eIF4A is an RNA helicase that works ATP-dependently (Rozen et al., 1990). This RNA helicase component of the multiprotein complex that forms on the virus RNA may introduce changes in secondary structure (Kolupaeva et al., 2003), which perhaps contribute to the translation initiation process. When the initiation factors are added separately to the IRES RNA, they bind only weakly to the IRES and only their synergistic interaction results in stronger binding of the entire eIF4 complex to the virus RNA (Kolupaeva et al., 1998). Consistently with this, we found here that binding of eIF4B is affected in parallel with binding of eIF4G.

In addition, so-called RNA chaperones, such as PTB, may augment the interaction of initiation factors with the IRES, although it is not known how they act at the molecular level. PTB stimulates the activity of the FMDV IRES (Niepmann, 1996; Niepmann et al., 1997) and the EMCV IRES (Kaminski et al., 1995; Kaminski & Jackson, 1998) and the RNA determinants that are contacted by PTB appear to be interspersed between the sites that are contacted by the standard factors. Accordingly, it is not surprising that the mutations in the eIF4G-binding site that most seriously affect the assembly of the complex of initiation factors on the virus RNA may, in turn, also affect binding of PTB, such as deletion of the unpaired dinucleotide stretches (Fig. 2d, lanes 2–4) and mutations in the 4 bp stack (Fig. 3d, lanes 2–4). Nevertheless, among all RNA determinants that provide contact sites for proteins, the highly conserved sequence element in subdomain 4-1 is involved directly in recruitment of eIF4G to the picornavirus RNA during the initial steps of translation of the virus genome.

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