In vivo footprint of a picornavirus internal ribosome entry site reveals differences in accessibility to specific RNA structural elements

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Internal ribosome entry site (IRES) elements were described in picornaviruses as an essential region of the viral RNA. Understanding of IRES function requires a detailed knowledge of each step involved in the internal initiation process, from RNA folding and IRES–protein interaction to ribosome recruitment. Thus, deciphering IRES accessibility to external agents due to RNA structural features, as well as RNA–protein protection within living cells, is of primary importance. In this study, two chemical reagents, dimethylsulfate (DMS) and aminomethylpsoralen, have been used to footprint the entire IRES of foot-and-mouth disease virus (FMDV) in living cells; these reagents enter the cell membrane and interact with nucleic acids in a structure-dependent manner. For FMDV, as in other picornaviruses, viral infection is dependent on the correct function of the IRES; therefore, the IRES region itself constitutes a useful target of antiviral drugs. Here, the in vivo footprint of a picornavirus IRES element in the context of a biologically active mRNA is shown for the first time. The accessibility of unpaired adenosine and cytosine nucleotides in the entire FMDV IRES was first obtained in vitro by DMS probing; subsequently, this information was used to interpret the footprint data obtained in vivo for the mRNA encompassing the IRES element in the intercistronic space. The results of DMS accessibility and UV–psoralen cross-linking studies in the competitive cellular environment provided evidence for differences in RNA structure from data obtained in vitro, and provided essential information to identify appropriate targets within the FMDV IRES aimed at combating this important pathogen.

INTRODUCTION

In mammalian cells, translation initiation of certain mRNAs can occur internally (Hellen & Sarnow, 2001). The process of internal initiation of translation in eukaryotic cells is dependent on a cis-acting element termed the internal ribosome entry site (IRES), which is usually located in the 5′ untranslated region of mRNAs. IRES elements were originally found in RNAs of picornaviruses (Sarnow, 2003), a virus family that includes important animal and human pathogens; one of its members is foot-and-mouth disease virus (FMDV). It is well established that the IRES element is a regulatory region that is absolutely required for successful picornavirus infection (Pilipenko et al., 1992). More recently, IRES elements have also been found in a large number of other viral and cellular mRNAs translated during conditions of cap-dependent inhibition (Baird et al., 2006; Stoneley & Willis, 2004). Therefore, efforts to understand IRES biology are of primary importance for increasing our knowledge of translational control in eukaryotes.

Determination of the RNA structure of several viral IRES elements by using in vitro approaches has been a major challenge in recent years (Costantino & Kieft, 2005; Jan, 2006; Martinez-Salas & Fernandez-Miragall, 2004; Pfingsten et al., 2006; Schuler et al., 2006). Structural analysis has revealed that IRES elements of picornaviruses, as well as that of hepatitis C virus (a flavivirus), are organized in complex RNA structures that contain stable stem–loops (Du et al., 2004; Fernandez-Miragall & Martinez-Salas, 2003; Kieft et al., 2002; Lukavsky et al., 2000; Phelan et al., 2004). Different stem–loops appear to perform specific roles during internal initiation, such that each of them is necessary, but not sufficient, to achieve efficient initiation of translation.

The basis of RNA structure-mediated mechanisms involved in ribosome recruitment during internal initiation is still poorly understood. Distantly related IRES elements do not share primary sequence and they appear to be organized in different RNA structures (Bonnal et al., 2003; Costantino & Kieft, 2005; Jan & Sarnow, 2002; Le Quesne et al., 2001; Piron et al., 2005; Serrano et al., 2007; Terenin et al., 2005). On the other hand, IRES elements present in closely related viral genomes might differ in their structural organization and/or trans-acting factor requirements (Pisarev et al., 2004). Thus, the definition of the structural requirements for IRES activity is so far unresolved.

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In addition to the RNA structure itself, different conserved motifs in the picornavirus IRES have been found to interact specifically with some translation initiation factors (eIFs) in reconstituted initiation complexes (Kolupaeva et al., 1998; Pilipenko et al., 2000). Furthermore, according to functional analysis, some of the eIFs are IRES-binding proteins essential for IRES activity (Clark et al., 2003; Lopez de Quinto & Martinez-Salas, 2000; Lopez de Quinto et al., 2001). Therefore, it is conceivable that, in the competitive environment of the cytoplasm, IRES function depends on the availability and coordinated interaction of eIFs and other IRES trans-acting factors (ITAFs) with the IRES RNA. These functional interactions may be compromised due to their preferential use in cap-dependent initiation, as well as in other processes related to RNA biology (Martinez-Salas et al., 2001; Sarnow, 2003).

Despite multiple efforts to understand IRES biology, information on the organization of IRES elements in living cells is still lacking. With the aim of filling this gap, we have sought to define the structural organization of a complex picornavirus IRES in vivo, when the IRES element is placed in the context of a functional mRNA active in translation. To this end, we have taken advantage of reagents that are permeable to the cellular membrane and recognize RNA molecules in a structure-dependent manner. Dimethylsulfate (DMS) enters living cells and reacts with RNA and DNA, providing information about the accessibility of individual residues in vivo (Mereau et al., 1997; Zaag & Cech, 1995). Psoralens are also membrane-permeable (Komura et al., 2001) but, in contrast to DMS, photoreact covalently with paired residues in nucleic acids to form interstrand cross-links (Kramer et al., 1999). Nucleic acids are highly specific targets of psoralens, and RNA–protein complexes are also accessible to this reagent (Wassarman & Steitz, 1992; Wellingher et al., 1999), allowing the study of ribonucleoprotein complexes in the natural context.

Here, we report complementary sets of data instrumental to understanding the organization in living cells of IRES ribonucleoprotein complexes in the context of a 3 kb bicistronic mRNA undergoing cap-independent translation. The pattern of in vivo DMS footprints revealed that a large proportion of the vulnerable residues were located in the central domain and, therefore, define accessible structural motifs. A diminished reactivity of IRES residues was in part consistent with IRES–protein interaction mapped previously in vitro, including several eIFs and auxiliary proteins. Notably, a reorganization of the FMDV IRES RNA structure of the central domain in vivo, compared with naked RNA, was inferred from UV–psoralen cross-linking. These results may be relevant to the development of efficient antiviral therapies aimed at inactivating this important pathogen.

**METHODS**

**Constructs and transcripts.** Plasmids pBIC, encompassing the FMDV IRES within a bicistronic RNA of the form chloramphenicol acetyltransferase (CAT)–IRES–luciferase, and pGEM-IRES, encoding the IRES region exclusively, have been described previously (Martinez-Salas et al., 1993; Ramos & Martinez-Salas, 1999). Prior to RNA synthesis, plasmid pGEM-IRES, encoding the full-length IRES, was linearized by using XhoI. Uncapped RNAs were produced in vitro by transcription from linearized templates, using a MEGAscript kit (Ambion) as recommended by the manufacturer.

**DMS treatment of transfected cells.** BHK-21 cells grown in 100 mm dishes were transfected with the bicistronic plasmid pBIC (9 μg DNA per dish) by using the T7 RNA polymerase expression system as described previously (Martinez-Salas et al., 1993). Mock-transfected cells were used as negative controls. To follow transfection efficiency, relative IRES activity was quantified as the expression of luciferase normalized to that of CAT. Measurement of CAT and luciferase protein accumulation indicated that a plateau was reached between 16 and 20 h post-transfection.

For DMS treatment, cells were washed in cold PBS at 20 h post-transfection, scraped into PBS (2 ml), pelleted and resuspended in PBS (100 μl). Then, DMS (1 μl) was added to the cell suspension, which was incubated for 2 min at room temperature with gentle shaking. Previously, optimal DMS concentration and time of cell treatment were determined by using a 10-fold DMS concentration range in parallel wells, according to described procedures (Antal et al., 2000; Inoue & Cech, 1985). The reaction was stopped by addition of β-mercaptoethanol (10 μl) prior to increasing the volume to 200 μl with PBS and extraction of total RNA by using Tripure reagent (Lopez de Quinto et al., 2002). As a control of untreated cells, an aliquot of pBIC-transfected cells was processed in the same way, except that no DMS was added.

**UV–psoralen cross-linking of transfected cells.** BHK-21 cells, transfected with plasmid pBIC as described above, were incubated with aminomethylpsoralen (AMT-psoralen; Sigma) (4 μg ml⁻¹), followed by UV irradiation (365 nm) for 30 min at 4 °C, rotating the cell suspension every 10 min (Hartshorne, 1998; Tyc & Steitz, 1992). The cell-suspension volume was increased to 200 μl with PBS and processed for total RNA extraction as described above. RNA concentration was determined by measurement of A₂₆₀.

**Primer-extension analysis.** Sites of DMS modification or UV–psoralen cross-linking were mapped by inhibition of reverse transcriptase (RT) elongation as described below, in triplicate experiments using total RNA prepared from transfected cells. DMS modifies unpaired A, C and G bases; however, only modified A and C bases halt the RT reaction at the base preceding the methylated residue (Mereau et al., 1997; Wells et al., 2000). Occasionally, pausing of the RT reaction before a G base is observed (Forstemann & Lingner, 2005; Pickering et al., 2004). In order to scan the total length of the FMDV IRES, four specific 5’ end-labelled antisense primers (termed a, b, c and d) were used to obtain overlapping RNA-mapping results. Primer a, 5’-CTTGTCCGCAAGGGAGGTCTC-3’, complementary to nt 185–165 of the IRES, was used to map part of domain 1 and the entire domain 1–2. Primer b, 5’-CCCGGGTGGGTGGTACCC-3’, complementary to nt 296–287, was used to detect RT stops in the central domain; primer c, 5’-CATTAAATGCTAAGGGATCCT-3’, complementary to nt 431–457, was used to analyse domain 4 and part of 3; and primer d, 5’-GCGCTTTTCTTATTGTTGGCGC-3’, priming downstream of the IRES sequence, was used to detect RT stops in domains 5 and 4.

The optimal amount of total RNA for primer-extension analysis was determined by using different RNA concentrations prepared from transfected cells; RNA prepared from mock-transfected cells was used as a negative control. For primer extension, total RNA (about 30 μg) was denatured for 3 min at 95 °C. Then, annealing and extension of the labelled antisense primer were carried out in 15 μl RT buffer
[20 mM Tris/HCl (pH 7.5), 15 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.01% (v/v) NP-40, 50% (v/v) glycerol] in the presence of 100 U SuperScript II RT (Invitrogen) and 1 mM each dNTP for 1 h at 45°C. Newly synthesized cDNA products were analysed in denaturing 6% acrylamide, 7 M urea gels (Fernandez-Miragall et al., 2006). In all cases, formation of full-length product that remained at the top of the gel was verified, irrespective of whether total RNA was prepared from treated or untreated cells.

Primer-extension reactions, using as template total RNA prepared from transfected cells, were performed strictly under the same conditions as RNA subjected to in vitro probing. In all cases, a sequence ladder, prepared by using a ThermoSequenase cycle sequencing kit (Amersham Biosciences) and the same 5’-labelled antisense oligonucleotide used for primer extension, was loaded in parallel to identify the RT extension products. Denaturing gels, 43 cm long, were allowed to run for different lengths of time to facilitate nucleotide identification. The modified residue was identified as the base preceding the RT stop by one position; the curvature of the gel was considered in a few cases to assign the position of the modified base.

**In vitro RNA structure probing.** For chemical probing, RNA (about 1.5 µg) was treated with DMS (Fluka) (1 µl DMS freshly diluted 1:5 in ethanol; 15 min at 20°C) using native conditions (buffer N; 50 mM sodium cacodylate (pH 7.5), 300 mM KCl, 10 mM MgCl₂), as described previously (Brunel & Romby, 2000; Fernandez-Miragall & Martinez-Salas, 2003) and then analysed by primer extension as described above.

**RESULTS**

**DMS probing of the entire FMDV IRES in vitro**

In this report, we have conducted studies to determine the structural organization of the FMDV IRES by combining DMS footprinting and UV–psoralen cross-linking. The FMDV IRES spans an internal region of the viral genome, which is organized in domains termed 2–5 (or H–L) in the 5’→3’ direction (Fig. 1a). These domains encompass different stem–loops according to partial RNA probing analysis and computer prediction programs (Fernandez-Miragall & Martinez-Salas, 2003; Pilipenko et al., 1989). However, the secondary structure of the complete FMDV IRES RNA is still unknown.

Therefore, RNA probing of the entire IRES was a requisite to interpret the results obtained by using mRNA complexes

Fig. 1. Chemical probing of the entire FMDV IRES. (a) Schematic drawing of the FMDV genome. The black box (enlarged below) represents the IRES region that directs translation of the single viral open reading frame. IRES nucleotide numbering, domain nomenclature and approximate position of primers used for RNA structure analysis are indicated. (b) A transcript encompassing the entire IRES sequence was incubated in vitro with or without DMS, as indicated in Methods, and then subjected to primer-extension analysis using three different 5’ end-labelled primers, a, b and c. cDNA products were analysed on denaturing 6% acrylamide gels in parallel with a sequence ladder obtained with the same labelled primer (lanes AGCU). The FMDV IRES sequence, every 10 bases apart, is indicated on the left side of each autoradiogram. The nucleotide marked on the right corresponds to the modified residue. The autoradiograms show examples of different regions of the IRES.
undergoing IRES-dependent translation in living cells. To fill this gap, a transcript encompassing the entire IRES was subjected to DMS chemical probing in vitro, followed by primer-extension analysis with three different antisense primers (termed a, b and c; Fig. 1a). Subsequent fractionation of the RT elongation products directed by the different 5' end-labelled primers provided information on the bases modified in overlapping regions of the entire IRES transcript. This approach has allowed the detection of specific DMS attacks on nucleotides of the IRES transcript, undetected in the non-treated RNA [examples of three different primer-extension assays are shown in Fig. 1(b)]. Primer-extension stops also observed in the untreated RNA were not taken in consideration, as they were presumably caused by compact RNA structure. The results obtained for the three different primers in duplicate experiments are shown in Fig. 2. A monocistronic IRES construct exhibited the same DMS pattern as the bicistronic RNA, strongly suggesting a similar folding of the IRES element, regardless of the upstream sequences in the bicistronic RNA. Consistent with these observations, the FMDV IRES is located in the viral RNA downstream of a heavily structured region (Serrano et al., 2006). Additionally, the FMDV IRES efficiently drives internal initiation of translation in mono- or bicistronic vectors (Reigadas et al., 2005).

According to the results obtained in this study, the RNA structure of the proximal region of domain 2, as well as specific residues in domain 4, have been modified relative to previous reports (Martinez-Salas & Fernandez-Miragall, 2004; Pilipenko et al., 1989) to accommodate DMS reactivity. Results obtained for domain 3 were in accordance with previously published data (Fernandez-Miragall et al., 2006).

**In vivo DMS footprint of the FMDV IRES reveals a specific accessibility pattern**

Subsequently, the organization of ribonucleoprotein complexes involved in IRES-dependent translation was investigated in BHK-21 cells transfected with a plasmid that produces a 3200 nt bicistronic RNA encompassing the FMDV IRES in the intercistronic space (Fig. 3a). The expression of reporter genes CAT (cap-dependent) and luciferase (IRES-dependent) indicated that IRES activity reached a plateau about 16–20 h after transfection (data not shown). Therefore, to investigate the FMDV IRES organization in translationally competent complexes, cells were treated with DMS 20 h post-transfection, coincident with the plateau of IRES-driven protein accumulation. Total cytoplasmic RNA was then isolated and subsequently used for primer-extension analysis with RT and four
specific primers (a, b, c and d) that allow scanning of the accessibility towards DMS of the entire IRES (Fig. 3a).

Comparison of the IRES primer-extension pattern obtained with total RNA prepared from DMS-treated cells indicated that different domains of the IRES showed distinct accessibility patterns. Primer-extension analysis carried out with primer b detected attacks to 12 residues in the apical region of the central domain, encompassing C141–C237 (filled arrows in Fig. 3(b), compare lanes 1 and 2). Attacks to residues 197–213 of the IRES were observed clearly in vivo, suggesting that the apical region of the central domain folds as a DMS-accessible structure (Fig. 4). We also noted a primer-extension pausing preceding base G178; however, as G is not expected to halt the RT reaction after DMS modification, the interpretation of this result is unclear. In contrast to the results obtained in vitro by using naked RNA, C237 was accessible to DMS attack, but no attacks were detected at the other bases of the C-rich loop.

Analysis of DMS accessibility in vivo carried out with 5’ end-labelled primer c revealed a large number of protected bases in a region spanning nucleotides C124–A399 within domain 4 (or J–K) [Fig. 3(c), lane 4; compare with Fig. 1(b), lane 6]. None of these protections were detected during in vitro probing using the same primer. The primer-extension stops that were detected at residues U395–A402 in both treated and untreated cells presumably arose as a consequence of the RNA structure. The IRES region immediately upstream of the translation initiation codon was analysed with primer d [Fig. 3(d), compare lanes 5 and 6]. A distinct pattern was observed in this region, with four bases accessible to DMS modification followed by an unreactive region, and then by six close DMS-sensitive nucleotides encompassing A448–A455 in the IRES 3’-end region.

The DMS footprints observed in vivo, summarized in Fig. 4, indicated that most of the attacks (78%) affected bases located in bulges throughout the entire IRES, validating the RNA structure mapped in vitro (compare with Fig. 2). However, attacks and protections were not distributed uniformly. Of the 28 DMS attacks observed, 14 were present in domain 3 and 12 in domain 5. Conversely, protections were located mainly in domain 4.

Significant differences were observed between in vivo and in vitro DMS accessibility. The region spanning the apical region of domain 3 was readily accessible to DMS modification in vivo and in vitro (compare Figs 2 and 4).
However, six attacks only observed in vivo affected bases that are presumably engaged in base-pair formation in vitro (thin arrows in Fig. 4). This result was indicative of a distinct RNA accessibility in the cellular environment.

A decrease in the in vivo DMS reactivity towards the C-rich loop was noticed. The unreactive bases located in bulges in the secondary structure generated in vitro suggested that the lack of reactivity observed in vivo could be due to the formation of RNA–protein complexes in most of the RNA molecules at the time of DMS treatment. In addition, residues in domain 5, comprising the right arm of the hairpin, and the stem–loop that constitutes domain 2 were not attacked by DMS in vivo. This result was consistent with their helical organization determined in vitro (Fig. 2).

**In vivo UV–psoralen cross-linking of the IRES indicates a local reorganization of the central domain**

The modification of the reactivity towards DMS in the central region of the IRES was of particular interest because the role performed by this structural element is still unknown. We therefore looked for independent approaches, capable of being applied to living cells, to confirm evidence of RNA structure organization in the cytoplasm. The photoreactive reagent AMT-psoralen reacts covalently with paired bases in RNA molecules, giving rise to interstrand cross-links (Wassarman & Steitz, 1992). Thus, we have made use of UV–psoralen cross-linking to verify the presence of paired nucleotides in the FMDV IRES structure that were not observed by using DMS reactivity. First, as described above for RNA probing, UV–psoralen cross-linking assays were performed in vitro with naked RNA as described in Methods. As shown in Fig. 5(a), specific UV–psoralen-dependent primer-extension stops were detected readily within the central domain of the FMDV IRES. The cross-linked nucleotides corresponded to pyrimidines, as expected.

Next, we took advantage of the cell-membrane permeability of AMT-psoralen to analyse the organization of the central domain of the FMDV IRES in transfected cells undergoing translation. A comparison of the primer-extension pattern obtained with RNA prepared from UV–psoralen-treated and -untreated cells is shown in Fig. 5(b). A hot spot of adduct formation was detected between positions A₁₈₄ and C₂₂₀. Five of these bases, A₁₈₄, G₁₈₅, C₁₉₂, C₂₁₉ and C₂₂₀, were below or close to the level of detection in vitro. Conversely, UV–psoralen cross-linking at residues C₁₅₆, U₁₆₉ and C₁₇₀, as well as C₂₃₇, appeared more prominent in vitro than in vivo.

A summary of the IRES UV–psoralen cross-linking data obtained in vivo is shown in Fig. 6. With the exception of C₂₃₇, the UV–psoralen adducts mapped in vivo were located in stems defined by RNA probing, consistent with the formation of interstrand cross-links in the secondary structure. However, the UV cross-link detected at C₂₃₇ corresponds to a loop that may be indicative of monoadduct formation (Kramer et al., 1999) or of tertiary RNA–RNA interactions (Fernandez-Miragall et al., 2006).
To establish whether there was a difference in RNA structure detectable by the preferential UV–psoralen cross-linking pattern in vivo, the cross-links were compared with those obtained with the IRES treated in vitro (Fig. 6). The most significant changes affected bases C156, U169 and C170, which were psoralen cross-linked in vitro, but not in vivo. Two of these residues, U169 and C170, are located in a loop in the secondary-structure model. These results suggested a local reorganization of RNA structure that modifies the base pairing within the apical region of the central domain.

**DISCUSSION**

Viral IRES elements exploit a specific mechanism of translation initiation that bypasses the requirement of one, or more, eIFs. This unique process, which occurs concomitantly with the inactivation of host protein synthesis in infected cells (Lloyd, 2006; Martinez-Salas et al., 2001; Sarnow, 2003), is still poorly understood at the cellular level. Understanding of IRES function requires a detailed deciphering of each step involved in the process, from RNA folding and IRES–protein interaction to ribosome recruitment. Thus, deciphering the accessibility of IRES RNA to external agents due to RNA structural features, as well as RNA–protein protections within living cells, is of primary importance. In this study, we describe the accessibility towards two different reagents of the entire IRES of the picornavirus FMDV in living cells. For many RNA viruses, such as FMDV, infection is dependent on the correct function of the IRES; therefore, the IRES region itself constitutes a useful target of drugs aimed at combating this important pathogen (Sobrino et al., 2001). Thus, our data provide essential information for identification of appropriate targets within the FMDV IRES.

We have analysed the ribonucleoprotein complexes involved in IRES-dependent translation in the context of...
a 3200 nt bicistronic mRNA encompassing the FMDV IRES in the intercistronic space. This study was achieved by using two membrane-permeable reagents that interact with nucleic acids in a structure-dependent manner to footprint the FMDV IRES. Our results showed that different regions of the IRES showed distinct DMS accessibility patterns. A marked DMS accessibility was apparent in vivo in a region including the apical region of the central domain (termed 3 or I). The pattern of DMS modification found in the cytoplasm of living cells differed from that obtained in vitro. Whilst several bases between C197 and C213 were more reactive towards DMS, the nucleotides around the C-rich loop were less reactive. These results provided evidence of a difference between data obtained in vivo and in vitro, reinforcing the importance of defining IRES structural features in living cells.

The central domain is a self-folding region that contains the conserved GNRA motif and the C-rich motif, organized in loops in vitro (Fernandez-Miragall & Martinez-Salas, 2003). Mutational analysis of the GNRA motif has shown its essential contribution to IRES activity in both FMDV (Lopez de Quinto & Martinez-Salas, 1997) and encephalomyocarditis virus (Robertson et al., 1999). Nucleotide substitutions that disrupt the GNRA motif abrogate IRES activity, presumably because of the critical role of the GNRA tetraloop in determining the structural conformation of the apical region (Fernandez-Miragall & Martinez-Salas, 2003; Fernandez-Miragall et al., 2006). In agreement with the involvement of structural elements in the role of this region during internal initiation, a change in RNA structure that involved an enhancement in DMS attack of residues 197–213 was inferred from our analysis (compare Figs 2 and 4). In addition, six new DMS-reactive bases in this region specifically revealed in vivo-affected nucleotides that are involved in the formation of G: C base pairs in the secondary-structure model generated in vitro. This observation suggests that the IRES structure differs in vivo from that generated by using naked RNA.

A decrease in DMS attack on residues in the C-rich loop in vivo suggested a potential RNA–protein interaction. This conserved motif is a candidate to interact with poly(rC)-binding protein (PCBP), a protein that interacts with the equivalent motif in entrovirus IRES elements (Gamarnik et al., 2000; Walter et al., 1999) and was found to interact with the FMDV IRES in depletion assays carried out with partial IRES transcripts encompassing the central domain (Stassinopoulos & Belsham, 2001). Similarly, analysis of DMS accessibility of domain 4 revealed a large number of protected bases. This RNA-protection pattern suggested the formation of RNA–protein complexes in most of the RNA molecules present in transfected cells at the time of DMS treatment. Notably, and in support of the RNA–protein interaction proposed in domain 4, residues protected from DMS attack in vivo encompassed a region described to interact with eIF4G in BHK-21 cells (Lopez de Quinto & Martinez-Salas, 2000). Moreover, this protection pattern is also expected to occur in reticulocyte lysates, an in vitro translation system that contains the IRES-binding proteins eIF4G, eIF4B and PTB (Bassili et al., 2004), where the FMDV IRES is very efficient. This is a possibility that can be addressed in the future.

The IRES region immediately upstream of the translation initiation codon, domain 5 (or L), exhibited distinct DMS accessibility. Reactive residues were located either in the single-stranded region around nt 450, downstream of the polypyrimidine tract, or in the bulged bases of the hairpin. Mutation of these two adenines disrupted eIF4B binding (Lopez de Quinto et al., 2001). However, residues in domain 5, comprising the right arm of the hairpin, including the polypyrimidine tract that contains a PTB-binding site (Luz & Beck, 1991), were not reactive towards DMS. Similarly, the stem–loop that constitutes domain 2 and contains the main binding site of PTB on its apical pyrimidine loop was not reactive.

Studies on the organization of the FMDV IRES in the cytoplasm using the photoactive reagent AMT-psoralen demonstrated a hot spot of cross-linked bases in the central domain. A large proportion of the cross-linked residues were pyrimidines located in stems as defined by RNA probing; this is therefore consistent with the formation of interstrand cross-links in the secondary structure. However, the cross-link found at C237, located in a loop, may be indicative of mono-adduct formation (Kramer et al., 1999) or a tertiary RNA–RNA interaction (Fernandez-Miragall et al., 2006). Regarding the latter possibility, we have recently proposed that the GNRA tetraloop controls the structural organization of the central domain by establishing a long-range interaction with a conserved sequence around G240, just downstream of C237.

Comparison of the UV–psoralen cross-link pattern observed in vitro with that seen in vivo showed significant changes. In particular, residues C156 and U169–C170 gave rise to the formation of cross-links in vitro, but not in vivo. Conversely, residues A187–G185, C192, C219–C220 and C243 were cross-linked in the cytoplasm, but not in vitro. Taken together, these results point to a local reorganization of RNA structure within the apical region of the central domain.

The modification of RNA accessibility detected in the central domain in vivo may be due to different scenarios. The interaction with host trans-acting factors in the cytoplasm may be responsible for this reorganization, as has been observed in vitro for other IRES elements (Kolupaeva et al., 2003; Mitchell et al., 2003). Another non-mutually exclusive possibility is the acquisition of a structural conformation that might facilitate a direct involvement of the apical region of the central domain with the ribosomal components; in favour of the latter hypothesis, a tRNA-like structural motif that serves as substrate for RNase P in vitro resides in this IRES region (Serrano et al., 2007). Both of these possibilities need further investigation.

Overall, the combination of DMS footprinting with UV–psoralen cross-linking in the FMDV IRES in living cells has
allowed us to define a map of accessible and protected regions. Accordingly, the distal region of the IRES, in particular the 3'‐end region, contained most of the protected bases. In contrast, the structural conformation adopted by the apical region in the central domain is widely accessible to chemical compounds that traverse the cellular membrane. The RNA accessibility map generated in living cells provides essential information needed to use the FMDV IRES as a target for specific drugs aimed at combating this animal virus.

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