AUGs are used as start codons for viral protein synthesis, although start at two in-frame AUG codons separated by 84 nt [10]. Both and a poly(A) tail, which stimulate IRES activity and participates viral RNA [5,6,7]. The 3(IRES) element that mediates cap-independent translation of the replication element (a variable region folding as two to four pseudoknots, the a long hairpin (termed S), a poly(C) tract of variable length, linked to the 5mRNAs at its 5 end. Instead, a viral protein (VPg) is covalently attached to the 5’ end of the viral genome [4]. The 5’UTR comprises a long hairpin (termed S), a poly(C) tract of variable length, a variable region folding as two to four pseudoknots, the cis-replication element (cre), and the internal ribosome entry site (IRES) element that mediates cap-independent translation of the viral RNA [5,6,7]. The 3’UTR consists of a region of about 90 nt and a poly(A) tail, which stimulate IRES activity and participates in viral RNA replication [8,9]. Replication of the viral genome occurs in the cytoplasm of infected cells. Polypeptide synthesis can start at two in-frame AUG codons separated by 84 nt [10]. Both AUGs are used as start codons for viral protein synthesis, although the second one is preferentially utilized in a variety of assays [11,12].

Picornavirus IRESes are cis-acting elements that recruit the 40S ribosomal subunits to the mRNA with the help of cellular trans-acting factors [13]. The FMDV IRES is distributed in structural domains (Fig. 1A) with the first 21 nt (domain 1) forming part of the cre element [14]. Domain 2 includes a polypyrimidine tract (UCUUU) that provides a polypyrimidine tract-binding protein (PTB) binding site [15]. Domain 3 consists of 210 nts, the most apical region of which is arranged as a cruciform structure that plays a crucial role in RNA-RNA interaction [16,17]. The proximal part is organized as a base-paired structure interrupted with bulges that include several non-canonical base pairs and a helical structure essential for IRES activity [18,19]. Domain 4, which is organized into two hairpin-loops with A-rich internal bulges conserved between FMDV and encephalomyocarditis virus (EMCV) [20] is responsible for the interaction with the translation initiation factor eIF4G [21,22], an essential step in FMDV and EMCV IRES-dependent translation initiation. Domain 5 is composed of a conserved hairpin-loop and a polypyrimidine-rich tract preceding the first functional AUG codon. Specific RNA motifs located in domains 2, 4 and 5 are responsible for the interaction with cellular proteins such as PTB, eIF4G, eIF3 and eIF4B, amongst other host factors controlling internal initiation [23].
The FMDV IRES showed significant differences in the accessibility in vitro and in vivo to dimethyl sulfate (DMS) [24], a reagent that is permeable to cell membranes and reacts with unpaired bases in the RNA structure. The differences in the accessibility of the IRES to DMS suggested that the IRES region adopts a different conformation in the cell cytoplasm compared to naked RNA. Similarly, results of UV-crosslinked amino-methyl psoralen-treated cells showed a local reorganization of RNA structure. Recent Selective 2’Hydroxyl Acylation analyzed by Primer Extension (SHAPE) structural analysis revealed a modular organization of the IRES region [25]. Additionally, clusters of SHAPE reactive nts indicated the presence of flexible regions, hairpin-loops and internal bulges within the IRES domains [26]. Accessibility to the FMDV IRES was also studied through hybridization of fluorescent-labeled IRES transcripts with complementary oligodeoxynucleotides printed on microarrays. It is worth noting that the accessible regions in the microarray were also reactive to SHAPE probing, except for specific nts within the apical region of domain 3, around the GNRA (where N is any nucleotide and R is a purine) stem-loop [26].

Accessibility of different regions of the IRES to 2’OMe AONs indicated the presence of flexible regions, hairpin-loops and internal bulges within the IRES domains [26]. Accessibility to the FMDV IRES was also studied through hybridization of fluorescent-labeled IRES transcripts with complementary oligodeoxynucleotides printed on microarrays. It is worth noting that the accessible regions in the microarray were also reactive to SHAPE probing, except for specific nts within the apical region of domain 3, around the GNRA (where N is any nucleotide and R is a purine) stem-loop [26].

Antisense oligonucleotides are single-stranded sequences that form a hybrid with their complementary RNA via Watson-Crick base pairing. The resulting hybrid can block gene expression by various mechanisms, depending on the chemical composition of the oligonucleotide and location of the hybrid. The strength of the hybrid depends on factors such as thermodynamic stability, the secondary structure of the target mRNA, and the proximity of the hybridization site to functional motifs on the designated transcript such as translational start site. Unmodified oligonucleotides are unstable within cells due to rapid nuclease degradation. By contrast, phosphorothioate [27] and 2’O-methyl oligoribonucleotides, carrying the 2’-OH residue of the ribose molecule replaced by a methyl group, are resistant to degradation by cellular nucleases [28]. 2’O-methyl antisense oligoribonucleotides (2’OMe AONs) form high melting temperature heteroduplexes with targeted mRNA [29] and induce antisense effect by a non-RNase H-dependent mechanism [30,31]. Earlier studies have been conducted to investigate the use of antisense transcripts to inhibit FMDV viral gene expression directed against the functional AUGs [32] and to both, the 5’ and 3’UTR [33,34]. An independent study using antisense morpholino oligomers targeting the viral RNA start codons also showed the capacity of these small molecules to inhibit viral FMDV multiplication [35]. The latter study showed controversial results with a previous work [36], in which phosphorothioate antisense oligodeoxynucleotides complementary to AUG2 inhibited virus multiplication with greater effect than those complementary to AUG1. Despite all these studies, a deep analysis of molecules targeting the entire IRES element to inhibit viral RNA expression in cells and in vitro was lacking.

Here, by taking advantage of the RNA structural analysis of the IRES-AUG region [12,16-18,23–26], we have designed a set of customized 2’OMe AONs to monitor the accessibility of FMDV in the context of full length RNA transcribed from an infectious cDNA clone. The results indicate that the AON targeting AUG2 inhibited FMDV viral multiplication more efficiently than that which targeted AUG1 in susceptible cells. By contrast, the results obtained using cell-free systems indicated that AUG1 was the best target to inhibit translation. Remarkably, four AONs (183, 207, 432 and 452) complementary to conserved motifs within the apical region of domain 3 and domain 5, that play key roles in internal initiation [17,37,38], were potent inhibitors in both systems, revealing accessible IRES regions which are candidate targets for small molecules interfering viral infectivity. Furthermore, the accessibility of different regions of the IRES to 2’OMe AONs exhibited important differences when comparing the results obtained in RNA-transfected cells or in vitro translation of the same RNA. These differences, which are likely due to the composition of the cell cytoplasm and the cell-free system, emphasize the need to use living cells to measure the inhibitory capacity of small molecules.

**Results**

**Differential inhibition of FMDV RNA infectivity by 2’O-methyl-antisense oligonucleotides targeting AUG1 and AUG2 in tissue culture cells**

FMDV RNA translation initiation is peculiar in that protein synthesis can be initiated at two start codons, AUG1 and AUG2 (Fig. 1A). While both AUGs are fully conserved in field isolates,
AUG2 is used more efficiently than AUG1 in infected cells [11,39]. Earlier works have addressed the study of AUG codon usage driven by the FMDV IRES using reporter genes [11,12,40,41]. However, little information was available concerning the influence of the complete viral RNA sequence on the accessibility of the translation start region to small molecules.

To fill this gap, we made use of an infectious cDNA clone [42] to study the capacity of 2′OMe AONs to inhibit FMDV RNA translation in BHK-21 cells. To ascertain the optimum inhibitory conditions, a concentration range (1–20 nM) of three oligonucleotides, AUG1, AUG2 and SCR (harboring a scrambled sequence, Table 1) were annealed to FMDV RNA for 20 min at 37°C prior to transfection of BHK-21 cells. Virus yield was determined 24 hpt relative to a parallel assay conducted without AONs (Fig. S1). Inhibition of FMDV titer was dose-dependent and sequence-specific, being 10 or 20 nM equally efficient in inhibiting the viral titer. Thus, 10 nM was used in all subsequent assays. Furthermore, no effect on the translation efficiency of the FMDV RNA was observed in the presence of increasing amounts of SCR AON relative to the FMDV RNA alone (Fig. S1); in addition no toxicity was observed when cells were incubated with any of these AONs during 48 hr by cell staining.

FMDV multiplication was inhibited in the presence of both, AUG1 and AUG2 AONs (Fig. 1B). Differences in FMDV titer inhibition induced by AONs were analyzed by a paired two-sided Student t-test; differences were considered significant when \( P<0.05 \). Overall, in agreement with the \( P \) values obtained in the t-test, 60% was the threshold separating inhibitor from non-inhibitor molecules. The oligonucleotide SCR had no effect on FMDV RNA infectivity (Fig. 1B), demonstrating that the impact of AUG1 and AUG2 AONs on viral gene expression was sequence specific. Moreover, the decrease of virus yield induced by AUG2 (22% relative to the control assay) was 3-fold higher than that of AUG1 (60% of the control RNA), indicating that the best target for inhibition of viral replication is by blocking the AUG2 region.

To confirm the inhibition of viral protein synthesis by AUG1 and AUG2 AONs, a western blot against the VP1 structural protein present in cytoplasmic extracts of BHK-21 transfected cells prepared prior to cell detachment was performed (Fig. 1C). In comparison to cells transfected with FMDV RNA incubated with the SCR AON, or the control without any AON, the intensity of VP1 confirmed that there was a strong inhibition of intracellular viral protein synthesized and accumulated 24 hpt in the presence of AUG2.

Response of FMDV RNA to 2′OMe AONs targeting the initiator codons using in vitro translation systems

Cell-free systems are often used to determine translation efficiency. Thus, to measure the ability of 2′OMe AONs to inhibit protein synthesis in the context of the complete viral RNA, FMDV RNA preincubated with SCR, AUG1 or AUG2 oligonucleotides under the same annealing conditions used in the RNA translation assays was used to program rabbit reticulocyte lysates (RRL) translation. The control RNA, without any AON, was efficiently translated, as expected. Analysis of the translation efficiency of FMDV RNA annealed to AONs AUG1 or AUG2 revealed a marked difference in protein synthesis (Fig. 2A). In contrast to the data observed in transfected cells, translation efficiency in the presence of AUG1 (15%) was significantly lower than that observed in the presence of AUG2 (Fig. 2B). Translation of viral proteins in the presence of AUG2 (75% of the value observed with the control RNA alone) was non-inhibitory (\( P<0.01 \)). No inhibition was observed in the presence of SCR, again showing a sequence-specific effect of AONs. Thus, we conclude that there is a contrast between cell-free systems and transfected cells in the response to AON interference depending upon the targeted initiation codon.

Impact of 2′OMe AONs targeting the IRES region on the FMDV RNA infectivity

The differential response of the functional start codons in the two systems used to analyze FMDV RNA translation prompted us to assess the impact of a panel of customized 2′OMe AONs (Table 1) targeted to the entire IRES region (Fig. 3). AONs design was carried out taking into account the structural analysis of the IRES region [16–18,25–26]. The capacity of each oligonucleotide to inhibit viral yield, measured by plaque forming units (PFU), was determined 24 hpt. A significant inhibition was noted in the 5′region of the IRES, where virus yield fell to 20% in the presence of the AON 40 (Fig. 4A). On the contrary, AONs 55, 66 and 83 were non-inhibitory. These results were confirmed by the reduction of VP1 intensity in a western blot assay using cytoplasmic cell extracts (Fig. 4B). The specific inhibition of AON 40 may be attributed to the pairing of the AONs to the IRES sequence adjacent to cre (Fig. 1A), an element necessary for viral replication [14].

The inhibitory capacity of AONs complementary to domain 3 is shown in Fig. 5. Gross differences were observed with molecules targeting the basal stem. While AONs 263, 282 and 300 inhibited virus yield (36%, 39% and 52%, respectively), no inhibitory effect was detected with AONs 104, 118, 135, and 134. The reason for these differences is not known. Interestingly, virus yield analysis revealed that AONs 183 and 207, complementary to the GNRA and the RAAA stem-loops respectively (Fig. 3), reduced FMDV RNA infectivity to about 40% (Fig. 5A). However, AONs 164 and 176, complementary to the sequences immediately upstream of the GNRA motif, did not affect virus yield. Moreover, the 3′region of the GNRA and RAAA motifs were resistant to the effect of AONs 192 and 213. Differences in the efficiency of VP1 viral protein synthesis 24 hpt were confirmed by western blot (Fig. 5B). The structural organization of the FMDV IRES that depends on interactions involving the GNRA motif and some nucleotides of the adjacent RAAA stem-loop [16,26] may form part of the basis of these differences.

AONs complementary to domain 4 behaved in a different way. With the exception of AON 349 for which a severe decline in virus yield (23% relative to the control RNA) was noticed (Fig. 6A), the yield observed in the presence of AONs 317, 331, 360, 384, 397, 407 and 419 were all above 60%, indicating that viral multiplication was not affected if AONs were pre-annealed to this IRES region. Domain 5, however, did not tolerate the disruption caused by AONs presumably due to the blocking of binding sites of RNA-binding proteins [23,43]. Specifically, the inhibition was slightly more pronounced in the hairpin (432, 24%) than in the single-stranded region (452, 32%) (Fig. 6A). The inhibition of protein synthesis by AONs complementary to domains 4 and 5 was also confirmed by a decrease in the amount of viral proteins shown in the western blot (Fig. 6B).

The capacity of AONs to reduce viral replication was further investigated by extending the incubation of the cells up to 48 hpt. Compared to control RNA, the virus yields revealed that five oligonucleotides (40, 183, 349, 432 and AUG2) remained inhibitory 48 hpt (Fig. 7) (\( P<0.01 \)). It is important to note that these AONs showed the highest potency in inhibiting the viral multiplication 24 hpt, with virus yields below 40%. The inhibition noticed at 48 hpt indicated the potential of the AONs to inhibit viral replication despite the growing number of viral RNA molecules during the viral multiplication process.
Interference of IRES activity in vitro denotes differences with cultured cells

After revealing the differential impact of the AUG1 and AUG2 oligonucleotides to down-regulate IRES activity, this work sought to further uncover the competence of AONs to intervene in the role of the IRES within a full-length FMDV genome using an in vitro translation system. The inhibition of polyprotein translation in vitro induced by AONs annealed to the FMDV RNA varied depending on each molecule (Fig. 8A, B). AONs 55 and 66, complementary to domain 2, induced a decrease in translation efficiency to 26% and 47%, respectively, relative to the control RNA. However, the stem of domain 2 was able to withstand the presence of AONs 40 and 83 (65% and 64%, respectively).

Variations were similarly observed with fifteen AONs complementary to the central IRES domain, with most of the inhibitions noted in molecules complementary to the apical region. In terms of IRES activity, inhibitions were prominent when conserved motifs GNRA, RAAA and C-rich motif were disturbed (ranging from 49 to 29%, with AONs 183, 192, 207, 213 and 247). In contrast, AONs targeting the stem were non-inhibitory (ranging from 64 to 118% with AONs 104 and 282, respectively). The inhibition observed by blocking the apical region of the central domain, reflected the susceptibility of the IRES conserved motifs and revealed the crucial role of the central domain in the translation process.

A striking result was noted in the lack of inhibition exerted by AONs complementary to domain 4, with values above 60% (Fig. 8A). However, and in agreement with the inhibition observed in transfected cells (Fig. 6A), the AONs 432 and 452 were strong inhibitors of protein synthesis.

Table 1. 2′O-methyl Antisense Oligoribonucleotides.

| Name   | Length | Target region | Sequence (5′–3′)            | GC | Tm  |
|--------|--------|---------------|-----------------------------|----|-----|
| 40     | 15     | D2: 40–26     | mUmUmCmAAGUUGCAmCmGmUmU     | 40 | 47  |
| 55     | 16     | D2: 55–40     | mAmAmGmACCAAGGCGGmAmGmUmU   | 56 | 57  |
| 66     | 16     | D2: 66–51     | mCmUmAmGACCUGGAAmAmGmAmC   | 50 | 46  |
| 83     | 15     | D2: 83–68     | mUmAmCmAAGUUGUUmAmCmCmC    | 40 | 47  |
| 104    | 19     | D3: 104–85    | mCmGmAmGCGGUGGAGCAAmCmAmCmA| 60 | 60  |
| 118    | 14     | D3: 118–105   | mAmCmUmCGCCAGUmGmGmAmU     | 57 | 59  |
| 135    | 18     | D3: 135–118   | mAmCmAmGUGCGUUAUCUmAmCmAmA | 39 | 55  |
| 154    | 18     | D3: 154–138   | mUmCmAmUGUCCCCGUACmGmAmGmC| 56 | 58  |
| 164    | 14     | D3: 164–150   | mCmCmAmGCGCCGUUmCmAmGmU    | 71 | 54  |
| 176    | 14     | D3: 176–163   | mAmAmGmAGAGGAGUmCmAmCmC    | 57 | 56  |
| 183    | 17     | D3: 183–167   | mUmGmGUmUAACAAAGGAAmGmAmGmU| 47 | 46  |
| 192    | 15     | D3: 192–178   | mGmUmGmGUCUCGUUmGmUmAmC    | 54 | 46  |
| 207    | 17     | D3: 207–191   | mGmUmGmGCUUUUGCCmCmCmGmU  | 65 | 64  |
| 213    | 15     | D3: 213–199   | mGmUmGmGCGGCGGUmUmGmUmU    | 60 | 53  |
| 226    | 17     | D3: 226–210   | mAmUmGAAGGCCCGGUUmGmGmGmG| 71 | 58  |
| 247    | 16     | D3: 247–233   | mUmCmAmGACGUGCGUUmGmGmGmU| 69 | 61  |
| 265    | 17     | D3: 265–249   | mUmGmGmGUUUUGCGAGUmAmAmG  | 47 | 56  |
| 282    | 18     | D3: 282–265   | mUmCmAmAUGCUACUUUmAmAmGmU | 27 | 45  |
| 300    | 17     | D3: 300–284   | mGmGmGmGuGUGGGGUAcmCmAmGmU| 53 | 62  |
| 317    | 17     | D4: 317–301   | mAmCmUmCUAGCCGUUmCmAmCmC  | 53 | 62  |
| 331    | 16     | D4: 331–317   | mGmUmGmGmUACCUAGGAmGmGmGmG| 63 | 60  |
| 349    | 18     | D4: 349–334   | mAmGmUmGmGCGGCGUUGUmAmCmAmC| 56 | 61  |
| 360    | 18     | D4: 361–344   | mAmCmAmGmAUCGCCAGGUmGmGmC | 61 | 63  |
| 384    | 18     | D4: 384–367   | mUmUmAmUAGAAGGCCTCmGmGmGmC| 50 | 56  |
| 397    | 18     | D4: 397–378   | mAmCmAmCmGAGGGCGUUUmAmAmAmG| 44 | 53  |
| 407    | 16     | D4: 407–392   | mGmAmGmGCUUUUAAmAmCmAmG    | 38 | 44  |
| 419    | 17     | D4: 419–404   | mUmAmUmCUAGGGCAUAGmAmAmGmC| 41 | 49  |
| 432    | 17     | D5: 432–415   | mCmCmAmGmCCGUACCUUmAmUmCmC| 59 | 59  |
| 452    | 19     | D5: 452–433   | mCmCmAmGmAAAGGAAAAAGGUmGmCmG| 47 | 48  |
| AUG1   | 18     | luciferase AUG| mUmUmCmCAAAUAAUACCAmAmCmAmG| 39 | 49  |
| AUG2   | 19     | FMDV AUG1     | mAmCmAmGmUUGJAUACUAAGmGmGmUmC| 42 | 52  |
| SCR    | 16     | Random sequence|mUmGmCmAGCUGGACGUmGmUmUAmA| 50 | 57  |

2′O-methyl modifications are designated with a letter ‘m’. GC, content of GC, Tm, melting temperature.

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Figure 2. Effect of 2′OMe AONs targeting the initiator AUGs of FMDV RNA in in vitro translation efficiency. (A). Autoradiograph of translation products obtained from in vitro synthesized FMDV RNA annealed, or not, with the indicated AONs and incubated with reticulocyte lysates. Translation assays were conducted as described in Material and Methods. The position of molecular weight markers is indicated on the left. (B). The intensity of the lanes were determined by densitometry and made relative to the total intensity of the lane indicated on the left. (B). The intensity of the lanes were determined by densitometry and made relative to the total intensity of the lane indicated on the left. (B). The intensity of the lanes were determined by densitometry and made relative to the total intensity of the lane indicated on the left. (B). A black bar denotes inhibitory capacity relative to the control translation reaction.

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Interference of virus multiplication by AONs AUG1 and AUG2

To extend our analysis of AON inhibition to virus infection, we used BHK-21 cell monolayers treated with AONs SCR, AUG1, AUG2 and mock-treated to carry out a virus infection as described in Material and Methods. Virus titer determined at time 0 was lower than 10 PFU/ml in all samples. However, the virus titer determined 6 hpi differed strongly between AUG1 and AUG2, while there was no effect of the SCR relative to the control mock-untreated cell monolayer (Fig. 9). The results indicated a significant inhibition in the case of cells treated with AUG2 (<20% relative to the control mock-untreated cells, P<0.01), while the decrease in virus titer in the presence of AUG1 was about 80% of the control cell monolayer. These results are in full agreement with the data obtained in infectious RNA transfections, manifesting a similar inhibitory effect in cells transfected with FMDV RNA than in cells infected with FMDV virus particles.

Discussion

Here we have made use of 2′OMe AONs complementary to the FMDV IRES-AUG translation start region to explore the capacity of these molecules to interfere viral RNA translation, and thus, viral multiplication. Specifically, we were interested in analyzing the response of the IRES-AUG region in the context of the viral RNA for several reasons. First, to take into account the cis-acting elements in the viral RNA that may contribute to IRES function that are absent in reporter sequences and second, to establish a comparison between the responses observed in cell-free systems and in tissue culture cells.

Our results reinforce the main role of AUG2 in viral RNA translation in tissue culture cells. This is supported by the strong inhibition of the virus yield measured 24 hpt with the AON complementary to AUG2, while that complementary to AUG1 only partially reduced virus multiplication (Fig. 1B). In both cases, the inhibition observed was dose-dependent and sequence specific (Fig. S1). These results are consistent with a preferential use of AUG2 in infected cells [39], as well as with the higher inhibitory capacity of antisense oligoribonucleotides targeting AUG2 [36], independently of using FMDV RNA-transfected cells or FMDV infection (Fig. 9). The reasons that would explain the different behavior of AUG1 and AUG2 AONs observed between in vitro translation and transfected cells are unknown; however they support a different accessibility of these two regions to external molecules despite being only 84 nt apart. Further, our results support the need to use in vitro assays to validate the interference of small molecules in viral gene expression.

The interference observed with a collection of thirty-two customized AONs targeting the entire IRES region revealed large differences in the capacity of these molecules to inhibit viral gene expression, and therefore multiplication of the viral RNA. Specifically, oligonucleotides complementary to domain 2, the apical region of domain 3, the basal region of domain 4 and domain 5 were the most efficient inhibitors in transfected cells, which were still detected 48 hpt (Fig. 7). This inhibition was confirmed when the viral protein VPI accumulated in the cell cytoplasm prior to cell detachment was measured by immunodetection, indicating that the low virus yield was a consequence of a decrease in viral RNA translation. In full agreement with these results, phosphorothioate derivatives targeting some of these regions were inhibitors of virus multiplication (Table S1).

Annealing of 2′OMe AONs to FMDV RNA prior to program in vitro translation indicated a differential response in some, but not all, IRES regions. Specifically, AONs 183, 207, 432 and 452 were inhibitory (as observed in transfected cells). However, several AONs complementary to the apical region of domain 3 were inhibitors in vitro but not in living cells. In agreement with this result, differences in DMS and amino-methyl psoralen accessibility to naked FMDV IRES or RNA expressed in the cell cytoplasm were already noticed [24]. Since RNA structure of this particular IRES region is constrained by tertiary interactions [16,26], it is likely that the composition of the cell-free system differs in cofactors influencing RNA structure. Further, the differences in gene expression observed with the FMDV RNA when transfected in susceptible cells or translated in vitro suggested important conformational changes, which may depend on the interaction of the IRES with transacting factors present in different concentrations in the cell cytoplasm. In addition, interactions with other cis-acting RNA elements such as the FMDV 3′UTR [44], modification of host factors in infected cells [45–50], or the specific intracellular environment of viral replication [51–54] may differentially affect expression in these systems. However, since hamster BHK-21 or swine IBRS-2 cell lines are known to have an inactive type I IFN system in response to FMDV infection [55,56] it is unlikely that the differences observed between the in vitro lysates and the transfected cells are due to type I interferon production.

As previously mentioned, domains 4 and 5 provide the binding site for eIFs and IRES-transacting factors (ITAFs) controlling internal initiation of translation [38,43,57]. Disruptions caused by the AONs targeting domain 5 were not tolerated, as reflected by the reduced infectivity of FMDV RNA in the presence of the AON complementary to the hairpin (432) and the single-stranded region (452) (Figs. 6A and 7). The inhibition induced by AONs complementary to domain 5 had similarities to those noted by AONs 40, 183 and 349, complementary to domain 2, the apical region of domain 3, or domain 4 (Fig. 3). Another possibility to explain the inhibition noticed in domain 5 could be the steric block caused by the AONs, interfering in the landing site of the translational machinery.
Our results are in partial disagreement with a previous study that used morpholino oligomers targeting domain 5, AUG1 and AUG2 to inhibit FMDV multiplication [35]. Furthermore, the concentration required to inhibit viral multiplication was higher (1–5 μM) and the interference produced by the morpholino complementary to domain 5 was lower than that of AUG1 or AUG2. Other IRES regions were not analyzed in the work by Vagnozzi et al. [35]. Morpholino-modified RNAs effectively inhibited replication of poliovirus [58], West Nile virus [59], dengue virus [60], or severe acute respiratory syndrome coronavirus [61], among other positive strand RNA viruses. More recently locked nucleic acid-based (LNA) RNAs were shown to be effective inhibitors of HCV [62]. Other RNA-based antiviral strategies were able to suppress virus multiplication in cell culture, as illustrated for HCV [63,64], FMDV [65] or poliovirus [58,66].

In summary, we have taken advantage of the stability of 2′OMe AONs to explore the accessibility of the entire IRES-AUG region in the context of the viral RNA. Overall, we have identified critical regions of the IRES element that led to a significant decrease of virus yield when targeted with 2′OMe AONs. Identification of these regions in tissue culture cells by monitoring the reduction in viral protein translation or reduced virus multiplication emphasizes the relevance of certain viral RNA region in controlling viral gene expression. In addition, they open up new avenues to develop novel tools aimed to inhibit viral infection.

**Materials and Methods**

**RNA synthesis**

The plasmid pMT28, encoding a cDNA copy of FMDV C-S8c1 genome inserted into pGEM-1 under the control of the SP6 promoter was described previously [42]. Following NdeI linearization FMDV RNA was transcribed in vitro using SP6 RNA polymerase, as described [8].

**2′-O-methyl antisense oligoribonucleotides design**

Thirty-two 2′O-Methyl antisense oligoribonucleotides designed to hybridize with the IRES, AUG1 and AUG2 of FMDV RNA (Table 1), with 2′O-methyl instead of the 2′OH of the ribose in the four flanking nucleotides, were purchased from SIGMA.
simplicity, 2′OMe AONs are named by the IRES nt position which is complementary to the 5′ end of each oligoribonucleotide (see Fig. 3). A scrambled sequence of 16 nt, with 50% GC content, was included as specificity control. The scrambled sequence was checked with NCBI-BLAST software (http://blast.ncbi.nlm.nih.gov/) to prevent any possible match in the FMDV RNA or the host cellular RNAs. MFold (http://www.tbi.univie.ac.at/ivo/RNA/) and oligo analyzer (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) were used to predict the secondary structure of each antisense oligoribonucleotide to prevent self-dimerization, formation of stable hairpins, and to optimize its hybridization to the IRES region avoiding perfect hairpin targets deduced from RNA probing data [16-18,25-26].

Annealing of 2′OMe AONs with the RNA and in vitro translation

The optimal inhibitory condition was determined using a concentration range (0.1, 1, 3 and 6 μM) of two AONs, AUG and SCR, annealed to RNA (250 ng) for 20 min at 37°C (data not shown). In vitro translation was performed by adding the RNA annealed with AON to a reaction mix containing 6.5 μl of nuclease-treated rabbit reticulocyte lysates (RRL) (Promega), 1 μl (1 mM) amino acid mix less methionine and 0.5 μl (6 μCi) 35S-methionine in a final volume of 10 μl. Translation reaction was carried out at 30°C for 60 min. The reaction was treated with 1 μl of RNase A, incubated for 10 min at room temperature and fractionated in polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) [11]. Inhibition of viral polypeptides synthesis was calculated by dividing the intensity of total polypeptides translated in the presence of the 2′OMe AON to that of the control RNA without ASO. Subsequent assays were carried out using 6 μM 2′OMe AON, annealed to the target RNA at 37°C, 20 min.

RNA transfection

The infectivity of viral RNA synthesized in vitro was determined using increasing amounts of FMDV RNA (25 pg to 2 ng) to transfet BHK-21 cell monolayers (5×10⁵ cells); virus yield was titrated 24 hpt in fresh cell monolayers. Typically, virus yield from 50 pg of FMDV RNA yielded about 1.6×10⁶ plaque forming units (PFU)/ml. In the RNA infectivity assay, and prior to RNA transfection, 50 pg of in vitro synthesized FMDV RNA was annealed with each oligonucleotide (10 nM) for 20 min at 37°C. Triplicates of BHK-21 cells grown in 35 mm well dishes, washed three times with Dulbeccos modified Eagle’s medium (DMEM), were transfected with a mixture of lipofectene-RNA annealed with each AON in 0.5 ml of DMEM, as described [9]. Three hours post-transfection (hpt), cell monolayers were washed with DMEM three times, and 2 ml of fresh DMEM supplemented with 5% FCS was added. At 24 and 48 hpt, 200 μl supernatant was collected. Cytoplasmic cell extracts were prepared for the determination of VP1 viral protein from transfected cells 24 hpt prior to cell detachment, using lysis buffer (100 μl of 50 mM Tris-HCl, pH 7.8, 120 mM NaCl, 0.5% NP40) and centrifuged at 14,000 RPM for 5 min to remove cellular debris. Transfection experiments were repeated at least 3 times.

Western blot analysis

Equal amounts of total protein prepared from transfected BHK-21 transfected cells were resolved in 10% SDS-PAGE, transferred to a PVDF membrane (Biorad) using a semidy electrotransfer device. VP1 protein was detected using the SD6 mouse monoclonal antibody (1:1000) [67] followed by goat-anti-mouse secondary antibody coupled to horseradish peroxidase (1:2000) (Thermo Scientific), and enhanced chemiluminescence (GE Healthcare). After stripping using restore western blot stripping device. VP1 protein was detected using the SD6 mouse mono-
clonal antibody (1:1000) [67] followed by goat-anti-mouse secondary antibody coupled to horseradish peroxidase (1:2000) (Thermo Scientific), and enhanced chemiluminescence (GE Healthcare). After stripping using restore western blot stripping buffer (Thermo Scientific) the same membrane was used to detect α-tubulin as a loading control using anti-tubulin antibody (1:5000) (Sigma).

PFU inhibition assay

Virus yield from three independent RNA infectivity assays were titrated in fresh susceptible IBRS-2 cells to determine the capacity of each 2′OMe AON to inhibit PFU. Monolayers of IBRS-2 cells were infected with serial dilutions of the supernatant from transfected BHK-21 cells. One hour after adsorption, the viral inoculum was removed and the cells were washed three times, overlaid with DMEM medium with 0.5% agar supplemented with 2% fetel calf serum. Virus titer (PFU/ml) was scored 24 hours post-infection (hpi) by fixing the cells with 2% formaldehyde solution and stained with 0.3% crystal violet in 2% formaldehyde solution. The viral titer from the 24 and 48 hpt supernatant was determined by counting the viral plaques that developed after 24 hpi [33]. The virus yield was calculated as the mean of PFU/
Figure 5. Analysis of virus yield in the presence of 2′OMe AONs complementary to domain 3. (A) FMDV RNA was annealed with the indicated AONs or a scramble (SCR), prior to transfect confluent BHK-21 cell monolayers in duplicate. Virus yield was determined as PFU/ml in the supernatant 24 hpt, and made relative to the value obtained in the control RNA alone. Values represent the mean and standard deviation of triplicate assays (** P < 0.01; *** P < 0.001). (B). Western blot of representative examples of viral protein VP1 accumulated in BHK-21 cells 24 hpt; anti-tubulin was used as a loading control.

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Figure 6. Effect of 2′OMe AONs complementary to domain 5 on virus multiplication. (A) FMDV RNA was annealed with the indicated AONs and a scramble (SCR), prior to transfect confluent BHK-21 cell monolayers in duplicate. Virus yield was determined as PFU/ml in the supernatant 24 hpt, and made relative to the value obtained in the control RNA. Values represent the mean and standard deviation of triplicate assays (** P < 0.01; *** P < 0.001). (B). Western blot of representative examples of viral protein VP1 accumulated in BHK-21 cells 24 hpt; anti-tubulin was used as a loading control.

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ml of three independent assays of FMDV RNA transfected BHK-21 cells with each 2’OMe AONs relative to the PFU/ml of FMDV RNA transfected without oligonucleotides, which was set at 100%. Cell monolayers were used to determine ASO cytotoxicity by cell staining at the end of the treatment.

Challenge of AON-treated cells with FMDV virus

BHK-21 cell monolayers (1x10^5 cells) were incubated with SCR, AUG1 or AUG2 ASO (0.4 μM) or DMEM alone 3 h prior to FMDV infection. Then, cell monolayers were infected with C-S8c1 FMDV at a multiplicity of infection (MOI) of 0.1 PFU/cell.

Figure 7. Monitoring time-effect of virus yield induced by 2’OMe AONs complementary to the IRES region. FMDV RNA was annealed with the indicated AONs and a scramble (SCR), prior to transfect confluent BHK-21 cell monolayers in duplicate. Virus yield was determined as PFU/ml in the supernatant 48 hpt, and made relative to the value obtained in the control RNA. Values represent the mean and standard deviation of triplicate assays. A black bar denotes inhibitory capacity (** P<0.01) relative to control transfection.
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Figure 8. Changes in FMDV RNA translation efficiency in vitro induced by 2’OMe AONs. (A). Autoradiograph of translation products obtained from FMDV RNA annealed, or not, with the indicated AONs in reticulocyte lysates. Translation assays were conducted as described in Material and Methods. (B). The intensity of each lane was determined by densitometry and made relative to the intensity of the lane without AON, which was set at 100%. Data correspond to the mean and standard deviation of the mean of three independent assays. A black bar denotes inhibitory capacity relative to control RNA.
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After 1 h of adsorption, the inoculum was removed by 3 washes with DMEM-HCl pH 6 to inactivate free virus, twice with DMEM to restore pH and then, incubated with DMEM supplemented with 2% FCS and the appropriate AON (0.4 μM) for 6 h. Supernatants were collected at 0 and 6 hpi for virus titer determination, as described [33].

Supporting Information

Figure S1 Determination of the optimum 2’OMe AON concentration required for virus yield inhibition. In vitro synthesized FMDV RNA (50 pg) was annealed with the indicated concentrations of AONs AUG1, AUG2 or the scramble SCR, prior to transfect confluent BHK-21 cell monolayers in duplicate. Virus yield was determined using fresh cells monolayers as the number of plaque forming units (PFU)/ml in the supernatant 24 hpt as described in Material and Methods, made relative to the control RNA which was set at 100%. Values represent the mean and standard deviation of triplicate assays. (TIF)

Table S1 Inhibition of FMDV RNA infectivity by phosphorothionate antisense oligonucleotides. (DOCX)

Author Contributions

Conceived and designed the experiments: EMS TF FS. Performed the experiments: TF MFR. Analyzed the data: TF MFR FS EMS. Wrote the paper: EMS.

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