trans Complementation by RNA of Defective Foot-and-Mouth Disease Virus Internal Ribosome Entry Site Elements

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A region of about 435 bases from the 5' noncoding region of foot-and-mouth disease virus RNA directs internal initiation of protein synthesis. This region, termed the internal ribosome entry site (IRES), is predicted to contain extensive secondary structure. Precise deletion of five predicted secondary structure features has been performed. The mutant IRES elements have been constructed into vectors which express bicistronic mRNAs and assayed within cells. Each of the modified IRES elements was defective in directing internal initiation when assayed alone. However, coexpression of an intact foot-and-mouth disease virus IRES complemented four of these defective elements to an efficiency of up to 80% of wild-type activity. No complementation was observed with the structurally analogous element from encephalomyocarditis virus. The role of RNA-RNA interactions in the function of the picornavirus IRES is discussed.

The initiation of protein synthesis on picornavirus RNAs occurs at AUG codons which are 600 to 1300 nucleotides (nt) downstream from the 5' termini of the molecules. No cap structure is present at the 5' terminus of the viral RNA, in contrast to cellular mRNAs. A number of features suggest that the initiation of protein synthesis on these RNAs must occur by a mechanism which is distinct from that described for cellular mRNAs (see reviews in references 11 and 16). The long 5' noncoding regions (NCRs) of picornavirus RNAs contain several unused AUG codons, and these regions are predicted to form extensive secondary structure. Translation of many picornavirus RNAs also has to occur when cap-dependent translation is abolished following the cleavage of the cap-binding complex (eIF-4F) component p220. In representatives of each of the genera of these viruses, evidence has been obtained for the presence of an element which directs internal initiation of protein synthesis (2, 5, 12, 17, 26). This element is usually termed the internal ribosome entry site (IRES).

There is considerable conservation of these structures within certain groups of picornaviruses. The IRES elements from aphthoviruses (foot-and-mouth disease viruses [FMDV]) and cardioviruses (e.g., encephalomyocarditis virus [EMCV]) are about 50% identical in sequence, and their predicted secondary structures are very similar (29) (Fig. 1). These structures have been defined on the basis of phylogenetic comparisons and biochemical probing of these RNA molecules. The enterovirus (e.g., poliovirus [PV]) and rhinovirus IRES elements also show extensive similarity in their predicted secondary structures (30, 33). However these elements are very different in sequence and predicted structure from the aphthovirus and cardiovirus elements. A conserved feature across all of these elements is the presence of a pyrimidine tract about 25 nt upstream from an AUG codon. In the case of the cardioviruses and aphthoviruses, this AUG codon is an initiation site of protein synthesis. In contrast, in the enteroviruses and rhinoviruses, a region of up to 120 nt is present between the AUG codon associated with the pyrimidine tract and the initiation codon. Mutagenesis of this cryptic AUG codon in PV has shown that it is not essential for virus viability. However, uniquely among the upstream AUG codons, its modification is deleterious to the virus. The mutant has a small-plaque phenotype (25). In FMDV, two initiation codons, some 84 nt apart, are used to initiate protein synthesis. It has been shown that some ribosomes scan through the region following the first initiation codon, presumably following recognition of the RNA at a point upstream of the first initiation codon (1).

Considerable attention has been focused on determining the mechanism by which the picornavirus IRES elements function. Several studies have identified cellular proteins which interact with them. The IRES elements of FMDV and EMCV cross-link principally to a protein of 57 or 58 kDa. Two binding sites have been mapped for this protein on the FMDV IRES (18). Only a single site has been identified in EMCV (13). The latter is analogous to the 5' proximal site in FMDV. A distinct protein of 52 kDa has been shown to cross-link to the PV IRES (21), although some evidence for interaction with the 57-kDa protein has also been presented (28). Recently evidence has been obtained (3) that the 58-kDa protein is the polyuridylic tract-binding protein (PTB), which has been previously assigned a function within the nucleus in the process of pre-mRNA splicing (24). The 52-kDa protein has been recently identified as Lα, a protein involved in maturation of RNA polymerase III transcripts, also located within the nucleus (20). The role of both of these proteins in IRES function is not yet clear. Mutagenesis of the 58-kDa protein binding site in the EMCV IRES indicated that loss of protein binding was associated with loss of ability to direct internal initiation of protein synthesis (13). However, other studies (7) suggested that this region of the IRES is not essential for the IRES to function, and hence the importance of this interaction is not certain.

Extensive analysis of the PV IRES has been performed. The sequence from nt 140 to 630 was initially defined as being necessary to direct internal initiation of protein synthesis. However, more recent studies (22, 27) have shown that two predicted stem-loop structures within this sequence are not essential for IRES activity. Indeed, the 3'-terminal structure, containing the cryptic AUG, can be deleted from full-length virion RNA without abolishing infectivity. However, the viruses recovered have regenerated an AUG codon in an appropriate position (31).

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Surprisingly, studies of the PV IRES showed that coexpression of full-length PV RNA within cells containing defective PV IRES elements partially restored activity to the defective elements (27). Furthermore, these studies recently have been extended to show that merely coexpression of the 5′ NC or PV with the defective IRES elements efficiently restored function (35). These studies suggested that complementation in trans between IRES elements occurred.

In this study, we have investigated the requirement for each of the major predicted stem-loop structures in the FMDV IRES for efficient internal initiation of protein synthesis. Furthermore, we have sought further evidence for the ability of an intact IRES element to complement defective IRES elements. Since the FMDV IRES is very different in sequence and predicted structure from the PV IRES, equivalent results would strengthen the view that such complementation may have a role in the biology of picornaviruses. We now show that four of five defective FMDV IRES elements tested can be efficiently complemented in trans by an intact FMDV IRES. The loss of the largest motif renders the IRES defective when assayed alone or in the presence of an intact IRES.

**MATERIALS AND METHODS**

**Plasmid constructions.** Standard methods (32) were used for the manipulation and growth of plasmid DNAs, which were purified by CsCl density gradient centrifugation prior to use in the transient expression assay. Plasmid pKSJC (Fig. 2) expresses a bicistronic mRNA transcript, encoding β-glucuronidase (GUS) (14) and chloramphenicol acetyltransferase (CAT) (10) reporter proteins, under the control of the T7 promoter. The FMDV IRES, as an EcoRI-ClaI fragment derived from pKSMHRM1.Cla (2), was inserted into pKS+ (Stratagene) to produce pSKRC1a and used as the template for deletion analysis. The same fragment was also inserted into EcoRI- and Clal-digested pSK+ to produce pSKRC1a, so that an antisense transcript is expressed from the T7 promoter (Fig. 2). The Smal-ClaI fragment from pSKRC1a containing the wild-type (wt) FMDV IRES was blunt ended and inserted into the vector pKSJC, previously digested with HindIII and blunt ended, to produce pKSJCSC (Fig. 2).

The PCR was performed essentially as described previously (32). The EMCV IRES element was produced by using primers GTGGCCATATAAGGATCCTG7'FF'ITTC and GG GACGGCCAGT) together with specific primers 1 (Table 1), which introduced the required deletions. Separate reactions using the complementary mutagenic primers (primers 2; Table 1) together with the reverse sequencing primer (AACAGCT ATGACCATG) were also performed. The half-reaction products were purified by agarose gel electrophoresis and mixed appropriately, and further PCRs were performed with only the forward and reverse primers. The fragments obtained were gel purified, digested with EcoRI and Clal, and ligated into EcoRI- and Clal-cut pSK+ or pKS+. Modified FMDV elements were constructed into the pKSJC vector as described above for the wt element except that blunt-ended EcoRI-ClaI fragments were used. All mutants were sequenced by direct double-stranded DNA sequencing using a T7 DNA polymerase-based sequencing system (Amersham).

**Transient expression assays.** Plasmids were assayed by transfection, using Lipofectin (Life Technologies), into HTK-143 cells infected with the recombinant vaccinia virus vTF7-3 (8), which expresses the T7 RNA polymerase as described previously (2). After 20 h, cell extracts were prepared and assayed for GUS activity in a fluorescence assay (14) and for CAT protein in a qualitative enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim).
TABLE 1. Deletions introduced into the FMDV IRES

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutagenic oligonucleotide</th>
<th>Sequence deleted*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKSGSCC</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>pKSGΔ1C</td>
<td>1, AGGAATTCCTCGTATACCTTA</td>
<td>-432 to -379</td>
</tr>
<tr>
<td></td>
<td>2, CGGGGTCGAGATCAAGCCAACTG</td>
<td></td>
</tr>
<tr>
<td>pKSGΔ2C</td>
<td>1, CACATTATGATGCTGACACGC</td>
<td>-375 to -164</td>
</tr>
<tr>
<td></td>
<td>2, AGCATCGTTCACGCAAGGATC</td>
<td></td>
</tr>
<tr>
<td>pKSGΔ3C</td>
<td>1, AGCAACTGGTGACGATATGCGA</td>
<td>-155 to -48</td>
</tr>
<tr>
<td></td>
<td>2, GCCCTCCGTCACCTATTGCTCACCAGCTTCT</td>
<td></td>
</tr>
<tr>
<td>pKSGΔ4C</td>
<td>1, TACCGCTGAATTTTTTTTTTA</td>
<td>-42 to -23</td>
</tr>
<tr>
<td></td>
<td>2, GTTGTATTTAAGGAAATTTTACG</td>
<td></td>
</tr>
<tr>
<td>pKSGΔ5C</td>
<td>1, GCAGGTAGTGACGCGCTGACCATCTATGCTG</td>
<td>-302 to -269,</td>
</tr>
<tr>
<td></td>
<td>2, CACACATGGTGGGATGCCCACCACATGCTCC</td>
<td>-266 to -258, and -253 to -244</td>
</tr>
</tbody>
</table>

*The numbering system used positions the A of the first initiation codon as nucleotide 1.

RESULTS

Determination of secondary structure motifs required for FMDV IRES function. The secondary structure prediction for the FMDV IRES, essentially as derived previously (29), has been used as the basis for precisely deleting predicted domains within the IRES (Fig. 1). Very similar models were proposed for the FMDV IRES and for the element from EMCV. Different laboratories have used alternative nomenclature (7, 13) for the various predicted secondary structure elements within the cardiovirus and aphthovirus IRES. A simpler system is shown in Fig. 1 and was used throughout this study. An alternative model for the EMCV IRES (7) is similar in many respects. However, at the 3' terminus, several short stem-loop structures (termed L, M, and N) which are not compatible with the FMDV sequence are predicted by the latter model. Within this region, the latter model (7) also includes the polyuridylic tract in secondary structure, in contrast to the models predicted for FMDV and EMCV previously (29). Alternative foldings of short sequences within the largest domain (domain 2) remain unresolved but do not affect the experiments undertaken in this study.

Each of the deletions indicated Fig. 1 and Table 1 was constructed by PCR as described in Materials and Methods. Each deletion was designed to precisely delete a predicted secondary structure feature. Note that deletion 5 removes only the hammerhead structure at the end of domain 2. The PCR fragments generated were cloned, sequenced, and constructed into vectors expressing bicistronic mRNAs transcribed from the T7 RNA polymerase promoter. The vectors contain the GUS gene as an indicator of cap-dependent translation, and this activity also verifies the expression of the plasmid within the cells. The efficient expression of CAT requires internal initiation of protein synthesis and is dependent on an active FMDV IRES.

Each of the plasmids expressing bicistronic mRNAs containing the wt or mutant IRES elements efficiently expressed GUS activity (Fig. 3a). However, marked differences in the level of CAT expression were observed (Fig. 3b). The expression of CAT directed by the wt IRES (plasmid pKSGSCC) served as a positive control, whereas the lack of expression from a plasmid (pKSGC) containing the GUS and CAT genes but lacking any IRES element (Fig. 2) served as a control for any

FIG. 3. Effects of coexpression of IRES elements on the expression of GUS activity. The indicated plasmids were transfected into vTF7-3-infected cells alone (a) or with pSKRCla (wt FMDV IRES; b), pSKRCla (antisense FMDV IRES; c) or pSKEMCRB (EMCV IRES; d). After 20 h, cell extracts were prepared and assayed for GUS activity. GUS activity from pKSGSCC assayed alone was set at 100% in each experiment, and other values were related to it. Mean values from four independent determinations are shown except for panel d, in which values are from a single experiment repeated twice with similar results.
expression resulting from reinitiation. Essentially no CAT expression was detected from this construct (Fig. 4a). Individual deletion of domains 2, 3, and 5 from the FMDV IRES reduced CAT expression to almost the background level. However, significant but low-level expression (ca. 3% of the wt level) of CAT was observed when domains 1 and 4 were individually deleted (Fig. 4a).

**Complementation of mutant FMDV IRES elements.** To determine whether the defects in the ability of the defective IRES elements were complementable in trans, each of the plasmids expressing the bicistronic mRNAs was cotransfected with a plasmid (pSKRCla) expressing the intact FMDV IRES. The results obtained for the IRES-directed expression of CAT are presented in Fig. 4. The results, mean values from four determinations, are presented as a percentage of the CAT expression obtained from plasmid pKSGGCC, which contains the wt FMDV IRES. The coexpression of the FMDV IRES, unlinked to any open reading frame, from plasmid pSKRCla enhanced expression of CAT from mutants with domains 1, 3, 4, and 5 individually deleted (Fig. 4b). The level of CAT expression observed was between 12 and 81% of that observed from the wt IRES. The deletion of domain 1 was particularly efficiently complemented. No enhanced CAT activity was ever observed from the construct lacking the large domain 2 or lacking all IRES sequences. It is noteworthy that the plasmid containing deletion 5 (deletion of only part of domain 2 [the hammerhead]) was very efficiently complemented. It may be that deletion of all of domain 2 interferes with the ability of the rest of the IRES to adopt its native conformation.

Coexpression of the intact FMDV IRES had some inhibitory effect on the expression of GUS from the plasmids (compare Fig. 3a and b), but this effect was very similar in each case and probably reflects competition for cellular components. The high efficiency of the complementation and also the inability to complement the deletion of domain 2 both argue strongly against the effect resulting from some form of recombination event between the plasmid DNAs. As a further control against this possibility, cotransfection with a plasmid (pSKRCla) containing the FMDV IRES in the opposite orientation to the T7 promoter was also performed. Under these conditions, no complementation, as indicated by enhanced CAT expression, of the defective mutants lacking domain 1, 3, 4, or 5 was observed (Fig. 4c). Very surprisingly, however, a consistent enhancement of CAT expression from the domain 2 deletion construct was observed. The antisense IRES also inhibited GUS expression (Fig. 3c). The latter finding was observed previously (35) in analogous studies with the PV IRES. The inhibition of GUS activity by the antisense IRES was most marked on the RNAs which contained the FMDV IRES. A substantially less severe inhibition of GUS expression from pKSGC was observed. This finding indicates that the interaction with the antisense RNA reduces the activity of the bicistronic mRNA in translation, perhaps by reducing its stability.

To determine whether the structurally related EMCV IRES was able to complement the defective FMDV IRES elements, we constructed a plasmid (pSKEMCRB; Fig. 2) containing this element unlinked to any open reading frame. The element used extends from the poly(C) tract to AUG10 just 8 bases upstream from the initiation codon. This EMCV IRES was unable to complement any of the defective FMDV IRES elements (Fig. 4d) but could complement deletions within the EMCV IRES when assayed with analogous plasmids (2a). It had an inhibitory effect similar to that of the FMDV IRES on the expression of GUS activity (Fig. 3d).

**DISCUSSION**

The FMDV and EMCV IRES are strikingly similar in predicted secondary structure. There is considerable agreement among several laboratories that a segment of about 450 bases is sufficient to direct efficient internal initiation of protein synthesis. However, some contradictory data on the precise 5' terminus of the element have been presented. Studies on the FMDV element (2) and the EMCV element (13) suggested that loss of sequence within domain 1 grossly impaired the
activity of the element. However, other studies (17) reported that partial loss of domain 1 of FMDV only had a modest effect (50% loss) on translational efficiency. Similarly, it has been reported that partial loss of sequence from this terminal stem-loop structure in EMCV impaired activity by about 70%, while complete loss of the element (and loss of a few bases from the next stem) resulted in a deficiency of only about 35% compared with the wt element (7). These results are of importance since this terminal structure is reported to be the major site of binding of the 58-kDa protein (PTB) which interacts with each of these elements. Resolution of these various results is not entirely clear. The data suggesting a minimal role for domain 1 have been obtained by using in vitro translation reactions on monomeric RNAs, whereas the evidence for a requirement for this structure has been obtained by using bicistronic mRNAs in cell culture. It has been suggested that partial motifs may interfere with other structures and hence give false answers (7). The presence of the upstream open reading frame in the bicistronic mRNAs may also perturb the structure of partial motifs. The precise deletion of this element in this study, without any predicted effect on the neighboring structures, severely inhibited the ability of the FMDV IRES to direct internal initiation, although some low-level (ca. 3% of the wt level) activity persisted. This result was consistent with our previous observations that the partial loss of this element made it severely defective (2).

Only limited mutagenesis of domains 2 and 3 has been performed previously. Linker insertions into the hammerhead structure at the end of domain 2 (as deleted in the domain 5 deletion) and into domain 3 completely abolished the function of the EMCV IRES (7). The results presented here show that precise deletion of the hammerhead structure and domain 3 within the FMDV IRES also totally abolished function.

We have used the secondary structure model for the 3'end of the IRES proposed by Pilipenko et al. (29), since the L, M, and N motifs suggested by Duke et al. (7) for the EMCV sequence are not compatible with the FMDV sequences. The stem-loop structure proposed by Pilipenko et al. (29) is compatible with both FMDV and EMCV sequences and leaves the polypyrimidine tract unstructured. Precise deletion of the predicted 3'-terminal motif (domain 4), leaving the polypyrimidine tract intact, also severely inhibited the activity of the IRES, although the activity was above the background level.

Complementation of defective IRES elements. The FMDV IRES is substantially different from that of PV. The sequence has little similarity, and the secondary structure prediction is completely different. However, they do have the same biological activity. In each case, not surprisingly, it has been shown that precise deletion of predicted secondary structure motifs can abolish the activity of the element when assayed alone. Deletion of domain IV from the PV IRES produces an element which retains significant activity, but none of major features of the FMDV IRES appear dispensable. More significantly, upon coexpression with an intact homologous element, unlinked to any other gene, enhanced activity is observed from the defective elements within bicistronic mRNAs. Recombination between the plasmid DNAs is ruled out by three lines of evidence. First, the efficiency of this unselected event is very high; up to 80% of the wt activity is observed from the defective elements when coexpressed with the intact IRES. Second, no enhanced CAT expression is observed when a plasmid containing the identical FMDV cDNA sequence, but in the opposite sense to the T7 promoter, is used (the one exception is discussed below). This plasmid should be able to recombine with the defective IRES elements in an equivalent manner to the construct containing the IRES in the opposite orientation. Third, the construct lacking domain 2 did not exhibit enhanced CAT expression when the wt IRES was coexpressed. If recombination was responsible for the restoration of CAT expression from the defective elements, one would expect that this defect would also be corrected.

It may be significant that the deletion of domain 1 is the most efficiently complemented (up to 81% of wt IRES activity). This is the region of the IRES to which different laboratories attribute very distinct properties (as discussed above) and to which p57/p58 (PTB) binds. It may be that this terminal region has an enhancing function as proposed for the stem-loop structure only on the 5' side of this domain in EMCV (13), which may also constitute a binding site for p57. Thus, its activity may be fully revealed only under stringent assay conditions, such as when the RNA is assayed in competition with other RNAs (cf. in vitro translation assays). However, since the EMCV IRES failed to complement even the deletion of this motif from the FMDV IRES, this element must do more than simply bind PTB.

The structurally related EMCV IRES was unable to complement any of the defective FMDV elements. This closely mirrors the inability of the rhinovirus 5' NCR to complement the defective PV IRES elements and the very limited ability of the more closely related coxsackievirus B4 5' NCR to perform the same function (35). These elements are about 60 and 70%, respectively, identical to the PV IRES over this region of the genome. This is a higher degree of identity than between the EMCV and FMDV IRES (about 50%).

Deletion of any sequence up to nt 528 within the PV 5' NCR (742 nt long) has proved to be complementable, but deletion of sequences at the 3'end of the IRES, including the region from nt 528 to 620, were not complemented by the cotransfection of the intact PV genome (27). Four independent deletions of the FMDV IRES have been complemented. However, it was not possible to complement, with the FMDV IRES, the deletion of the largest element, domain 2. It may be that the tertiary structure of the IRES is severely compromised by this large deletion. It is of interest that a single point mutation at the base of this large domain enhanced the activity of the type C FMDV IRES (19), suggesting that this domain is key to the structure and/or activity of the IRES.

The strangest result that we observed was the enhancement of CAT expression due to the deletion of the defective element lacking domain 2 when coexpressed with the antisense transcript of the FMDV IRES. We have no very satisfactory explanation for this phenomenon at present. Antisense oligonucleotides have been shown previously to enhance the translation of masked mRNAs (34). It was proposed that this resulted from competition with cellular proteins which were binding to the RNA and inhibiting its translation. It is difficult to adapt this concept to the enhanced activity of the defective IRES, however. An alternative possibility is that the antisense transcript, when folded by itself, forms a structure which allows the defective IRES to partially anneal to it and form a scaffold for the residual features of the defective IRES to function.

All of the data so far obtained indicating complementation of defective IRES elements by the coexpression of an intact IRES have been obtained by using the vaccinia virus-T7 RNA polymerase expression system (this report and reference 35). The cellular environment is clearly modified by the vaccinia virus infection. However, there is considerable accord between the results obtained in this assay system for other aspects of IRES function and those obtained by other workers using alternative strategies (2, 17, 22, 27, 35). An advantage of the vaccinia virus system is that it produces high levels of cytoplas-
mic transcripts as obtained with a natural picornavirus infection.

Two models for complementation of the PV IRES were suggested (35). One model suggests an RNA-RNA interaction in which the defective (and missing) element in one IRES is substituted by that domain from the intact element so that a functional unit is re-formed. This would be analogous to the regeneration in vitro of a functional EMCV IRES from two molecules by the melting and annealing of overlapping transcripts (4). The cosynthesis of the RNA transcripts within cells in our studies could obviate the need for such a melting procedure. The second model involves the transfer of a functional initiation complex from the functional IRES element to some point on the defective IRES structure in trans. In the case of FMDV and EMCV, this seems particularly attractive since previous studies (15) suggest that the viral RNA is recognized with respect to its ability to initiate protein synthesis from a point within 8 nt of the initiation codon itself. Thus, the region of the RNA in the immediate vicinity of the initiation codon may serve as an acceptor site for a preformed initiation complex. Very recently, it was shown (9) that a nonlinear migration of ribosomes on cauliflower mosaic virus RNA, which was demonstrated on individual RNA molecules, could also be achieved between two RNA molecules. These authors suggested analogous donor/acceptor regions for the ribosome shunt mechanism proposed.

Since the complementation of defective IRES elements has been observed for both major types of element found within the picornavirus family, it seems likely that this activity is important in the life cycle of these viruses.

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