Identification and Characterization of a *cis*-Acting Replication Element (*cre*) Adjacent to the Internal Ribosome Entry Site of Foot-and-Mouth Disease Virus

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Over the last few years, an essential RNA structure known as the *cis*-acting replicative element (*cre*) has been identified within the protein-coding region of several picornaviruses. The *cre*, a stem-loop structure containing a conserved AAACA motif, functions as a template for addition of U residues to the protein primer 3B. By surveying the genomes of representatives of several serotypes of foot-and-mouth disease virus (FMDV), we discovered a putative *cre* in the 5' untranslated region of the genome (contiguous with the internal ribosome entry site [IRES]). To confirm the role of this putative *cre* in replication, we tested the importance of the AAACA motif and base pairing in the stem in FMDV genome replication. To this end, *cre* mutations were cloned into an FMDV replicon and into synthetic viral genomes. Analyses of the properties of these replicons and genomes revealed the following. (i) Mutations in the AAACA motif severely reduced replication, and all viruses recovered from genomes containing mutated AAACA sequences had reverted to the wild-type sequence. (ii) Mutations in the stem region showed that the ability to form this base-paired structure was important for replication. Although the *cre* was contiguous with the IRES, the mutations we created did not significantly reduce IRES-mediated translation in vivo. Finally, the position of the *cre* at the 5' end of the genome was shown not to be critical for replication, since functional replicons and viruses lacking the 5' *cre* could be obtained if a wild-type *cre* was added to the genome following the 3D^{pol} coding region. Taken together, these results support the importance of the *cre* in replication and demonstrate that the activity of this essential element does not require localization within the polyprotein-encoding region of the genome.

Foot-and-mouth disease, one of the most important known pathogens of livestock, is caused by a small RNA virus of the family *Picornaviridae*. Foot-and-mouth disease virus (FMDV) is the prototype member of the *Aphthovirus* genus of this family, and although aspects of FMDV replication resemble those of many other picornaviruses, there are notable differences between FMDV and other viruses that include FMDV's broad host range and several unique genetic features.

The FMDV genome is over 8,300 bases in length and is covalently bound at its 5' terminus to a 23- to 24-amino-acid genome-linked protein, 3B. The genome encodes three copies of 3B, all of which are apparently utilized (8, 15). No natural isolates of FMDV with fewer than three 3B coding regions have been identified, suggesting that there is a strong selective pressure maintaining this redundancy, since homologous recombination within the FMDV genome (22) should readily eliminate redundant copies of 3B. Despite the observed retention of three 3Bs in all natural FMDVs, viruses lacking one of the 3Bs or with two nonfunctional 3Bs have been derived by genetic engineering (7). Recently, we have observed that viruses lacking two of the three 3Bs can be generated by similar methodology, and these "single-3B" viruses display a reduced ability to replicate in fetal bovine kidney cells, although they grow well in BHK cells and porcine kidney cells (J. M. Pacheco, T. M. Henry, and P. W. Mason, unpublished data).

Sequencing of the FMDV genome revealed a 5' untrans-

lated region (UTR) of over 1,300 bases in length (9), longer than the UTRs found in the genomes of other picornaviruses, such as the enteroviruses (poliovirus, 740 bases [16]) and cardioviruses (encephalomyocarditis virus, 850 bases [6]). The FMDV UTR begins with a 360-base hairpin loop (the S fragment), which is separated from the remainder of the genome by a 150- to 250-nucleotide RNase T₁-resistant tract containing approximately 90% C residues, with a small number of U and A residues (28). The poly(C) tract is followed by three to four tandemly repeated pseudoknots of unknown function (5) and the FMDV internal ribosome entry site (IRES) (4, 17).

Pilipenko et al. (30) derived a secondary-structure model for the 5' end of the FMDV genome by analysis of published nucleotide sequences and chemical and enzymatic treatment of RNAs derived from the same region of the genome of encephalomyocarditis virus (which has an IRES element similar to that of FMDV). This model identified five domains in the 5' UTR, including several IRES domains that have been shown to be important in binding host cell proteins, such as polypyrimidine tract-binding protein (19, 20, 23, 31) and an FMDV IRES-specific *trans*-activating factor, ITAF45 (31). Figure 1 shows a structure of the 5' end of the FMDV genome of serotype A12, derived from the model of Pilipenko et al. (30).

Several lines of investigation have indicated the importance of the highly structured 5' end of picornaviral genomes in replication (1–3, 13, 38). However, McKnight and Lemon reported in 1996 the surprising result that an RNA structure within the P1-encoding region of the human rhinovirus 14 genome was required for replication (26). These studies demonstrated that this element was able to act only in *cis*, since

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FIG. 1. Two-dimensional representation of a portion of the 5' UTR of FMDV serotype A12. The structures of domains 2 to 5 are based on the model of Pilipenko et al. (30). The *cre* (*cis*-acting replicative element) and its conserved AAACA sequence (boxed) are described in the text.

replicons lacking this portion of the genome could not replicate in the presence of helper viruses (26).

In a subsequent study, these investigators coined the term *cis*-acting replicative element (*cre*) and demonstrated that the *cre* was a short hairpin loop which contained the sequence AAACA in the unpaired loop that did not require translation to exert its activity (27). Since these reports, there have been reports of similar *cre* structures in the genomes of other picornaviruses. Surprisingly, these elements, although apparently responsible for the same function, are located in different regions of the genomes of these other viruses: 1B in the case of three different cardioviruses (18), 2C for poliovirus (11, 34), and 2A for human rhinovirus 2 (10). Analyses of *cre* activity of

wild-type and mutant genomes indicate that this essential structure forms in the positive-strand genome, not in the negative-strand copy (11, 27).

Recently, work on poliovirus replication suggested that the *cre* serves as the template for 3D^{pol}-mediated uridylylation of 3B (29, 34), a critical step in RNA replication, since the pUpU-3B serves as the primer for RNA synthesis. These studies showed that the *cre* stimulated the uridylylation of 3B by the picornaviral polymerase (3D^{pol}). Furthermore, the authors of these studies suggested that the conserved AAACA present in all *cre*s identified to date (see above) serves as a template for addition of the U residues to 3B (29, 34). Since uridylylation of 3B is a requisite for RNA replication, these studies help to

explain why the *cre* is essential for replication, but they do not explain why the activity must be supplied in *cis*.

Here we report the presence of a *cre* structure in the 5' UTR of FMDV in a region adjacent to the IRES that differs in location from a putative *cre* predicted to occur in the 2C region of the FMDV genome (37). Our *cre* structure was initially identified through its conservation among a number of different serotypes of FMDV, and its functionality was confirmed by examining the properties of viral genomes and replicons containing a panel of deliberately engineered *cre* mutations. Evaluation of the properties of the mutant genomes and replicons confirmed that the AAACA is absolutely required for FMDV genome replication but suggested that some changes in the stem structure could be tolerated. In addition, we found that replicating genomes and viruses could be recovered from synthetic genomes that contained an inactive (or deleted) *cre* in the natural 5' position and a wild-type *cre* added at the 3' end of the viral genome, downstream of the last coding region in the viral polyprotein.

MATERIALS AND METHODS

Cell lines, plaque assays, and cDNAs. Baby hamster kidney (BHK) cells, strain 21, clone 13 (American Type Culture Collection, Manassas, Va.), were maintained as previously described (33). These cells were used to propagate viruses and to perform plaque assays by standard techniques (33). Two genome-length cDNA clones encoding FMDV genomes for serotype A12 viruses [pRMC2, poly(C) tract of 2 bases, and pRMC35, poly(C) tract of 35 bases] and a heparan-sulfate binding serotype O1 chimera created from pRMC35 (pCRM4) have been described previously (33, 35).

RNA isolation, cDNA synthesis, PCR amplification, and sequencing. RNA was isolated from infected cell lysates, culture fluids harvested from infected cells, or individual viral plaques with Trizol (Life Technologies, Gaithersburg, Md.). Viral cDNAs were synthesized with Moloney murine leukemia virus reverse transcriptase (Life Technologies) with random hexamers as primers, and cDNA fragments were amplified by PCR (14) with specific oligonucleotides. PCR-amplified fragments were purified by with the Qiaquick kit (Qiagen, Valencia, Calif.) directly from the amplification reactions or from bands excised from agarose gels. Sequence analyses of these fragments were performed with selected primers and asymmetric amplification with Big-Dye terminators (ABI, Foster City, Calif.), followed by resolution on an ABI 3700 sequencer.

Construction of genome-length infectious cDNAs of type A12 containing the β -galactosidase gene. An FMDV replicon was created from the FMDV serotype A12 genome-encoding plasmid pRMC35 by using the following strategy. A DNA fragment containing the *Escherichia coli* β -galactosidase coding region was amplified by PCR (with *Taq* polymerase; ABI) from the p β gal-Control plasmid (Clontech, Palo Alto, Calif.) with a forward-sense oligonucleotide containing an *EcoRI* restriction endonuclease site present in codons 2 and 3 of the Lb form of the FMDV L^{pro} protein, followed by one additional L^{pro} codon and the first six codons of the β -galactosidase coding region, and an antisense oligonucleotide containing an artificial *XmaI* site (created by silent mutation at codons 17 and 18 for the 18-amino-acid 2A protein), the entire sequence of the 2A protein, a single Gln codon from the C terminus of 1D, and seven C-terminal codons of β -galactosidase. Following endonuclease digestion with *EcoRI* and *XmaI*, the fragment was inserted into these sites in a derivative of pRMC35 containing the artificial *XmaI* site in 2A, replacing the L^{pro}, 1A, 1B, 1C, and 1D coding regions. The resulting plasmid was sequenced through the junction regions, and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside staining of cells transfected with RNA transcribed from this plasmid (see below) confirmed that the replicon specified the production of β -galactosidase.

This β -galactosidase-containing plasmid was further modified to contain a 3' hepatitis delta virus ribozyme similar to one described previously (36). However, in this case the cleavage site was positioned to coincide precisely with the last base of the 15-nucleotide poly(A) tract, and a unique *SwaI* site was inserted downstream of the ribozyme. Finally, a 5'-end hammerhead ribozyme (identical to one used by Herold and Andino [12] to enhance replication by poliovirus replicons) was added to the 5' end of the FMDV S fragment of our β -galactosidase replicon plasmid, producing a plasmid designated pA12- β gal that could be used to produce RNA transcripts that began exactly at the predicted 5'-end UU

of the viral genome and terminated with 15 A residues. The 5' end of the genome of this plasmid with the hammerhead ribozyme was transferred to plasmid pCRM4 (encoding a tissue culture-adapted form of FMDV; see above) to create a hammerhead ribozyme derivative, pCRM4H.

Mutations in the *cre* were made by PCR overlap mutagenesis with standard techniques (14). Following reconstitution of the mutated fragments into the genome-length clones, the sequences of the amplified regions were obtained to confirm that only the desired mutations had been inserted. To create a replicon with a shortened poly(C) tract, this portion of the genome of a plasmid containing a poly(C) tract of only two C residues was substituted for the equivalent region of pA12- β gal. The *cre* was removed from the 5' end of the genome and added to the 3' end of the genome with similar PCR overlap mutagenesis procedures (14).

Derivatives of replicon plasmids that were able to transcribe RNAs capable of translation in the absence of replication were created by removing a 3,228-base fragment (precisely 1,076 codons) extending from a *BglII* site at codons 106 to 108 of 2B to a *BamHI* site at codons 273 to 275 of 3D.

In vitro RNA synthesis and transfection. Plasmids containing genome-length cDNAs were linearized at the *SwaI* site following the 3' hepatitis delta virus ribozyme of pA12- β gal or *StuI* following the poly(A) tract of pCRM4H and used as templates for RNA synthesis with the MegaScript T7 kit (Ambion, Austin, Tex.) following the manufacturer's instructions. Transcripts were purified from unincorporated nucleotides by precipitation with LiCl and then quantitated by measuring their optical density at 260 nm. BHK cells were transfected with these synthetic RNAs by an electroporation method (24) adapted to an Electroporation Manipulator 600 (BTX Corp., San Diego, Calif.). Briefly, 0.5 ml of BHK cells at a concentration of 1.5×10^7 cells/ml in phosphate-buffered saline (PBS) were mixed with 10 μ g of RNA in a 4-mm-gap BTX cuvette. The cells were then pulsed once at 330 V, infinite resistance, and a capacitance of 1,000 μ F and diluted in cell growth medium, and a portion of the cells were allowed to attach to 35-mm-diameter tissue culture plates. The dead cells and medium were removed after 2.5 to 4 h, and fresh medium was added to the cultures, followed by incubation at 37°C for up to 24 h (24). In some cases, RNAs were introduced into cells with Lipofectin (Life Technologies) following the manufacturer's recommended procedures.

In vivo translation experiments. To determine whether mutated 5' UTRs could direct translation in BHK cells (in the absence of replication), truncated replicon plasmids created as described above were transfected into BHK cells along with a plasmid encoding cytomagalovirus promoter-driven bacteriophage T7 polymerase (pCB-T7). For these experiments, Lipofectamine Plus reagent (Life Technologies) was used to transfect 12.5-mm-diameter monolayers of cells with a total of 800 ng of DNA (consisting of an equimolar ratio of FMDV and T7 polymerase plasmids), as recommended by the manufacturer.

Virus recovery. Following overnight growth at 37°C, tissue culture plates containing the 35-mm-diameter monolayers of electroporated cells were frozen and thawed to lyse the cells. Ten percent of the combined lysate/culture fluid was used to infect fresh BHK monolayers, which were incubated for up to 48 h prior to freezing. In some cases, lysed monolayers from this BHK passage 1 were used to perform subsequent passages in a similar manner. In other cases, the passage 1 material was used as an inoculum to infect cells for the isolation of individual plaques, which were picked from agarose-overlaid monolayers and amplified once by growth on 35-mm-diameter monolayers by standard techniques.

β -Galactosidase activity assay. Following electroporation of β -galactosidase-expressing replicon RNAs or transfection of DNAs encoding truncated replicons with Lipofectamine Plus, monolayers of cells were incubated for the indicated times and then washed twice with PBS and lysed in β -galactosidase lysis buffer (Clontech Labs, Palo Alto, Calif.). Samples were then frozen, thawed, clarified, and assayed for the presence of β -galactosidase with a luminescent β -galactosidase assay system, as described by the manufacturer (Clontech Labs). The units of β -galactosidase present in each sample were determined by using a standard curve created with known concentrations of β -galactosidase, and the protein content of each lysate was also determined (Coomassie protein assay reagent; Pierce, Rockford, Ill.), permitting expression of data as units of β -galactosidase per nanogram of cell protein. In some cases, the protein-standardized activity was then divided by the values obtained from wild-type constructs used in side-by-side electroporations or transfections to present mutant expression as a percentage of the wild-type values.

RESULTS

Analyses of sequence data from the L fragment [poly(C) to poly(A)] of the genomes of SAT2/KEN/3/57 (GenBank accession no. AJ251473) and O1 Kauffbueren (GenBank accession

FIG. 2. Schematic diagram of replicon and infectious FMDV genomes encoded by plasmids pA12-βgal and pCRM4H, respectively. Open boxes indicate protein-coding regions, and lines indicate RNA structures. The hatched box shows the position of the *E. coli* β-galactosidase-coding region in the replicon plasmid pA12-βgal. βgal, β-galactosidase; S, short fragment of the genome; poly(C), polycytosine tract; PKs, pseudoknots; *cre*, *cis*-acting replicative element; IRES, internal ribosome entry site. Hairpin loop structures below the genome contain the wild-type and mutant versions of the *cre*, with altered nucleotides circled.

no. X00871) and the complete genomes of C3 Argentina (GenBank accession no. AJ007347), A12 (GenBank accession no. M10975 and L11360), and O/TAW/97 (GenBank accession no. AF308157) revealed the presence of several AAACA motifs in the genomes of these viruses. However, among these genomes, there was only a single AAACA that was common to all, and it was located following the pseudoknot region of the A12 genome [nucleotides 242 to 246 following the poly(C) tract of infectious cDNA plasmid pRMC35]. Furthermore, folding of this segment [nucleotides 220 to 273 following the poly(C) tract] with mFold program version 3.1 (25, 40) produced a stem-loop structure with an exposed loop containing the AAACA for all of these genomes (results not shown).

A schematic diagram depicting the putative *cre* of the A12 genome is shown in Fig. 1 along with domains 2, 3, 4, and 5 drawn as derived by Pilipenko et al. (30). Interestingly, the descending strand of the stem of the *cre* element overlaps structural domain 1 of the 5' UTR predicted by Pilipenko et al. (30) based on computer modeling and biochemical analyses of the homologous cardiovirus 5' UTR. The domain 1 defined by Pilipenko et al. (30) has been previously shown to have little influence on the translational activity of the FMDV IRES in vitro (17), but it is often included in published representations of the FMDV IRES.

To analyze the function of the putative *cre* in RNA replica-

tion in the absence of viral replication and transmission, we used the β-galactosidase-expressing replicon RNA shown in Fig. 2. Evaluation of this β-galactosidase replicon in electroporated BHK cells showed that the replicon produced large amounts of β-galactosidase, with a peak in activity detected approximately 12 h postelectroporation (Fig. 3). This β-galactosidase activity was substantially reduced by the addition of guanidine HCl, which blocks FMDV multiplication (32) through inhibition of RNA synthesis, consistent with inhibition

FIG. 3. Time course of β-galactosidase (βgal) production by cells transfected with β-galactosidase-expressing replicons from pA12-βgal (wild-type *cre*; ♦), pA12-C2-βgal (wild-type *cre*; ▲), and pA12-βgal in the presence of 5 mM guanidine HCl (■).

FIG. 4. Replication and in vivo translation activity of replicon RNAs electroporated into BHK cells and truncated replicon-derived cDNAs transfected into BHK cells, respectively. The structures of the wild-type (WT) and mutant replicons in the pA12- β gal-derived RNAs shown along the bottom are illustrated in Fig. 2. Open bars show “in vivo” β -galactosidase (β gal) translation activity from the average of duplicate transfections containing equimolar amounts of plasmid DNA encoding replication-incompetent (truncated) genomes and the bacteriophage T7 polymerase (harvested 22 h posttransfection; see the text). Hatched bars show β -galactosidase activity recovered from a single representative experiment in which cells were transfected by electroporation with 10 μ g of LiCl-purified, in vitro-transcribed RNA (harvested 12 h postelectroporation; see the text).

of replication with no effect on translation. However, we were not able to conclude that all of the residual β -galactosidase activity (Fig. 3) was due to translation of the transfected RNAs, since we did not determine if the level of guanidine HCl used completely inhibited replication.

In the same experiment, we included a replicon containing a poly(C) tract of only two C's (pA12-C2- β gal), since our wild-type replicon contains a poly(C) tract of only 35 residues and the poly(C) tracts of synthetic FMDV RNAs elongate during passage in culture (33, 39). The C2 replicon replicated to levels similar to those of the replicon containing 35 C's, suggesting that a very long poly(C) tract is not required for genome replication, consistent with studies showing that poly(C) tract length affects the specific infectivity of viral particles but not viral replication (E. Rieder and P. W. Mason, unpublished data). In the particular experiment shown in Fig. 3, the C2 replicon replicated better than the pA12- β gal replicon, but other experiments showed a slightly better performance by the C35 replicon (results not shown). The fall-off in β -galactosidase activity at 12 h postelectroporation is likely the result of cytopathic effect induction within cells replicating the β -galactosidase-expressing genomes, which results in their detachment from the monolayer and loss of β -galactosidase activity from the cell lysates being tested.

All replicon RNAs encoding *cre* mutants showed a significantly reduced ability to replicate in transfected cells (Fig. 4). Among these, the only mutant that showed activity significantly above the background level was mutant 5. To confirm that this reduction in activity was not due to an effect of these mutations on IRES-mediated translation, we evaluated the translational activity of these mutant 5' UTRs by quantitating β -galactosi-

dase activity in BHK cells transfected with plasmid DNAs encoding truncated genomes (Fig. 4). These data clearly show that the mutations in the *cre* had only modest effects on the translational activity (Fig. 4), demonstrating that these mutations exerted their effects at the level of genome replication.

The same panel of *cre* mutations were inserted into full-length genomes specifying a cell culture-adapted virus to further evaluate the importance of this segment on genome replication. Recovery of virus from cells electroporated with these genomes showed that by the second or third passage of lysates from electroporated cells, live virus could be recovered from all mutant genomes except for the double mutation (ACCCA, mutant 6; results not shown). Evaluation of the *cre* sequences from the recovered viruses revealed a reversion to the wild-type AAACA for all point mutants in our panel (mutants 1, 3, 4, and 5; results not shown). Evaluation of the sequences of the *cre* from the viruses recovered from the first passage of the “stem” mutants 2a and 2b revealed that the mutated bases and their predicted partners in the putative stem-loop structure shown in Fig. 2 were present as mixtures.

To investigate the mixtures of viruses in the first passage recovered from cells transfected with RNA from pCRM4H mutant 2a, the BHK passage 1 material obtained from cells transfected with RNA containing mutation 2a was used as a source of viruses for plaque purification. Following purification, the 5' UTRs of six plaque-picked viruses were sequenced through a 475-base region commencing 15 bases downstream from the poly(C) tract (encompassing the 4 pseudoknots, the *cre*, and most of the IRES of these genomes). These sequence analyses revealed that one of the six plaque-purified isolates contained a mixture of genomes, while the remaining five contained one predominant genotype (no mixtures were detectable at any position).

The *cre* sequences detected in these five plaque isolates are shown in Fig. 5. In the case of plaques 2a.2 and 2a.4, identical sequences were revealed in the *cre*, although one of these isolates had point mutations within the pseudoknot region of the genome, indicating that they were separate clones. The other four plaque isolates had wild-type sequences throughout the sequenced region except for the substitutions shown in Fig. 5. Evaluation of the structures shown in Fig. 5 suggests that the stem-loop structure is a critical requirement for viral replication. Interestingly, one of the recovered plaques (plaque 2a.3) had four U residues in the mutated region, which does not appear to produce a strongly base-paired stem. However, computer folding (mFold) (25, 40) of a larger RNA sequence, extending from pseudoknot 4 through domain 2, indicated that the favored conformation of the wild-type and all four mutant 2a revertants shown in Fig. 5 formed the *cre* stem-loop, whereas mutants 2a and 2b (Fig. 2) did not (results not shown).

To determine how fit these mutant plaques were, we performed plaque assays on BHK cells with the same stock of virus that was used to obtain the sequence data shown in Fig. 5. Photographs of these plaques are shown in Fig. 6. All of the revertant viruses produced plaques that were considerably smaller than those obtained with wild-type virus, although mutant 2a.1, predicted to have the most stable structure at the base of the loop (Fig. 5), formed the largest plaques (Fig. 6).

To determine if the 5' UTR localization of the *cre* was a requirement for FMDV replication, we undertook a study to

FIG. 5. Predicted structures of the *cre*s of five different plaque isolates recovered from cells transfected with a viral genome encoding *cre* mutant 2a. Substitutions relative to the transfected RNA are shown in lowercase letters and identified with arrowheads (wild-type and mutant 2a structures are shown at the left for comparison).

determine if we could translocate the element to the 3' end of the polyprotein. To this end, a wild-type form of the *cre* element was inserted immediately following the stop codon found at the end of the viral polyprotein (Fig. 7) in replicons and genomes containing either the wild-type 5' UTR *cre* or a mutant 6 5' UTR *cre*. Both replicons were able to replicate well compared to wild-type virus (51% and 34% of wild-type activity, respectively, measured as shown in Fig. 4), strongly suggesting that the 3' UTR *cre* was functioning, since the mutant 6 *cre* was completely inactive in the replicon context (Fig. 4). Studies with the double *cre*-containing viral genomes showed similar results. Viruses were readily harvested from the ge-

nomes containing the wild-type form of the *cre* in the 3' UTR and either the wild-type or nonfunctional mutant 6 in the 5' UTR. More importantly, we found that viable viruses with a complete deletion of the *cre* element could be created (mutant 7 [Fig. 7]) if a *cre* was added at the start of the 3' UTR (Fig. 7). Viruses recovered from cells transfected with these 3' *cre* viruses, containing either a double-mutated 5' *cre* (mutant 6) or no 5' *cre* (mutant 7), were able to form plaques on BHK cells, although the plaques were considerably smaller than those produced by the wild-type virus (Fig. 8). Sequence analyses of the double *cre* viruses revealed that both maintained the 3' *cre* for two passages without any modification. Additionally,

FIG. 6. Photographs of plaques of the indicated viruses on BHK cell monolayers. Plaques from the wild-type virus (derived from pCRM4H) were produced by the second passage from the electroporated stock. Plaques from the plaque-picked isolates were produced by the BHK-amplified plaques picked from the first passage postelectroporation (this is the same passage used for the sequence analyses; see text for details).

FIG. 7. Schematic diagram of replicon and infectious FMDV genomes showing the positions of the *cre* deletion (mutant 7) and the insertion of the *cre* at the 3' end of the genome. For this portion of the figure, lowercase letters indicate specific mutations to the genome to facilitate construction, and underlined lowercase letters indicate bases added to the genome to facilitate construction. Other abbreviations and nomenclature are explained in the legend to Fig. 2.

the virus with the mutant 6 *cre* at the 5' end maintained this mutated 5' *cre* during passage, further confirming the functionality of the 3' UTR *cre*. Consistent with these studies, we found that in vivo translation activity of the mutant 7 5' UTR (measured as described in the legend to Fig. 4) was 89% of the value obtained with the wild-type UTR.

DISCUSSION

Upon entering a susceptible host cell, the picornavirus genome must be translated by host ribosomes, and the resulting protein products must then be used to produce the negative-sense form of the genome that is needed for subsequent pro-

FIG. 8. Photographs of plaques produced by the indicated viruses on BHK cell monolayers. Plaques for all three viruses were produced by virus recovered from the second passage of lysates of cells transfected with synthetic genome-length RNAs by using Lipofectin (see the text).

duction of the positive-stranded genomic RNA. An orchestration of the initial translation and replication activity is likely to be required to ensure efficient viral replication, and hence these viruses must have evolved a method to ensure that translation and replication (which take place in different directions on the infecting genome) do not interfere with each other (3, 13). The fact that picornavirus genomes are infectious in the absence of other viral factors supports the assumption that factors controlling this orchestration are likely to reside in primary or secondary RNA structures. However, it is clear that these structural elements are altered by their interaction with cellular proteins, which are known to influence translation (see the introduction).

The *cre* has been identified in several picornavirus genomes, but to date it has always been found within the polyprotein-encoding region (see the introduction). However, data from McKnight and Lemon (27) indicated that translation of the sequences encoded by the human rhinovirus 14 *cre* element was not a requirement for genome replication. Furthermore, McKnight has recently shown that a replicating human rhinovirus 14 genome with the *cre* moved to the 3' end of the genome can be obtained (K. McKnight, personal communication). These data argue against a model recently proposed by Barton and coworkers, which suggests that the localization of the *cre* within the open reading frame contributes to the ability of the virus to control translation and replication by ensuring that the *cre* does not function to provide uridylylated 3B until the polyprotein-encoding region of the genome is cleared of translating ribosomes (3). Although the translation of the *cre* may serve as a source of this type of control for other picornaviruses, the same function must be provided by a different mechanism for FMDV, since the FMDV *cre* is in the 5' UTR upstream of the ribosome entry site, and the FMDV *cre* can also function when translocated to the 3' UTR.

On the surface, our ability to translocate the *cre* element from one end of the genome to the other appears to be a dramatic accomplishment. However, models for genome replication indicate that the 5' and 3' ends of the picornavirus genome are noncovalently linked to each other by host cell and viral proteins to facilitate efficient translation and replication (3, 13). Furthermore, Lyons et al. have recently demonstrated that the 5' end of the poliovirus genome is required in *cis* for efficient VPg uridylation and negative-strand RNA synthesis, suggesting that the 5' and 3' ends of the genome must interact at this crucial step in the replication cycle (21). If there is a similar requirement for bringing together the 5' and 3' ends of the FMDV genome, then our ability to translocate the *cre* may not result in significant movement of the *cre* from the three-dimensional context in which it normally functions.

Data obtained in our characterization of mutated forms of genetically engineered replicons, as well as the phenotypes and genotypes of viruses recovered from mutated forms of genetically engineered viral genomes, confirmed that the FMDV *cre* is required for replication. Interestingly, the *cre* is found adjacent to the IRES, suggesting that this location could be important for orchestrating the relationship between translation and replication, although this mechanism is apparently not used by other picornaviruses which have the *cre* located within the polyprotein-encoding portion of their genomes (see above). Although our translation studies indicated a minimal effect of

the *cre* mutations (including a complete deletion) on translation, it is possible that there is a subtle interaction between these contiguous replication and translation elements that is important for efficient viral replication and spread in the animal host. This supposition is consistent with the small-plaque phenotype of viruses in which the *cre* has been translocated to the 3' end of the genome.

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