JOURNAL OF VIROLOGY, Sept. 1993, p. 5139–5145
0022-538X/93/095139-07$02.00/0

Vol. 67, No. 9

Genetically Engineered Foot-and-Mouth Disease Viruses with Poly(C) Tracts of Two Nucleotides Are Virulent in Mice

ELIZABETH RIEDER, THOMAS BUNCH, FRED BROWN, AND PETER W. MASON*

Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture, Greenport, New York 11944-0848

Received 16 February 1993/Accepted 20 May 1993

To determine the role of the poly(C) tract found at the 5′ end of the genome of foot-and-mouth disease virus, synthetic RNAs (in vitro transcripts) with poly(C) tracts of different lengths have been produced and evaluated. RNAs with poly(C) tracts of 35, 25, 16, 6, or 2 residues displayed similar specific infectivities in baby hamster kidney (BHK) cells. Viruses recovered from cells transfected with in vitro transcripts containing 6 to 35 Cs had properties similar to those of the wild-type virus in cell culture, and poly(C) tracts present in the synthetic RNA-derived viruses ranged from 75 to 140 bases in length. Viruses recovered from transcripts containing only two Cs showed very different properties. Specifically, viruses grew to much lower levels in cell culture and maintained a poly(C) tract of only two residues. The pool of viruses harvested from cells transfected with the synthetic C_2 RNA also contained a small amount of a virus with a 42-base deletion in the region of the poly(C) tract, which appeared to have arisen by recombination. Taken together, these data suggest that recombination provides the mechanism of poly(C) elongation and that viruses with poly(C) tracts over 75 bases in length have a selective advantage in cell culture. Interestingly, all of the in vitro transcript-derived viruses [including viruses with poly(C) tracts of only two residues] were equally virulent in mice, indicating that poly(C) tract length has no effect on virulence in this animal model.

Foot-and-mouth disease virus (FMDV), a member of the family *Picornaviridae*, causes a devastating disease of domestic animals. Despite the successful application of inactivated vaccines in controlling the disease, there are some risks associated with the use of these products. In particular, the escape of virus from vaccine production plants and failure to completely inactivate the vaccine have led to outbreaks (1). Furthermore, the virus is subject to extensive variation, and antigenic variants which are not recognized by animals immunized with vaccine viruses often arise. Consequently, alternative approaches to vaccines need to be investigated. One approach involves the possibility of producing a deliberately attenuated vaccine virus by genetic engineering. This strategy has recently been employed by Duke et al. (9), who showed that cardioviruses with shorter-than-natural poly(C) tracts were dramatically attenuated.

Picornaviruses contain a single-stranded positive-sense RNA genome of 7,100 to 8,300 nucleotides that is polyadenylated at the 3′ terminus and covalently bound at its 5′ end to a small protein, VPg. The single long translational open reading frame in the viral genome is flanked at each end by untranslated regions (UTR) which carry signals for replication. The 5′ UTR, which can range from 610 to 1,300 nucleotides, also encodes the internal ribosome entry site, where host cell ribosomes initiate translation (17).

The 5′ UTR of cardioviruses and aphthoviruses contains a homopolymeric poly(C) tract of unknown function (4, 27). The poly(C) tract separates the 150- to 370-base 5′ portion of the genome (small fragment) from the remainder of the genome (large fragment), which contains a series of pseudoknots, the internal ribosome entry site, and the open reading frame (6, 7, 14, 25, 30). For FMDV, poly(C) tracts ranging from 100 to 420 residues interspersed with an occasional U residue have been reported (2, 11, 14). On the basis of their demonstration that a virulent strain of FMDV type SAT1 had a longer poly(C) tract than the attenuated strain derived from it, Harris and Brown (15) suggested that the tract may have a role in determining virulence. However, in a study of two viruses of serotype C, Costa Giomi et al. (8) found that poly(C) tract length could not be directly related to virulence in cattle. Further uncertainty over the role of poly(C) in attenuation has arisen from a recent paper by Escarmis et al. (11) indicating that an FMDV strain rescued from persistently infected cells, which was highly attenuated for cattle and mice, had a poly(C) tract of approximately 420 residues (145 residues longer than its parental strain).

Recently, the engineering of cardioviruses with shorter-than-natural poly(C) tracts has been reported (10). These viruses maintain short poly(C) tracts in HeLa cells and display growth properties similar to those of the wild-type virus in cell culture. However, viruses with poly(C) tracts shorter than 30 nucleotides are dramatically attenuated in mice (9), providing a direct link between the length of the poly(C) tract and virulence in the cardioviruses.

It has not been possible to address the role of poly(C) length in virulence by using a full-length cDNA clone of FMDV type O because viruses derived from transcripts of this cDNA [which had shorter-than-natural poly(C) tracts] had acquired much longer poly(C) tracts (38). Furthermore, the in vitro RNA derived from this cDNA was 5 orders of magnitude less infectious than the authentic viral RNA, suggesting that (i) FMDV RNAs containing short poly(C) tracts were inherently poorly infectious and (ii) progeny of these transfections resulted from infrequent events which produced longer poly(C) tracts (38).

We report here the production of synthetic RNAs of FMDV type A_{12} that differ in the length of their poly(C) tracts and the properties of viruses recovered from BHK cells transfected with these RNAs. Viruses derived from RNAs containing poly(C) tracts of 6 or more nucleotides had acquired much longer poly(C) tracts and were virulent in

* Corresponding author.

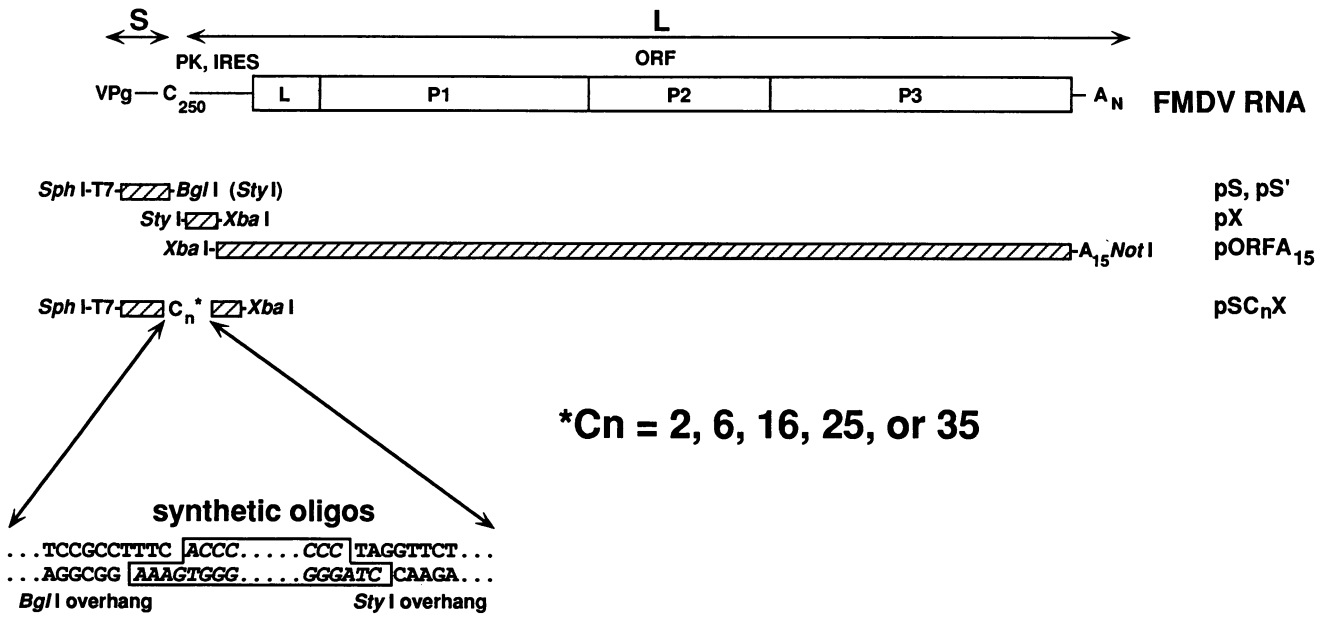

FIG. 1. Schematic diagram showing the strategy for assembly of full-length infectious clones of FMDV type A_{12}. S, short fragment; L, long fragment; PK, pseudoknot; IRES, internal ribosome entry site; ORF, open reading frame.

mice. Viruses derived from RNAs containing poly(C) tracts of 2 nucleotides maintained this poly(C) tract length during growth in cell culture and grew poorly in BHK cells but were virulent in mice. These results are discussed with reference to the differences between cardioviruses and aphthoviruses with regard to poly(C) tract length, mechanism of elongation, and role in virulence.

MATERIALS AND METHODS

Cell lines, viruses, and plasmids. Baby hamster kidney cells (BHK-21 clone 13; ATCC CCL10) were maintained in Eagle's minimal essential medium containing 10% calf serum and 10% tryptose phosphate broth, supplemented with antibiotics. FMDV type A_{12} 119ab (large-plaque variant of strain 119) was provided by B. Baxt (Plum Island Animal Disease Center, Greenport, N.Y.). Plaque assays were performed on BHK cells by using the tragacanth overlay technique previously described (12), and titers in PFU were determined by using monolayers stained at 48 or 72 h postinfection (or posttransfection). Viral plaques were picked from infected monolayers overlaid with Seaplaque agarose (FMC, Rockland, Maine). Plasmid pTRP123 containing most of the coding region of FMDV type A_{12} (34) was supplied by M. Grubman (Plum Island Animal Disease Center). The low-copy-number plasmid pCL1921 (20) was supplied by M. Inouye (Robert Wood Johnson Medical School, Piscataway, N.J.), and pGEM plasmids were obtained from Promega (Madison, Wis.).

Preparation of viral RNA and production, cloning, and assembly of cDNAs. Viral RNA was prepared as described by Grubman et al. (12), except that proteinase K treatment was not used and RNA was not purified on sucrose gradients. cDNAs were synthesized with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, Md.) by the manufacturer's protocols, using 90 to 500 ng of virion RNA and 100 pmol of antisense oligonucleotide. cDNA fragments were amplified by using the polymerase chain reaction (PCR) (31, 32), with Amplitaq polymerase (Perkin-Elmer, Norwalk, Conn.), the buffer recommended by the manufacturer, and 0.5 μM each primer. Thermal cycling was performed on a Coy (Ann Arbor, Mich.) thermal cycler, and typical profiles included 20 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 75°C. PCR fragments were digested with restriction enzymes, ligated to cleaved plasmid DNAs, and transformed into *Escherichia coli* DH5α (GIBCO BRL) by standard techniques. Sequences were determined by the dideoxy chain termination method (33, 37), using Sequenase (U.S. Biochemicals, Cleveland, Ohio).

cDNA fragments from the 5′ UTR of FMDV were amplified by using an oligonucleotide primer identical to the first 19 nucleotides of the genomes of strains A_{10}, C_1, C_2, and $Asia_1$ (13, 23) and an oligonucleotide complementary to sequences 313 nucleotides 5′ of the first functional translation initiation site in the A_{12} genome (29). Sequence analysis of plasmids containing these PCR products revealed poly(C) tracts of only 8 to 14 nucleotides (1/20th of the length found in virion RNA), consistent with internal recombination during amplification or slippage by *Taq* polymerase (3, 19). Analysis of the sequence data from these cDNAs revealed two different cDNA sequences adjoining the poly(C) tract, C_nTAGG and C_nTAAG, with the former sequence being found in two of six plasmids sequenced. We presumed that both sequences represented viable polymorphisms, consistent with the quasispecies nature of FMDV (21), and selected the sequence containing the *Sty*I restriction enzyme site (CCTAGG; Fig. 1) for all further work. In addition, the sequence preceding the poly(C) tract included a portion of a *Bgl*I restriction site (Fig. 1). Using these two naturally occurring restriction sites, we devised a strategy to insert synthetic poly(C) tracts (Fig. 1). cDNA fragments including a synthetic T7 promoter, the entire small fragment, a *Bgl*I site, and the *Sty*I site were generated by using chimeric oligonucleotides and PCR. These cDNA fragments were cloned into a pGEM3 derivative, and two different plasmids, pS and pS′ (which were subsequently shown to differ by a single nucleotide at position 200 [see below]), were selected

for further constructions (Fig. 1). DNAs from these plasmids were digested at the *Sty*I and *Bgl*I sites and ligated to synthetic poly(dC-dG) oligonucleotides containing either 25 or 35 C/Gs (Fig. 1). These ligations yielded multiple recombinant plasmids, and two of these, which were confirmed by sequence analysis to contain the correct number of Cs, were designated pSC_{25} and $pS'C_{35}$.

A plasmid containing cDNA spanning the 5′ end of the large fragment (pX [Fig. 1]) was produced by a similar strategy. The cDNA portion of pX was released with *Sty*I and *Xba*I and ligated to plasmids pSC_{25} and $pS'C_{35}$ to produce recombinant plasmids ($pSC_{25}X$ and $pS'C_{35}X$) which contained sequences surrounding the poly(C) tract that were identical to the authentic A_{12} genome. Three additional plasmids containing poly(C) tracts of 2, 6, or 16 Cs (pSC_2X, pSC_6X, and $pSC_{16}X$) were generated by similar procedures. Sequence analyses of the cDNAs found in all five recombinant plasmids confirmed the expected poly(C) tract lengths and revealed a polymorphism at position 200 in the genome (A in pSC_6X, $pSC_{16}X$, and $pSC_{25}X$ and C in pSC_2X and $pRS'C_{35}X$), which is in an unpaired region of the small fragment stem-loop structure (5).

A cDNA molecule terminating with poly(A) and a unique restriction site was produced by using PCR with a sense oligonucleotide at position 7573 in the FMDV A_{12} open reading frame (29) and an antisense oligonucleotide containing a *Not*I site followed by 15 Ts. The cDNA product from this amplification reaction was cleaved at the internal *Bam*HI site and in the primer-encoded *Not*I site and ligated to DNA from a derivative of the low-copy-number plasmid pCL1921. Several plasmids were sequenced, and one which contained 15 A residues (pA_{15}) was selected for further use. DNA from pA_{15} was cleaved with *Xba*I and *Bam*HI, and the 6.8-kb fragment encoding the bulk of the open reading frame from pTRP123 (34) was added, yielding plasmid $pORFA_{15}$ (Fig. 1).

Full-length FMDV A_{12} cDNAs were assembled by ligating the 7.5-kb *Xba*I-*Not*I fragment of plasmid $pORFA_{15}$ into plasmid $pSC_{25}X$, producing plasmid $pRMC_{25}$. Four additional full-length cDNA clones ($pRMC_{35}$, $pRMC_{16}$, $pRMC_6$, and $pRMC_2$) were then derived from $pRMC_{25}$ by substituting the *Sph*I-*Xba*I fragments from $pS'C_{35}X$, $pSC_{16}X$, pSC_6X, and pSC_2X (Fig. 1).

In vitro RNA synthesis and transfection. RNA was synthesized from restriction endonuclease-linearized plasmid DNA templates by using T7 polymerase (GIBCO BRL) (35). T7 reaction mixtures were then treated with RNase-free DNase I (Boehringer Mannheim, Indianapolis, Ind.), extracted with phenol and chloroform, and precipitated twice with ethanol. RNA integrity was evaluated by electrophoresis in agarose gels, and concentrations were determined by optical density at 260 nm. BHK cells plated in 35-mm-diameter dishes were transfected with RNA by using Lipofectin (GIBCO BRL) and overlaid with medium containing 1% serum, and samples of culture fluid were collected at 72 h posttransfection. For determination of specific infectivity, transfected monolayers were overlaid with tragacanth, and plaques were visualized as described above.

Determination of poly(C) tract length. The lengths of the poly(C) region of the genome present in in vitro transcripts and viral RNAs were determined by primer extension. Briefly, in vitro transcripts or crude virion RNAs (prepared from culture fluid by ultracentrifugation through a sucrose cushion) were annealed to a ^{32}P-labeled oligonucleotide complementary to sequences 3′ of the poly(C) tract, and reverse transcription was carried out at 42°C with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The primer extension products were then treated for 2 h at 60°C with 0.3 M NaOH to hydrolyze the RNA and analyzed on a 4% polyacrylamide sequencing gel. Regions encompassing the poly(C) tracts were amplified by using a slight modification of the procedure described above and analyzed by polyacrylamide gel electrophoresis, sequenced by the methods of Higuchi and Ochman (16) or Winship (36), or cloned into pGEM plasmids and sequenced as described above.

Mouse virulence studies. Tenfold dilutions of virus were prepared in phosphate-buffered saline containing 1% calf serum, and aliquots were simultaneously titrated by plaque assay or by intraperitoneal injection into groups of 5 to 10 7-day-old mice. Mice were observed for 14 days, and 50% lethal doses (LD_{50}s) were determined by the method of Reed and Muench (28).

Quantitation of relative abundances of C_2 and $C_2\Delta_{42}$ genomes. PCR products were prepared from the crude viral RNAs, using an oligonucleotide identical to the first 19 bases of the FMDV genome and a ^{32}P-labeled oligonucleotide complementary to bases 50 to 67 following the poly(C) tract in the A_{12} genome. After *Hae*III digestion, the samples were resolved by polyacrylamide gel electrophoresis, and the relative proportions of PCR products from the C_2 or $C_2\Delta_{42}$ genome were determined by phosphoimaging. Internal controls prepared from mixtures of two plaque-purified viruses confirmed that the proportions of PCR products correlated with the relative amounts of the two genomes.

RESULTS

Full-length cDNA clones of FMDV A_{12} were assembled by using a construction strategy (Fig. 1) which produced sequences surrounding the poly(C) tract identical to those of the authentic virus (see Materials and Methods for details). Five cDNA clones, designated $pRMC_2$, $pRMC_6$, $pRMC_{16}$, $pRMC_{25}$, and $pRMC_{35}$, were produced; these clones differed only in the lengths of their poly(C) tracts. These full-length cDNAs were preceded by the T7 polymerase promoter and two nongenomic guanosine residues (added to ensure high-level transcription by T7 polymerase [22]) and followed by a poly(A) tail of only 15 residues and a *Not*I site (Fig. 1).

Infectivity of FMDV RNA produced by in vitro transcription. To establish the effect of poly(C) tract length on infectivity, RNAs containing poly(C) tracts of 2, 6, 16, 25, or 35 nucleotides were produced from the pRMC plasmids and transfected into BHK cells. T7 transcripts were produced in similar high amounts from all five *Not*I-linearized pRMC DNAs, and these RNAs were indistinguishable in size from viral RNA in agarose gel electrophoresis (results not shown). All five in vitro transcripts were capable of producing some cytopathic effect in BHK cells, and DNase and RNase treatments demonstrated that RNA, but not DNA, was the infectious agent under these conditions (results not shown). A cytopathic effect was clearly visible at 24 h posttransfection with $pRMC_{35}$ and $pRMC_{25}$, at 48 h posttransfection with $pRMC_{16}$, and at 72 h posttransfection with $pRMC_6$. Interestingly, cells transfected with the RNA from $pRMC_2$ showed a much-less-marked cytopathic effect, which was barely detectable at 72 h posttransfection. Under similar conditions, complete cell destruction was detected at 24 h posttransfection with authentic virion RNA. Since the specific infectivities of all transcripts were indistinguishable (Table 1), the differences in cytopathic effect did not reflect the differential ability of these transcripts to establish an infectious cycle but

TABLE 1. Specific infectivity of virion RNA and in vitro transcripts

Source of RNA	PFU/μg[a]
Virion	4.2×10^5
$pRMC_{35}$	4.7×10^3
$pRMC_{25}$	9.2×10^3
$pRMC_{16}$	4.0×10^3
$pRMC_6$	7.3×10^3
$pRMC_2$	7.4×10^3

[a] PFU were determined by using duplicate dilutions of RNA as described in Materials and Methods.

rather reflected different properties of the transfectant viruses.

Growth characteristics of viruses recovered from in vitro transcripts. To more clearly define differences among the viruses recovered from these five RNAs, we examined their growth characteristics following transfection and passage and examined their plaque morphologies in BHK cells. At 72 h posttransfection, roughly equivalent amounts of infectious virus were released from cells transfected with 2 to 2.5 μg of RNA from $pRMC_{16}$, $pRMC_{25}$, or $pRMC_{35}$. However, 10- to 100-fold-lower amounts of infectious virus were recovered from cells transfected with RNA from $pRMC_6$ or $pRMC_2$. Following one passage in BHK cells, viruses obtained from $pRMC_6$ through $pRMC_{35}$ had essentially identical properties, whereas the $pRMC_2$ virus grew much more poorly in BHK cells (data not shown). These differences in growth in culture were also reflected in the sizes of the plaques produced by the transfectant viruses; viruses obtained from $pRMC_6$ through $pRMC_{35}$ all produced large plaques (5 to 8 mm at 48 h), while the passaged $pRMC_2$ transfectant exhibited a microplaque phenotype (<1 mm at 48 h). One-step growth curves of the wild-type and the $pRMC_2$ and $pRMC_{35}$ transfectant viruses show that the $pRMC_{35}$ virus was essentially identical to the wild-type virus with respect to growth in BHK cells, whereas the $pRMC_2$ transfectant virus grew much more poorly, consistent with its smaller plaque size and lower titer under overnight growth conditions (Fig. 2).

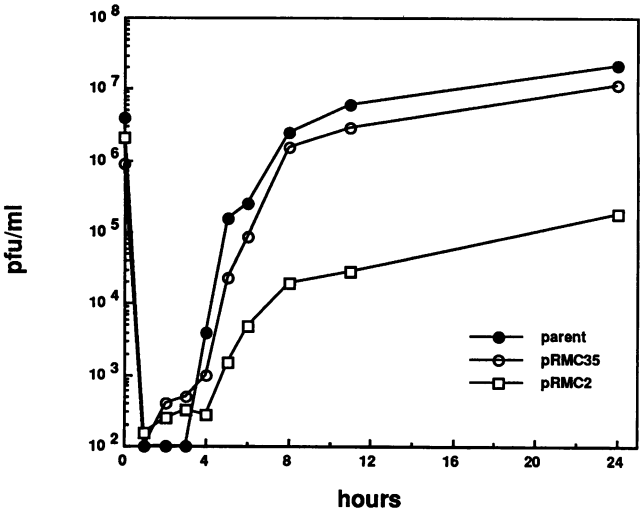

FIG. 2. One-step growth curves of the wild-type, $pRMC_2p2$, and $pRMC_{35}p2$ viruses. Cell monolayers were infected for 1 h at 37°C at a multiplicity of infection of 5 to 10, rinsed with 25 mM morpholineethanesulfonic acid–150 mM NaCl, pH 6.0 (to eliminate any residual extracellular virus), and then incubated in Eagle's minimal essential medium for an additional 23 h. At the times indicated, the amount of virus released from the infected cells was determined by plaque assay.

Cells transfected with synthetic RNAs containing poly(C) tracts of 6 or more nucleotides yielded viruses with much longer poly(C) tracts. The lengths of the poly(C) region present in in vitro RNAs and viral RNAs were determined by primer extension. The autoradiogram in Fig. 3 establishes the fidelity of this method by demonstrating that it can accurately size the poly(C) tracts present in the in vitro transcripts. In addition, Fig. 3 shows that the transfectant virus derived from $pRMC_2$ maintained a poly(C) tract equivalent to that of its in vitro transcript. All of the other transfectant viruses contained considerably longer 5′ UTRs, consistent with poly(C) tracts of 75 to 140 nucleotides in length, with one or more predominant species (Fig. 3). The presence of individual viruses with distinct 5′ UTR lengths within these populations was demonstrated by performing primer extension analyses on two plaque-purified viruses obtained from the $pRMC_{35}$ transfectant (Fig. 3). Sequence analysis of PCR products containing the poly(C) tracts of the $pRMC_{16}$, $pRMC_{25}$, and $pRMC_{35}$ viruses revealed the presence of shortened poly(C) tracts (see above) and revealed bordering sequences identical to those of the original pRMC plasmids.

The viruses recovered from transfections with RNAs containing six or more Cs presumably arose as a result of recombination within the homopolymeric tract or by slippage of the FMDV polymerase, as suggested by Zibert et al. (38). The fact that only viruses with poly(C) tracts longer than those of the in vitro transcripts were found in transfections with RNAs containing 6 to 35 Cs is consistent with the enhanced growth rates of these viruses relative to that of the $pRMC_2$ transfectant virus.

The primer extension experiments demonstrated that the poly(C) tract of the genome of the $pRMC_2$ transfectant virus had not increased in length following transfection, and sequencing of PCR products containing this region confirmed that the transfectant virus contained the same sequence found in the plasmid template.

Virulence of the synthetic RNA-derived viruses and identification of a virus with a deleted pseudoknot. The virulence of the transfectant viruses was evaluated with 7-day-old mice. These experiments showed no significant differences in virulence between synthetic RNA-derived viruses with short (C_2) or long (C_{75} to C_{140}) poly(C) tracts or the parental (C_{185}) virus (Fig. 3, Table 2). For all of these viruses, the lethal doses were within 1 order of magnitude of each other (Table 2). Since the association of poly(C) length and virulence in the cardioviruses (9) is so compelling, we measured the lengths of the poly(C) tracts of viruses harvested from three moribund mice; mouse D0 received approximately 1,000 PFU of unpassaged $pRMC_2$ transfectant virus ($pRMC_2tx$), mouse G3 received approximately 1,000 PFU of the second passage of this virus ($pRMC_2p2$), and mouse G6 received approximately 1 PFU of $pRMC_2p2$. PCR was utilized to screen the lengths of the poly(C) tracts of these viruses, since *Taq* polymerase could effectively replicate poly(C) tracts of up to six Cs, and it gave a characteristic smear for products containing poly(C) tracts with 16 or more Cs (data not shown).

Surprisingly, a shorter-than-expected PCR product was obtained from viruses recovered from all three mice. In the case of mouse G6, the shortened PCR product appeared to be the only one present. Direct sequence analysis of this

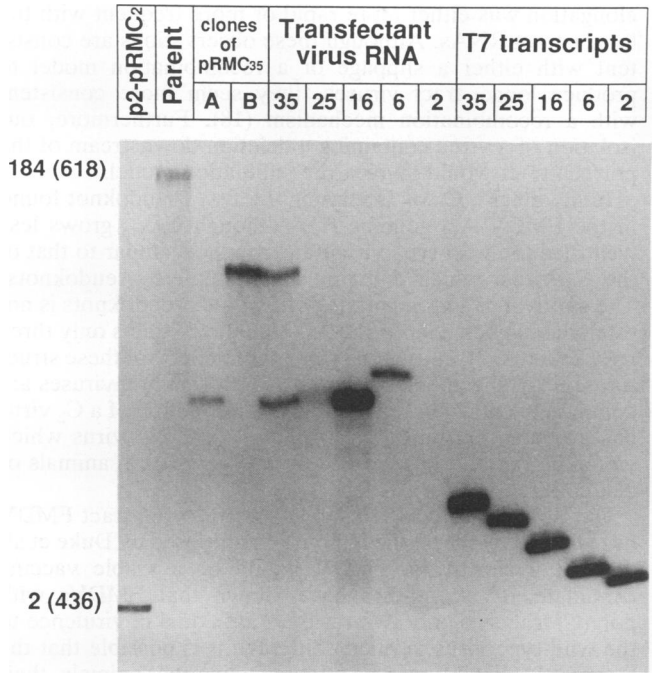

FIG. 3. Primer extension analysis of poly(C) length. ^{32}P-labeled primer extension products prepared from the indicated RNA samples were electrophoresed on a 4% polyacrylamide sequencing gel. The numbers on the left side of the autoradiogram correspond to the predicted lengths (nucleotides) of the poly(C) tracts of the parent and C_2 viruses followed by the actual lengths of the primer extension products (in parentheses). "clones of $pRMC_{35}$" denotes two plaque-picked viruses (A and B) obtained from the transfectant virus population shown in the next lane.

shortened product showed that it corresponded to a genome (designated $C_2\Delta_{42}$) with a deletion of 42 bases downstream of the two-residue poly(C) tract (Fig. 4). Interestingly, this deleted region contains one of the four potential pseudoknot structures found between the poly(C) tract and the internal ribosome entry site region of the genome of A_{12} by Clarke et al. (7). Since the PCR product corresponding to the deleted genome appeared in animals inoculated with both passaged and unpassaged transfectant viruses, the deleted genome

TABLE 2. Comparative virulence of transcript-derived and parental viruses in 7-day-old mice

Virus[a]	PFU/LD_{50}[b]
Parent	3
$pRMC_{35}$tx	1.5
$pRMC_{25}$tx	1.5
$pRMC_{16}$tx	2.0
$pRMC_{6}$tx	0.5
$pRMC_{2}$tx	ND[c]
$pRMC_{35}$p2	4.5
$pRMC_{6}$p2	1
$pRMC_{2}$p2	1
C_2 (plaque picked)	1.5
$C_2\Delta_{42}$ (plaque picked)	0.5

[a] Viruses included the parent, the direct transfectant viruses (tx), second-passage virus stocks (p2), and plaque-picked $pRMC_2$ derivatives with the genomic sequences shown in Fig. 4 (see text).
[b] PFU and LD_{50} were determined as described in Materials and Methods.
[c] ND, not determined.

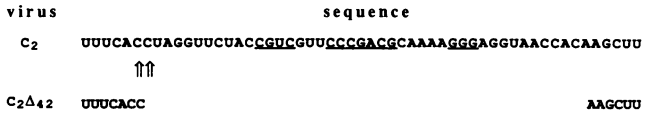

FIG. 4. Sequences in the region of the poly(C) tract of the C_2 viral genome (identical to the original cDNA $pRMC_2$) and the deleted viral genome ($C_2\Delta_{42}$; see text). Arrows denote the position of the poly(C) tract in the C_2 genome, and underlining denotes the regions involved in base pairing in the pseudoknot structure (7).

appears to have arisen as the result of a single recombination event early after transfection.

The proportions of C_2 and $C_2\Delta_{42}$ viruses in the $pRMC_2p2$ preparation and in the three virus populations recovered from inoculated animals were determined by a quantitative PCR assay (see Materials and Methods). Interestingly, these data confirmed that the $pRMC_2p2$ virus preparation contained a small proportion of the $C_2\Delta_{42}$ genome (4%) and showed that the viruses recovered from mice G3, D0, and G6 contained 4, 31, and 100% $C_2\Delta_{42}$ genomes (respectively). These latter data, which suggested that both the C_2 and the $C_2\Delta_{42}$ viruses were able to kill mice, were supported by experiments showing that plaque-picked viruses with the C_2 or $C_2\Delta_{42}$ genome (see below) were equally virulent in mice (Table 2).

The C_2 and $C_2\Delta_{42}$ viruses display similar properties in cell culture. Since the $C_2\Delta_{42}$ genome appeared to hold a slight selective advantage over the C_2 genome in 7-day-old mice, we investigated whether it displayed a selective advantage in cell culture. PCR analysis of the virus obtained following six passages of the $pRMC_2tx$ virus showed that over 20% of the genomes present in this preparation corresponded to the $C_2\Delta_{42}$ virus, suggesting that viruses with the deleted genome grew slightly better. To obtain a pure population of this virus, we plaque purified 14 viruses from the $pRMC_2p6$ population and found two clones containing the deleted genome. Although the $C_2\Delta_{42}$ virus formed plaques which were slightly larger than those obtained with the C_2 virus, both produced plaques much smaller than those of either the wild-type virus or the viruses recovered from cells transfected with RNA from $pRMC_6$ through $pRMC_{35}$. Furthermore, both the $C_2\Delta_{42}$ and C_2 viruses grew to much lower titers than the wild-type or $pRMC_6$ through $pRMC_{35}$ viruses.

The sequences of the region surrounding the poly(C) tract of the plaque-purified cloned viruses, which had been subjected to a total of nine passages in BHK cells, confirmed that they were identical to either $pRMC_2$ or the $C_2\Delta_{42}$ virus recovered from the moribund mouse G6 (Fig. 4).

DISCUSSION

Classical biochemical approaches have identified long poly(C) tracts as distinctive features of the genomes of cardioviruses and aphthoviruses. For FMDV, descriptive studies have suggested that changes in poly(C) tract length may be associated with changes in virulence, but these studies have been unable to relate categorically poly(C) tract length to any biological function of the virus, either in tissue culture or in vivo (see the introduction). Studies on cardioviruses, which have made use of genetic engineering, have clearly demonstrated that the poly(C) tract plays a role in virulence and prompted Duke et al. (9) to suggest that a similar approach could be used to produce an attenuated FMD vaccine. However, evidence supplied by Zibert et al. (38), using genetically engineered FMDV type O_1, suggested

that FMDV had an absolute requirement for a poly(C) tract of 60 bases in order to replicate in cell culture (see the introduction).

We have successfully engineered infectious RNA transcripts of FMDV type A_{12} which contain poly(C) tracts of 2 to 35 nucleotides. These transcripts all display similar specific infectivities and are approximately 1,000-fold more infectious than those reported for in vitro transcripts of FMDV type O (38; see the introduction). Although it is unclear why our transcripts should be so much more infectious than those reported for type O, these differences could be related to the specific properties of individual isolates used to prepare the cDNA clones, or they could be explained by differences in the engineering of the 5′ and 3′ ends of the genomes of the full-length cDNAs.

Specifically, our full-length cDNA of type A_{12} was derived from a highly passaged, tissue culture-adapted virus, whereas Zibert et al. (38) used a low-passage virus of type O_1 as the source of their cDNA. Furthermore, we engineered our plasmid so that the poly(C) tract was in the context identical to that of the authentic viral genome, while Zibert et al. (38) introduced nongenomic sequences on either side of the poly(C) tract of their full-length cDNA. Finally, our in vitro RNA contained only two nongenomic residues at the 5′ end, whereas the in vitro transcripts generated by Zibert et al. (38) contained 12 additional residues at their 5′ ends. The accuracy of our transcripts at the 5′ end and surrounding the poly(C) tract may be particularly important in establishing the first replicative cycle of the transfected genomes, since the 5′ UTR undoubtedly contains structures critical for genome replication.

Interestingly we discovered that viruses containing poly(C) tracts of only two nucleotides were viable, showing that FMDV, like cardioviruses (9), does not need a long poly(C) tract to replicate. However, viruses containing poly(C) tracts of 75 to 140 nucleotides rapidly grew out of transfections with RNAs containing six or more Cs, and these viruses achieved titers in cell culture that were similar to those of wild-type virus after a single passage in BHK cells. The selective advantage of a long poly(C) tract explains why we were unable to detect C_6, C_{16}, C_{25}, or C_{35} genomes in those viruses recovered from cells transfected with synthetic RNAs containing poly(C) tracts with these lengths; the long-tract viruses which presumably arose from these synthetic genomes apparently rapidly outgrow the short-tract viruses in the transfected cultures. The mechanism of production of these viruses must be very efficient, however, since transfection with the C_{16} through C_{35} RNAs produced large plaques, suggesting that each primary infection was able to produce progeny viruses with long poly(C) tracts. In the case of transfection with $pRMC_6$, the evolution of viruses with longer poly(C) tracts must be slower since we were able to obtain a small-plaque C_6 virus from BHK cells transfected with small amounts of synthetic RNA (results not shown). However, on subsequent passage, the poly(C) tract of this virus increased in length (results not shown).

The rapid elongation of the poly(C) tract of FMDV viruses stands in sharp contrast to the situation with cardioviruses, in which shorter-than-natural poly(C) tracts of the synthetic RNAs were maintained during growth in HeLa cells (9) and in vivo (24). Although these differences may relate to the cell types used in the experiments, it is also possible that the poly(C) tract of FMDV is required for growth to high titer. The fact that infectious viruses were proliferated more rapidly from transfections with RNAs containing 35 Cs than from those with RNAs containing 6 Cs suggests that this elongation was either more rapid or more frequent with the longer-tract RNAs. Although these observations are consistent with either a slippage or a recombination model to produce longer-tract viruses, they seem more consistent with a recombination mechanism (18). Furthermore, our isolation of a virus containing a deletion downstream of the poly(C) tract would favor a recombination model.

Interestingly, $C_2\Delta_{42}$ is missing the first pseudoknot found in the FMDV A_{12} genome (7). Although $C_2\Delta_{42}$ grows less well than the wild-type virus, its growth is similar to that of the C_2 virus, which contains four complete pseudoknots. The survival of a virus missing one of the pseudoknots is not totally unexpected since the A_{10} genome contains only three pseudoknots (7), suggesting some redundancy of these structures. Rearrangements in the 5′ UTRs of picornaviruses are common events in nature (26), and our creation of a C_2 virus has probably permitted us to identify a $C_2\Delta_{42}$ virus which would be rapidly outgrown by wild-type virus in animals or cell culture.

Our ability to produce a stable short-poly(C)-tract FMDV has allowed us to test the hypothesis proposed by Duke et al. (9) that a short-tract FMDV would be a viable vaccine candidate. These studies have shown that FMDVs with poly(C) tracts of only two residues are equal in virulence to the wild-type virus in mice. Although it is possible that the C_2 or $C_2\Delta_{42}$ FMDV may be attenuated in other animals, their virulence in mice demonstrates that the role of poly(C) tract length in virulence established for cardioviruses does not apply to the murine model for FMD. The reasons for this difference are unclear, but it may relate to the mechanisms by which these viruses induce disease in their hosts.

ACKNOWLEDGMENTS

We thank A. Palmenberg, Institute for Virology, University of Wisconsin, Madison, for helpful suggestions and discussions on the design of the infectious clone of FMDV A_{12} and for a critical reading of the manuscript; B. Baxt, Plum Island Animal Disease Center, for supplying the seed of FMDV A_{12} 119ab; and M. Grubman, Plum Island Animal Disease Center, for supplying pTRP123.

REFERENCES

1. **Beck, E., and K. Strohmaier.** 1987. Subtyping of European foot-and-mouth disease virus strain by nucleotide sequence determination. J. Virol. **61**:1621–1629.
2. **Black, D. N., P. Stephenson, D. J. Rowlands, and F. Brown.** 1979. Sequence and location of the poly C tract in aphtho- and cardiovirus RNA. Nucleic Acids Res. **6**:2381–2390.
3. **Brakenhoff, R. H., J. G. G. Schoenmakers, and N. H. Lubsen.** 1991. Chimeric cDNA clones: a novel PCR artifact. Nucleic Acids Res. **19**:1949.
4. **Brown, F., J. Newman, J. Stott, A. Porter, D. Frisby, C. Newton, N. Carey, and P. Fellner.** 1974. Poly(C) in animal viral RNAs. Nature (London) **251**:342–344.
5. **Bunch, T., E. Rieder, and P. Mason.** Unpublished data.
6. **Chumakov, K. M., and V. I. Agol.** 1976. Poly(C) sequence is located near the 5′-end of encephalomyocarditis virus RNA. Biochem. Biophys. Res. Commun. **71**:551–557.
7. **Clarke, B. E., A. L. Brown, K. M. Currey, S. E. Newton, D. J. Rowlands, and A. R. Carroll.** 1987. Potential secondary and tertiary structure in the genomic RNA of foot and mouth disease virus. Nucleic Acids Res. **15**:7067–7079.
8. **Costa Giomi, M. P., I. Gomes, B. Tiraboschi, P. Auge de Mello, I. E. Bergmann, E. A. Schodeller, and J. L. La Torre.** 1988. Heterogeneity of the polyribocytidilic acid tract in aphthovirus: changes in the size of poly(C) of viruses recovered from persistently infected cattle. Virology **162**:58–64.
9. **Duke, G. M., J. E. Osorio, and A. C. Palmenberg.** 1990. Attenuation of Mengo virus through genetic engineering of the 5′ noncoding poly(C) tract. Nature (London) **343**:474–478.

10. **Duke, G. M., and A. C. Palmenberg.** 1989. Cloning and synthesis of infectious cardiovirus RNAs containing short, discrete poly(C) tracts. J. Virol. **63**:1822–1826.
11. **Escarmis, C., M. Toja, M. Medina, and E. Domingo.** 1992. Modification of the 5′ untranslated region of foot-and-mouth disease virus after prolonged persistence in cell culture. Virus Res. **26**:113–125.
12. **Grubman, M. J., B. Baxt, and H. L. Bachrach.** 1979. Foot-and-mouth disease virion RNA: studies on the relation between the length of its 3′-poly (A) segment and infectivity. Virology **97**:22–31.
13. **Harris, T. J. R.** 1980. Comparison of the nucleotide sequence at the 5′ end of RNAs from nine aphthoviruses, including representatives from seven serotypes. J. Virol. **36**:659–664.
14. **Harris, T. J. R., and F. Brown.** 1976. The location of the poly(C) tract in the RNA of foot-and-mouth disease virus. J. Gen. Virol. **33**:493–501.
15. **Harris, T. J. R., and F. Brown.** 1977. Biochemical analysis of a virulent and an avirulent foot-and-mouth disease virus. J. Gen. Virol. **34**:87–105.
16. **Higuchi, R. G., and H. Ochman.** 1989. Production of single-stranded DNA templates by exonuclease digestion following the polymerase chain reaction. Nucleic Acids Res. **17**:5865.
17. **Jang, S. K., H. G. Krausslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. H. Wimmer.** 1988. A segment of the 5′-untranslated region of encephalomyocarditis virus directs internal entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.
18. **Kirkegaard, K., and D. Baltimore.** 1986. The mechanism of RNA recombination in poliovirus. Cell **47**:433–443.
19. **Klug, J., M. Wolf, and M. Beato.** 1991. Creating chimeric molecules by PCR directed homologous DNA recombination. Nucleic Acids Res. **19**:2793.
20. **Lerner, C. G., and M. Inouye.** 1990. Low copy number plasmids for regulated low-level expression of cloned genes in Escherichia coli with blue/white insert screening capability. Nucleic Acids Res. **18**:4631.
21. **Mateu, M. G., M. A. Martinez, E. Rocha, D. Andreu, J. Parejo, E. Giralt, F. Sobrino, and E. Domingo.** 1989. Implications of a quasispecies genome structure: effect of frequent, naturally occurring amino acid substitutions on the antigenicity of foot-and-mouth disease virus. Proc. Natl. Acad. Sci. USA **86**:5883–5887.
22. **Milligan, J. F., D. R. Groebe, G. W. Witherell, and O. C. Uhlenbeck.** 1987. Oligonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. **15**:8783–8798.
23. **Newton, S. E., A. R. Carroll, R. O. Campbell, B. E. Clarke, and D. J. Rowlands.** 1985. The sequence of foot-and-mouth disease virus RNA to the 5′ side of the poly(C) tract. Gene **40**:331–336.
24. **Osorio, J. E., and A. C. Palmenberg (University of Wisconsin).** 1992. Personal communication.
25. **Perez-Bercoff, R., and M. Gander.** 1977. The genome of mengo virus. I. Location of the poly(C) tract. Virology **80**:426–429.
26. **Pilipenko, E. V., V. M. Blinov, and V. I. Agol.** 1990. Gross rearrangements within the 5′-untranslated region of the picornaviral genomes. Nucleic Acids Res. **18**:3371–3375.
27. **Porter, A., N. Carey, and P. Fellner.** 1974. Presence of a large poly(rC) tract within the RNA of encephalomyocarditis virus. Nature (London) **248**:675–678.
28. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
29. **Robertson, B. H., M. J. Grubman, G. N. Weddell, D. M. Moore, J. D. Welsh, T. Fischer, D. J. Dowbenko, D. G. Yansura, B. Small, and D. G. Kleid.** 1985. Nucleotide and amino acid sequence coding for polypeptides of foot-and-mouth disease virus type A12. J. Virol. **54**:651–660.
30. **Rowlands, D. J., T. J. R. Harris, and F. Brown.** 1978. More precise location of the polycytidylic acid tract in foot-and-mouth disease virus RNA. J. Virol. **26**:335–343.
31. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
32. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
34. **Vakharia, V. N., M. A. Devaney, D. M. Moore, J. J. Dunn, and M. J. Grubman.** 1987. Proteolytic processing of foot-and-mouth disease virus polyproteins expressed in a cell-free system from clone-derived transcripts. J. Virol. **61**:3199–3207.
35. **Van Der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:2330–2334.
36. **Winship, P.** 1989. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucleic Acids Res. **17**:1266.
37. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
38. **Zibert, A., G. Maass, K. Strebel, M. M. Falk, and E. Beck.** 1990. Infectious foot-and-mouth disease virus derived from a cloned full-length cDNA. J. Virol. **64**:2467–2473.