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Infectious Foot-and-Mouth Disease Virus Derived from a Cloned Full-Length cDNA

A. ZIBERT,[1]† G. MAASS,[2] K. STREBEL,[1]‡ M. M. FALK,[1] AND E. BECK[1]*

Zentrum für Molekulare Biologie Heidelberg, Universität Heidelberg, D-6900 Heidelberg,[1] and Bundesforschungsanstalt für Viruskrankheiten der Tiere, D-7400 Tübingen,[2] Federal Republic of Germany

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A full-length cDNA plasmid of foot-and-mouth disease virus has been constructed. RNA synthesized in vitro by means of a bacteriophage SP6 promoter inserted in front of the cDNA led to the production of infectious particles upon transfection of BHK-21 cells. These particles were also found to be highly infectious for primary bovine kidney cells as well as for baby mice. The difficulty in cloning the foot-and-mouth disease virus cytidyl tract in *Escherichia coli* was circumvented by joining two separate cloned parts, representing the S and L fragments of the genome, and, in a second step, inserting a dC-dG homopolymer. Homopolymeric sequences of up to 25 cytidyl residues did not lead to the production of virus. Replicons containing poly(C) tracts long enough to permit virus replication were first established in yeast cells. One of these constructs could also be maintained in *E. coli* and was used to produce infectious RNA in vitro. The length of the poly(C) sequence in this cDNA plasmid was 32 nucleotides. However, the poly(C) tracts of two recombinant viruses found in transfected BHK-21 cells were 60 and 80 nucleotides long, respectively. Possible mechanisms leading to the enlargement of the poly(C) tract during virus replication are discussed.

Within the family *Picornaviridae*, two groups, the aphthoviruses (foot-and-mouth disease virus [FMDV]) and the cardioviruses (e.g., mengovirus and encephalomyocarditis virus), are outstanding with respect to their long homopolymeric tract of cytidyl residues near the 5′ end of the genomic RNA (4). The length of this poly(C) tract among isolates varies between 50 and 250 nucleotides. It is, however, characteristic for individual isolates and has been used as a tool to identify different variants of FMDV (19). Alterations in the length of the poly(C) tract have been observed during serial passages of FMDV in BKH-21 cells (6, 12).

Complete infectious cDNA clones have been established for several members of the picornaviruseslike poliovirus (25), rhinovirus (22), hepatitis A virus (5), and coxsackievirus B (16). In contrast to these viruses, the presence of the poly(C) tract in the cardiovirus-aphthovirus group has for a long time hampered the construction of full-length cDNA clones. Long poly(dC-dG) sequences are believed to interfere with the replication of plasmids in *Escherichia coli* (7). The biological function of the poly(C) tract is unknown.

In the case of cardioviruses, an infectious encephalomyocarditis virus cDNA clone was obtained recently (8). The length of the homopolymeric cytidyl sequence in the cloned cDNA was reduced, and virions with poly(C) tracts as short as eight cytidyl residues could be stably propagated in HeLa cells.

This situation does not apply to FMDV, as shown in this report. Homopolymeric cytidyl sequences of up to 25 nucleotides in length, which were easily maintained in *E. coli*, proved to be too short to permit replication of the virus. We succeeded, however, in constructing cDNA clones with poly(C) tracts sufficiently long for virus replication in yeast cells, using a 2μm circle-derived vector system. Unexpectedly, one of the 2 μm circle constructs led to the production of infectious RNA even after replication in *E. coli*. The poly(C) tract in this plasmid has a length of 32 nucleotides. However, the minimal length of the poly(C) found in recombinant viruses derived from this plasmid was approximately 60 nucleotides. Possible mechanisms leading to the enlargement of the poly(C) tract during establishment of the recombinant viruses are discussed.

MATERIALS AND METHODS

Virus growth and RNA isolation. FMDV strain O1K was isolated in Kaufbeuren, in southern Germany, in 1966. The virus used to derive the infectious cDNA was cloned three times from single plaques and passaged seven times in BHK-21 cell cultures. Viral RNA was prepared essentially as described by Grubman et al. (10).

Construction of plasmid pFMDV-L. First-strand cDNA was synthesized essentially as described previously (18). The RNA-cDNA hybrid was heat denatured, and second-strand cDNA was primed with a synthetic oligonucleotide homologous to the sequence of the first 29 nucleotides downstream of the poly(C) tract (positions 92 to 120 of the published nucleotide sequence; 9) and an additional *Nhe*I site at the 5′ end: 5′-d(GCTAGCAAGTTTTACCGTCGTTC CCGACGTAAAAG)-3′. The double-stranded cDNA was preparatively separated by electrophoresis on a 1% agarose gel, and only full-length cDNA (ca. 8 kilobase pairs [kb]) eluted from the gel. After addition of *Hpa*I linkers, the cDNA was ligated into an *Hpa*I site previously inserted into the *Sma*I site of vector pSP64 (21). *E. coli* HB101 cells were transformed, and ampicillin-resistant colonies were screened with the radiolabeled primer of the second-strand cDNA synthesis. Positive clones were characterized by restriction endonuclease analysis and determination of the nucleotide sequences at the ends of the inserted cDNA. One clone containing the complete primer sequence (including the *Nhe*I site) adjacent to the SP6 promoter, the entire FMDV cDNA downstream of the poly(C) tract, and a homopoly-

* Corresponding author.
† Present address: State University of New York, Stony Brook, NY 11794-5208.
‡ Present address: Laboratory of Molecular Microbiology, National Institutes of Health, Bethesda, MD 20892.

meric adenosyl tract of approximately 90 nucleotides at the 3′ end was chosen for further constructions.

Construction of plasmid pFMDV-S. First-strand cDNA was primed with the synthetic oligonucleotide 5′-d(GCTA GCTGAAAGGCGGGCGCCGG)-3′. The nucleotide sequence preceding the poly(C) tract had been previously determined by extension of the 5′-labeled oligonucleotide 5′-d(GGGGGGTGAAAGGC)-3′, which is complementary to the conserved 3′ end of this region (so-called S fragment; 13, 24). Second-strand cDNA was synthesized by using the 51-mer oligonucleotide 5′-d(TTGAAAGGGGGCATTAGGGT CTCACCCCTAGTAAGCCAACGACAGTCCCTG)-3′ as a primer. The nucleotide sequence at the 5′ end of the RNA of strain O1K used here has been established in an approach similar to that described above, using a 15-mer primer oligonucleotide homologous to the conserved 5′-terminal sequence of the viral genome (11). After addition of synthetic *Eco*RI adaptors (plus strand, 5′-OH-d[AATTCCAT GGATGCATGC]-3′; minus strand, 5′-PO_4-d[GCATGCAT CCATGG-3′]), the double-stranded cDNA was ligated into pSP64 and used to transform *E. coli* HB101 cells. Colonies were screened in parallel, using the two ^{32}P-labeled oligonucleotide primers as probes. Of several hundred clones reactive with either one of the two oligonucleotides, only two were positive with both probes. Their cDNA inserts were characterized by nucleotide sequence analysis, and only one contained the complete S fragment. Part of the artificial linker sequence between the SP6 promoter and the viral 5′ end was eliminated by linearization of the plasmid with *Hin*dIII and partial digestion with *Sph*I (the *Sph*I site is contained in the adaptor sequence). After incubation with DNA polymerase I (Klenow fragment) and the four deoxynucleoside triphosphates, the DNA was recircularized with T4 DNA ligase. In the final construction, in vitro transcripts are thus preceded by 12 artificial nucleotides in front of the viral genome.

Construction of plasmid pFMDV-ff. Plasmid pFMDV-ff, representing a complete cDNA clone without poly(C), was obtained as follows. pFMDV-L was linearized with *Nhe*I and digested partially with *Pvu*I, the cleavage products were separated on a 1% agarose gel, and a 8.7-kb fragment containing the viral cDNA was eluted from the gel. Similarly, a 2.7-kb DNA fragment containing the viral S fragment and the SP6 promoter was isolated from a *Pvu*I (total)-*Nhe*I (partial) digest of pFMDV-S. The two fragments were combined to a single plasmid with T4 ligase.

A 20-μg amount of this plasmid was linearized by partial cleavage with *Nhe*I and, after fill-in of the protruding ends, ligated with synthetic poly(dC-dG) fragments with average lengths of 50 to 150 nucleotides. These homopolymeric fragments had been prepared from high-molecular-weight commercial material (Boehringer GmbH, Mannheim, Federal Republic of Germany) by partial DNase digestion and repair synthesis with DNA polymerase I. After phenol extraction and ethanol precipitation, the crude ligase mixture was transcribed in vitro with SP6 RNA polymerase (21) and used for transfection of BHK-21 cells without further purification.

Construction of plasmids pFMDV-YEP and pFMDV-YEP-polyC. Plasmid pFMDV-YEP was constructed by blunt-end ligation of a 9-kb DNA fragment containing the complete viral cDNA and the SP6 promoter into the yeast vector YEp51 (2). The 9-kb fragment was obtained by complete digestion of pFMDV-ff with *Hpa*I and partial digestion with *Nhe*I, followed by a DNA polymerase I fill-in reaction. The shuttle vector YEp51 (7.3 kb), which contains a ColE1 origin, an ampicillin resistance gene for replication and selection in *E. coli*, the autonomous replication portion of the 2 μm circle, and the *LEU2* marker for growth in *Saccharomyces cerevisiae*, had been linearized previously by digestion with *Bam*HI and *Hin*dIII and rendered blunt end with DNA polymerase. Transformed *E. coli* colonies were screened for full-length cDNA inserts with the nick-translated 800-base-pair *Nhe*I fragment covering the viral 5′ end and the SP6 promoter. A clone containing the complete viral genome without poly(C) and the SP6 promoter in tandem with the *GAL10* promoter of the vector was characterized by restriction endonuclease analysis. This construction was cleaved at the remaining unique *Nhe*I site, and poly(C) and poly(G) tails, respectively, with lengths of between 50 and 150 nucleotides were created at the filled-in 3′ ends of one half each of the preparation, using terminal nucleotidyl transferase (7). The tailed DNAs were cleaved with *Not*I in two fragments of 14 and 2.7 kb and separated on a 1% agarose gel. The eluted C-tailed 14-kb fragment and the G-tailed 2.7-kb fragment were recombined with T4 ligase and used to transform yeast or *E. coli* cells.

Cloning in yeast cells. Lithium-treated *S. cerevisiae* cells (strain GY92; *MAT*α *ade2 leu2 ura3 trp1* [cir$^+$]; kindly provided by G. Cesareni, European Molecular Biology Laborator, Heidelberg, Federal Republic of Germany) were transformed (15) with the poly(C)-containing plasmid pFMDV-YEP-polyC. Recombinants were selected on 2% agarose plates containing synthetic complete medium without leucine (29). Isolated clones were grown to a density of 10^7 cells per ml and then transferred in synthetic complete medium with galactose instead of glucose as a carbon source in order to activate transcription from the *GAL10* promoter. Six hours after induction, the cells were harvested and total RNA was isolated by the guanidinium-hot phenol method (20).

Transfection of BHK-21 cells. Semiconfluent monolayers of BHK-21 cells grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum and 2 mM L-glutamine (23) were transfected either with crude RNA isolated from yeast clones or with RNA transcribed in vitro from plasmid DNA with SP6 RNA polymerase (21) by the calcium phosphate coprecipitation method (28).

Infection of baby mice. Three-day-old mice in groups of five animals each were inoculated intraperitoneally with 0.1 ml of virus dilution in phosphate-buffered saline. Dead animals were determined 72 h after infection.

ELISA. The sandwich enzyme-linked immunosorbent assay (ELISA) technique used is a modification of the method of Roeder and Le Blanc Smith (27). A microdilution plate was coated with rabbit anti-FMDV serum and incubated with dilutions of the individual viruses for 60 min at 37°C. After washing, seven different types of strain O1K-specific monoclonal antibodies (kindly provided by E. Pfaff, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Federal Republic of Germany) were added and incubated for another hour at 37°C. The plate was washed again and incubated with peroxidase-conjugated anti-mouse immunoglobulin G (Dianova, Hamburg, Federal Republic of Germany) for 30 min at 37°C. Bound antibodies were visualized by *o*-phenylenediamine and hydrogen peroxide.

RESULTS

Construction of the infectious cDNA. Initial attempts to obtain an infectious cDNA clone by combining our previously isolated FMDV subclones (9) failed. These clones had

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                                    SP6 promoter          RNA
                                    ATTTAGGTG ACACTATAGA ATACAAGCTC
  ⌐ FMDV ⟶
  1 TTGAAAGGGG GCATTAGGGT CTCACCCCTA GTAAGCCAAC GACAGTCCCT
 51 GCGTTGCACT CCACACTTAC GTTGTACACA CGCGGGACCC GATGGGCTAT
101 CGTTCACCCA CCTACAGCTG GACTCACGGC GCCGCGTGGC CATTTAGCTG
151 GATTGTGCGG ACGAACACGC TTGCGCACCT CGCGTGACCG GTTAGTACTC
201 TTACCACTCT CCGCCTACTT GGTCGTTAGC GCTGTCTTGG GCATTCCTGT
251 GGGGGCCGTT CGACGCTCCA CGGGAACTCT CCTGTGTGAC ATCTACGGTG
301 ATGGGGCCGT TTCGCGTGGG CTGGTCGTTT GGACTGCTTC GGCTGTCACC
                          Nhe I
351 CGGCGCCCGC CTTTCAGCTA GCCCCCCCCC CCCCCCCCCC CCCCCCCCCC
    [92]
    CCCTAGCAAG TTT...
```

FIG. 1. Nucleotide sequence of the viral 5′ region in plasmid pFMDV-YEP-polyC. The start site of RNA synthesis by SP6 RNA polymerase and the beginning of the virus-specific sequence are indicated (arrows). The SP6 promoter and the artificial *Nhe*I linker sequences are boxed. [92] refers to position 92 of the published FMDV O1K sequence (9).

been derived in part from a highly passaged stock of the virus (64 passages in BHK-21 cells) that is no longer infectious for cattle (unpublished result). In addition, we could not exclude the possibility that the lack of infectivity was due to a cloning artifact during construction of the full-length cDNA from seven different subclones even though the reading frame was intact. Therefore, the complete region downstream of the poly(C) tract was recloned, starting from RNA of strain O1K, which had been passaged only seven times in BHK-21 cells. Construction of this clone (pFMDV-L) is described in Materials and Methods.

Several approaches to synthesize cDNA of the genomic region in front of the poly(C) tract, using a 15-mer oligonucleotide as a primer, did not result in complete clones, probably because the extended secondary structure of this region impaired proper extension of the primer. Only when we used a long primer homologous to the sequence of the first 51 nucleotides could a clone corresponding to the complete S fragment be obtained, as concluded from comparison of the derived nucleotide sequence with the sequences of strains A10 and O1BFS (24). The sequence is shown in Fig. 1.

The cloned 5′ and 3′ ends of the viral genome were combined to form a single plasmid (Fig. 2). This construction (pFMDV-ff) contains a complete cDNA copy of the FMDV O1K genome except for the poly(C) tract, which is replaced by an *Nhe*I linker. We subsequently inserted synthetic double-stranded poly(dC-dG) fragments with a heterogeneous length of 50 to 150 base pairs into this *Nhe*I site (see Materials and Methods).

To determine whether this construction contained all of the genetic information essential for virus replication, the crude ligation mixture was transcribed in vitro and used to transfect BHK-21 cells. After 3 days the cells were completely lysed, whereas in control assays with RNA derived from pFMDV-ff without poly(C), the cells grew to high densities. A sample of the cell-free supernatant medium added to fresh BHK-21 cells led to lysis within 12 h, indicating the presence of viable viruses.

The same ligation mixture was then used to transform *E. coli* cells. The resulting clones were found to contain homopolymeric cytidyl sequences of 17 to 25 nucleotides only. Longer homopolymers were not detected, although the average size of the poly(dC-dG) fragments used in the

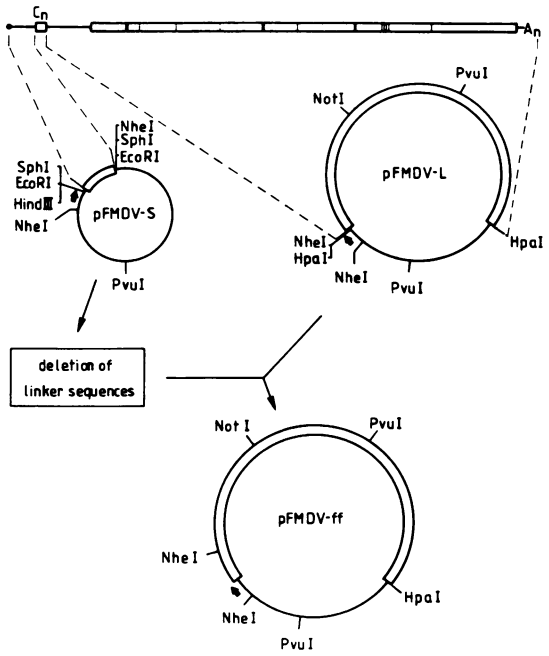

FIG. 2. Construction of plasmid pFMDV-ff. cDNA of the viral genome was cloned in two separate parts (pFMDV-S and pFMDV-L) and then combined to form a single plasmid (pFMDV-ff). In this clone, the poly(C) tract is replaced by an *Nhe*I linker sequence. For details, see Materials and Methods.

ligation assay was larger. None of the RNAs derived in vitro from these clones led to the lysis of BHK-21 cells after transfection. From these results, we concluded that it would probably be impossible to establish FMDV cDNA clones with a sufficiently long poly(C) sequence in *E. coli*.

Infectious cDNA clones in yeast cells. In a search for alternative vector systems, we tested whether derivatives of the 2μm circle, which replicate autonomously in both *E. coli* and *S. cerevisiae*, were capable of maintaining long poly(C) sequences in yeast cells. Vector YEp51 (2) was found to tolerate inserts of homopolymeric dC-dG tracts up to 100 nucleotides, although we observed that the number of vector copies per yeast cell decreased under these conditions (data not shown). Consequently, we inserted the FMDV part of plasmid pFMDV-ff, including the SP6 promoter region, into this yeast vector as described in Materials and Methods. The resulting plasmid, pFMDV-YEP, contained a single *Nhe*I site, allowing insertion of a synthetic poly(dC-dG) sequence in a directed orientation, using terminal deoxynucleotidyl transferase as outlined schematically in Fig. 3.

Most of the yeast clones obtained by transformation with pFMDV-YEP DNA containing 50 to 150 nucleotides of synthetic poly(C) stably maintained the recombinant 2μm replicon, although smaller deleted forms were also observed by Southern blot analysis (data not shown). Sufficient amounts of FMDV-specific RNA could not be derived in vitro from total yeast DNA or from DNA enriched for the 2μm circle by gel filtration. Therefore, we took advantage of the *GAL10* promoter located approximately 400 nucleotides upstream of the FMDV sequence in the vector to synthesize FMDV-specific RNA in vivo.

Approximately half of the BHK-21 cell cultures transformed with crude RNA from individual yeast clones were lysed, and the presence of infectious viruses was demonstrated by the induction of lysis of further cell cultures with supernatants from the primary lysates.

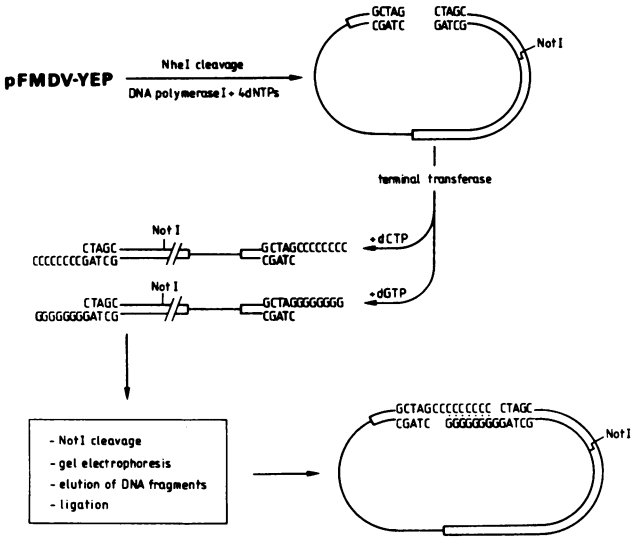

FIG 3. Insertion of poly(C) into plasmid pFMDV-YEP. The plasmid was cleaved with *Nhe*I; then poly(C) or poly(G) tails, respectively, with average lengths of 50 to 150 nucleotides were added to the filled-in 3′ ends, using terminal nucleotidyl transferase. The DNAs were cleaved with *Not*I, and the resulting short (2.7-kb) G-tailed fragment was ligated with the long (14-kb) C-tailed fragment as described in Materials and Methods. dNTP, Deoxynucleoside triphosphates.

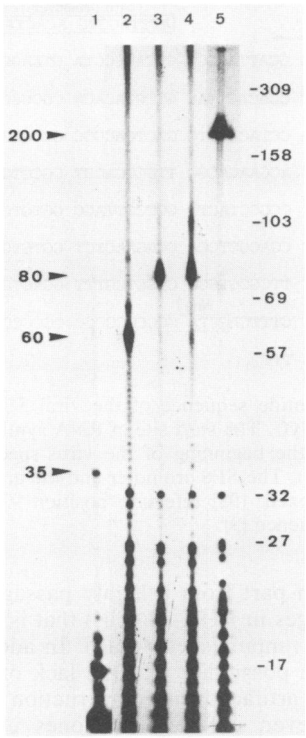

FIG. 4. Determination of the length of the poly(C) tract. ^{32}P-labeled RNA was digested with RNase T_1, and the products were analyzed on a 10% sequencing gel. Lanes: 1, in vitro transcript of pFMDV-YEP-polyC DNA [the DNA was linearized with *Hin*dIII ca. 50 base pairs downstream of the poly(C) tract]; 2, recombinant virus 1; 3 and 4, recombinant virus 2, passages 3 and 30, respectively; 5, FMDV O1K wild type.

Infectious cDNA clone in *E. coli*. The use of yeast cells to propagate infectious FMDV cDNA is limited by the fact that there is no simple protocol to purify the 2μm replicons, which represent less than 1% of the total cellular DNA. For future manipulations of the cDNA to study gene functions or construct attenuated strains, it was desirable to have larger amounts of purified cDNA available. Therefore, we made another attempt to establish an infectious cDNA clone in bacteria and transformed *E. coli* cells with the same batch of polycytidylated pFMDV-YEP DNA as had been used to transfect the yeast cells. Plasmid DNA was prepared from 20 individual ampicillin-selected clones and transcribed into RNA. One of these RNAs led to the production of infectious viruses after transfection of BHK-21 cells. Nucleotide sequence analysis of primer extension using the RNA of the recombinant virus as a template unequivocally excluded contamination with another virus. By analysis of the RNA sequence of two independent transformation-derived virus preparations, we detected the filled-in *Nhe*I linker sequence downstream of the poly(C) tract, which demonstrates the derivation of these viruses from recombinant DNA (data not shown).

It was possible to expand this clone (pFMDV-YEP-polyC) under standard conditions, and the isolated plasmid DNA could also be used to transform other *E. coli* cells without complications. Transfection of BHK-21 cells with RNA transcribed from these second-generation plasmids did not always lead to the production of viruses. We initially interpreted this fact as a certain instability of the infectious clone. More careful analysis revealed, however, that this result was due to the low relative infectivity of the in vitro-derived RNA of this clone (see below).

Correlation of poly(C) length and infectivity. To determine the differences between the infectious and the noninfectious cDNA clones, we characterized the plasmids by cleavage with several different restriction endonucleases and by translation of in vitro-derived RNA in rabbit reticulocyte extracts. No changes in the pattern of the DNA fragments or in the protein bands were detected (data not shown). Analysis of the poly(C) region by DNA sequencing revealed that the infectious cDNA clone contained probably more than 30 cytidyl residues, whereas the homopolymer in noninfectious clones ranged from 17 to 25 nucleotides.

The exact nucleotide sequence of the infectious clone in the region beyond the poly(C) tract could not be analyzed in several approaches using different plasmid preparations derived from independently isolated transformants. The length of the poly(C) tract of this clone was therefore determined by an alternative approach at the RNA level. ^{32}P-labeled in vitro transcripts of the plasmid were incubated with RNase T_1, and the sizes of the digestion products were analyzed by electrophoresis on a 10% sequencing gel. The largest RNase-resistant fragment was found to be 35 nucleotides in length (Fig. 4). Since RNase T_1 cleaves specifically 3′ of guanosine, three nucleotides must be subtracted according to the nucleotide sequence (Fig. 1), resulting in a homopolymeric stretch of 32 cytidyl residues in this clone.

The poly(C) tracts of two individual virus preparations derived from the infectious *E. coli* clone were also analyzed by using in vivo-labeled viral RNA (19). Both samples displayed a single, defined length of the poly(C) tract, but it was markedly longer than the homopolymer in the original cDNA (Fig. 4). Lengths of 60 and 80 nucleotides, respectively, were determined by using single-stranded *Hae*III fragments of bacteriophage fd (1) as size markers.

The recombinant virus containing 80 cytidyl residues was

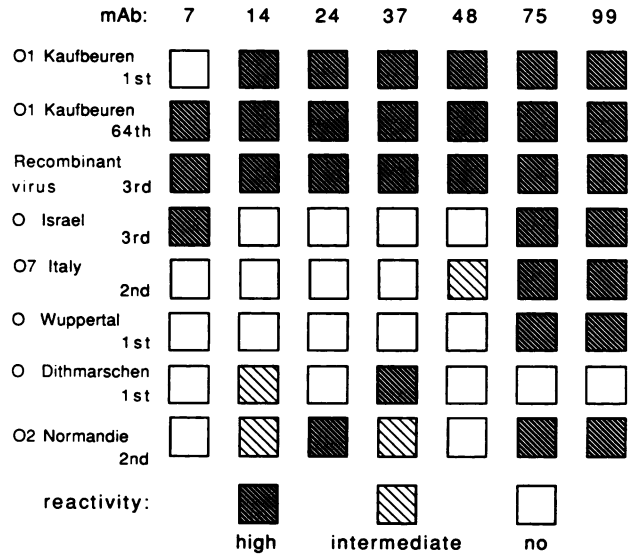

FIG. 5. Characterization of the recombinant virus by ELISA. The reaction of several different FMDV strains of serotype O with a set of strain O1K-specific monoclonal antibodies (7 to 99) was tested in an ELISA as described in Materials and Methods. The ordinal numbers below the names of the viruses indicate the number of passages in BHK-21 cells. Reactivity was subdivided in three categories (high, intermediate, and no reaction). The derivation of the recombinant virus from strain O1K is clearly seen and excludes the possibility of contamination with strain O Israel, which was used as a control during transfection. The first passage of strain O1K shows a slightly different reaction pattern, suggesting a heterogeneous virus population in the original field isolate.

propagated in 30 consecutive passages in BHK-21 cells. The poly(C) tract did not change during these passages. The length of the poly(C) tract of the O1K wild-type virus determined under the same conditions was found to be approximately 200 nucleotides (passage 7 as well as passage 64; Fig. 4).

Biological and immunological characterization of the recombinant virus. The plaque sizes of the wild-type and passage 30 recombinant viruses were indistinguishable on BHK-21 and bovine kidney (BK) cells. Earlier passages of the recombinant virus produced smaller plaques on BHK-21 but not BK cells (not shown). Using a set of type O1-specific monoclonal antibodies in an ELISA, the recombinant virus could unequivocally be identified as serotype O1K (Fig. 5). This finding excluded a potential contamination with strain O Israel, which was used as a transfection control. Interestingly, the first passage of the O1K virus in BHK-21 cells showed a slightly different recognition pattern of the different monoclonal antibodies, suggesting a heterogeneous virus population in the original isolate. (The virus used for construction of our cDNA clone derives from a later passage; see Materials and Methods.)

To analyze the viability of the viruses in an environment other than BHK-21 cells, baby mice and primary BK cells were infected. The virulence of the recombinant virus for baby mice (50% lethal dose [LD_{50}] = $10^{4.2}$ PFU per mouse) was reduced by approximately 1 order of magnitude compared with the value for the virus stock used to derive the cDNA clone (LD_{50} = $10^{3.1}$ PFU per mouse). Although no differences in viral proteins were seen by immunological means, we cannot exclude the possibility that mutations besides the shorter poly(C) length and the artificial nucleotides account for this decrease in virulence. Nevertheless, the reduced virulence for baby mice may not reflect the virulence for natural hosts, since infectivities for different species are not necessarily correlated. A highly passaged batch of FMDV O1K (64 passages in BHK-21 cells) that is avirulent for cattle (unpublished observation) was relatively infectious for baby mice (LD_{50} = 10^3 PFU per mouse).

TABLE 1. Virus titers on BK and BHK-21 cells

Virus	Passage no.	PFU/ml	
		BK cells	BHK cells
Recombinant[a]	2	3×10^7	2×10^5
	3		4×10^5
	5		4×10^5
	10	2×10^7	
	15		1×10^6
	20	1.2×10^7	
	30	1.2×10^7	1.5×10^7
O1K wild type	7	1.8×10^7	1.3×10^7
	64	1×10^6	2.5×10^7

[a] Recombinant virus 2 containing 80 cytidyl residues.

Initially, the growth pattern of the recombinant viruses in primary BK cells in comparison with growth in the BHK-21 cell line was puzzling. Complete lysis of BHK-21 cells after transfection usually could not be observed, and the virus titers were very low in the first few passages (on the order of 10^5 PFU/ml; Table 1). However the titers in primary cells were high in these passages (>10^7 PFU/ml). During passage in BHK-21 cells, the titer of the recombinant virus increased by 2 orders of magnitude (1.5×10^7 PFU/ml), which is comparable with the values for normal BHK-21 cell-adapted FMDV. Inversely, the titer in primary cells dropped during these passages by a factor of 2.

Increase in the virus titer in consecutive tissue culture passages is a frequently observed phenomenon in field isolates of FMDV and reflects the adaptation of a wild-type virus to growth under artificial conditions. In this respect, our recombinant virus behaves like a wild-type virus, which is not unexpected since it derives from an early passage in BHK-21 cells. The higher titer of the natural virus in the seventh BHK-21 cell passage (1.3×10^7 PFU/ml; Table 1) can be explained by the presence of a mixture of viruses with different (higher) capabilities to grow in tissue culture, whereas the infectious clone was derived from a virus not yet adapted to these cells.

DISCUSSION

This report contains the first description of a full-length cDNA clone of FMDV. RNA derived in vitro from this DNA proved to be infectious for tissue culture cells. Although infectious cDNA clones for picornaviruses other than FMDV have been constructed in the past (5, 8, 16, 22, 25), this technological breakthrough was not achieved for FMDV despite the fact that most regions of the FMDV genome had been cloned by several groups (3, 18, 26). Some regions of the untranslated 5′ region had, however, resisted cloning in *E. coli*. The major difficulty in obtaining clones from this region of the viral genome was thought to be the presence of a long homopolymeric cytidyl sequence characteristic of cardioviruses and aphthoviruses. In the case of cardioviruses, this homopolymer seems to be dispensable, as recently shown by Duke and Palmenberg (8) for a mengovirus-encephalomyocarditis virus chimera. Oligo(C) as short as

eight nucleotides was shown to allow replication of the virus in tissue culture, in which case this short C sequence was stably maintained.

Our results indicate that such short C sequences are not sufficient for the replication of FMDV. The lowest number of nucleotides in the poly(C) tract necessary for infectivity of plasmid-derived RNA was found to be 32 in our experiments. This number of C residues seems to be crucial in two respects. On the one hand, it seems to be at the upper limit to be tolerated in a high-copy-number plasmid in *E. coli*. We do not know why we were unable to establish a correspondingly long C tract in the smaller vector pSP64. Possibly, the large size of the yeast vector construct (17 kb) or the resulting reduced plasmid copy number per cell led to maintenance of this sequence. On the other hand, a poly(C) tract of 32 nucleotides seems not to be sufficient for efficient replication of infectious virus particles, since no viruses with poly(C) sequences of this short size were detected after transfection. Until now, the shortest poly(C) tract of a virus produced by transfection of in vitro-synthesized RNA consisted of 60 residues, which is in agreement with the findings in natural virus isolates (6; our unpublished results). Whatever mechanism underlies this amplification of the poly(C) tract, it seems to be a prerequisite for establishing viral replication.

In transfection assays with BHK-21 cells, we observed that lysis occurred considerably earlier in controls with viral RNA than with the plasmid-derived RNA. Some of the cell cultures transfected with the same batch of SP6 RNA did not lyse at all. Finally, only one specific size of the poly(C) tract was observed in each of the progeny viruses derived from a successful transfection. Taken together, these results indicate that the enlargement of the poly(C) sequence is a rare event. The additional 12 nucleotides present at the 5′ end of the viral sequence of the in vitro-synthesized RNA certainly reduce the relative infectivity (30). However, the low frequency at which virus replication is established upon transfection cannot be explained solely by this fact.

Spontaneous changes in the length of the poly(C) tract have been also observed in natural isolates of FMDV (6). In principle, there are two possible mechanisms leading to the size variation of the homopolymer. The poly(C) sequence could be enlarged by "chattering" or "slippage" of the viral RNA polymerase during transcription, a mechanism postulated for the production of long poly(A) sequences at the ends of vesicular stomatitis virus mRNAs, which are coded for by only short oligo(U) stretches on the genome (14). Alternatively, the elongation of the poly(C) tract could be achieved by strand switching of the RNA polymerase during replication, a mechanism proposed to underly recombination in picornaviruses (17). The former mechanism would presuppose specific characteristics of the RNA polymerase and should lead to heterogeneous lengths of the poly(C) tract in viral RNA preparations, which is clearly not observed. The relatively low rate at which virus replication is established argues for the recombination model.

Because production of an infectious virus presupposes enlargement of the poly(C) tract, which occurs only with low frequency, the relative infectivity of the plasmid-derived RNA is not comparable with that of the viral RNA or RNA derived from other recombinant picornaviruses. For standard transfection assays, approximately 1 μg of synthetic RNA was used, which on average led to the production of infective viruses in two of three parallel assays. Supposing that lysis of the transfected tissue culture was induced by a single infectious particle, the relative infectivity would thus be below 1 PFU/μg of RNA.

One could argue that the artificial *Nhe*I linker sequence flanking the poly(C) tract would prevent efficient multiplication of the virus, and only mutants having exchanged some of the "wrong" nucleotides into correct C residues were able to grow. We cannot fully exclude this possibility, since the nucleotide sequence 5′ to the poly(C) has not been determined for the recombinant viruses. However, the linker sequence 3′ to the poly(C) tract was found to be unchanged. The reduced virulence of the recombinant viruses for baby mice could point to a certain interference of the linker sequences with viral replication. This could, however, also be due to the shorter poly(C) tract (80 nucleotides) of the recombinant virus than of the wild-type virus (200 nucleotides).

The relatively high titer in primary cells argues against interference of the additional nucleotides with the viability of the recombinant virus. Rather, it indicates that BHK-21 cells do not represent the ideal tissue for recovery of wild-type viruses from transfection. As it is not possible to maintain primary bovine cells over several passages, we did not try to transfect these cells directly. For future constructions of recombinant FMDV clones, it will be advisable to derive the cDNA from a plaque-purified virus fully adapted to growth on BHK-21 cells rather than from the wild-type form.

The availability of an infectious cDNA clone of FMDV will allow us to address many questions on the functions of viral gene products involved in infection by and replication of this virus group. It will also allow the design of a new approach to the development of a recombinant FMDV vaccine. The introduction of deletions in specific positions of the viral genome should lead to attenuated strains that cannot easily revert to virulence during the highly inaccurate replication of the virus.

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