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Cloning and Synthesis of Infectious Cardiovirus RNAs Containing Short, Discrete Poly(C) Tracts

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Mengovirus RNA transcripts with 5′ noncoding poly(C) tracts of C_8, C_{12}, and $C_{13}UC_{10}$ have been synthesized in vitro from cDNA clones and shown to be infectious to HeLa cells. A chimeric clone has also been constructed which links the 5′ end from one mengovirus clone (299 nucleotides, containing $C_{13}UC_{10}$) to a 7,424-base fragment derived from the 3′ end of encephalomyocarditis (EMC) virus. Progeny virus isolated after transfection with the clone-derived RNAs had the same poly(C) tracts, mengovirus-specific sequences, or EMC virus-specific sequences as the transcript from which it was derived. Although the cloned poly(C) tracts were considerably shorter than those found in viral RNA from mengovirus ($C_{50}UC_{10}$) or EMC virus ($C_{115}UCUC_3UC_{10}$), the growth characteristics of the progeny viruses in HeLa cells were indistinguishable from those of the parental viruses, indicating the length of this tract does not play a significant restrictive role for cardiovirus infectivity in tissue culture.

The positive-sense RNA genomes of all picornaviruses contain a single long open reading frame encoding the viral polyprotein. Unusual among eucaryotic mRNAs, the open reading frame typically begins a minimum of 700 nucleotides from the 5′ end of the genome. In cardioviruses (e.g., mengovirus and encephalomyocarditis [EMC] virus) and aphthoviruses (foot-and-mouth disease), the 5′ noncoding sequences are even longer (750 to 1,300 nucleotides) and are further distinguished by the presence of a homopolymeric poly(C) tract located about 150 to 330 bases from the 5′ end. The sizes of (60 to 250 bases) and specific sequences (e.g., discontinuities) within the poly(C) tracts are characteristic of particular viral isolates (2).

By virtue of its existence, the poly(C) tract has been assumed to play an essential role in the life cycle of those viruses which contain it. Therefore, attempts at recombinant engineering in cardioviruses and aphthoviruses have necessarily concentrated on reproducing these regions into accurate cDNA copies. Unfortunately, long poly(dC-dG) segments are difficult to clone into viable bacterial vectors (4, 5). Short oligomeric dC fragments (10 to 20 bases) are commonly used to "tail" desired DNA fragments before cloning (5, 6), but it has been consistently observed that longer tracts (>30 nucleotides) can severely limit the yield of resulting bacterial colonies (4; unpublished observations).

Complete infectious cDNA copies of several picornavirus RNAs (e.g., poliovirus, rhinovirus, and hepatitis A virus) have been constructed and characterized and are now being widely used in a variety of important mutagenesis and genetic engineering experiments (1, 3, 7, 11). However, none of these viruses contains a natural poly(C) tract, and to date, there have been no descriptions of analogous infectious clones for any picornavirus containing such a tract. We now report construction of three DNA plasmids containing complete copies of the mengovirus genome and of a chimeric plasmid combining sequences from both mengovirus and EMC virus.

The nucleotide sequence of the complete genome of mengovirus strain M has been determined (manuscript in preparation). According to our numbering conventions, bases 1 to 147 represent the 5′ viral S fragment; bases 148 to 208 include the poly(C) tract and its discontinuities; bases 759 to 7637 are the polyprotein-coding region; and the 3′ poly(A) tract begins at base 7762. For EMC virus (strain Rueckert), the analogous base numbers are: 1 to 148 (S fragment), 149 to 280 [poly(C) and discontinuities], 834 to 7709 (polyprotein-coding region), and 7836 [beginning of poly(A)].

First-strand cDNA for our mengovirus plasmids was synthesized using viral RNA (vRNA) oligo(dT_{12}) and avian myeloblastosis virus reverse transcriptase (8). Second-strand cDNA was synthesized in a replacement reaction (9) by using the annealed vRNA as the primer. After addition of *Bam*HI linkers, the double-stranded cDNA was ligated into phosphatase-treated, *Bam*HI-digested pUC9 vector and then used to transform competent JM101 cells to ampicillin resistance. Ampicillin-resistant transformants were screened for the size of their *Bam*HI inserts. The largest insert (contained in plasmid pM6) was a nearly full-length copy of mengovirus RNA [from viral base 21 to the poly(A) stretch].

To obtain the remaining 5′-end fragment, a synthetic oligodeoxynucleotide complementary to vRNA bases 360 to 371 was used to prime cDNA synthesis on vRNA with reverse transcriptase. After the addition of 10 to 20 dG residues to the 3′ ends of the cDNA by terminal transferase, second-strand cDNA was synthesized in a replacement reaction by using dC_{12-18} as a primer. The resulting double-stranded cDNA was ligated into phosphatase-treated, *Sma*I-digested M13mp19 and then used to transform competent JM101 cells. Single-stranded DNA from the resulting plaques was sequenced by the dideoxy method. One clone, mM10, contained an insert with 14 dG residues, followed by bases 1 to 142 derived from the mengovirus genome. By using a unique *Eco*RV site within the mengovirus cDNA sequence, bases 1 to 45 from mM10 were joined to base 46 to the poly(A) tract from pM6. The cDNA fragment was placed between the *Eco*RI and *Bam*HI sites of Bluescribe M13+, and the resulting plasmid was designated pM16 (Fig. 1A).

Sequence analysis of the mengovirus 5′ noncoding region of pM16 DNA showed that it contained a much shorter

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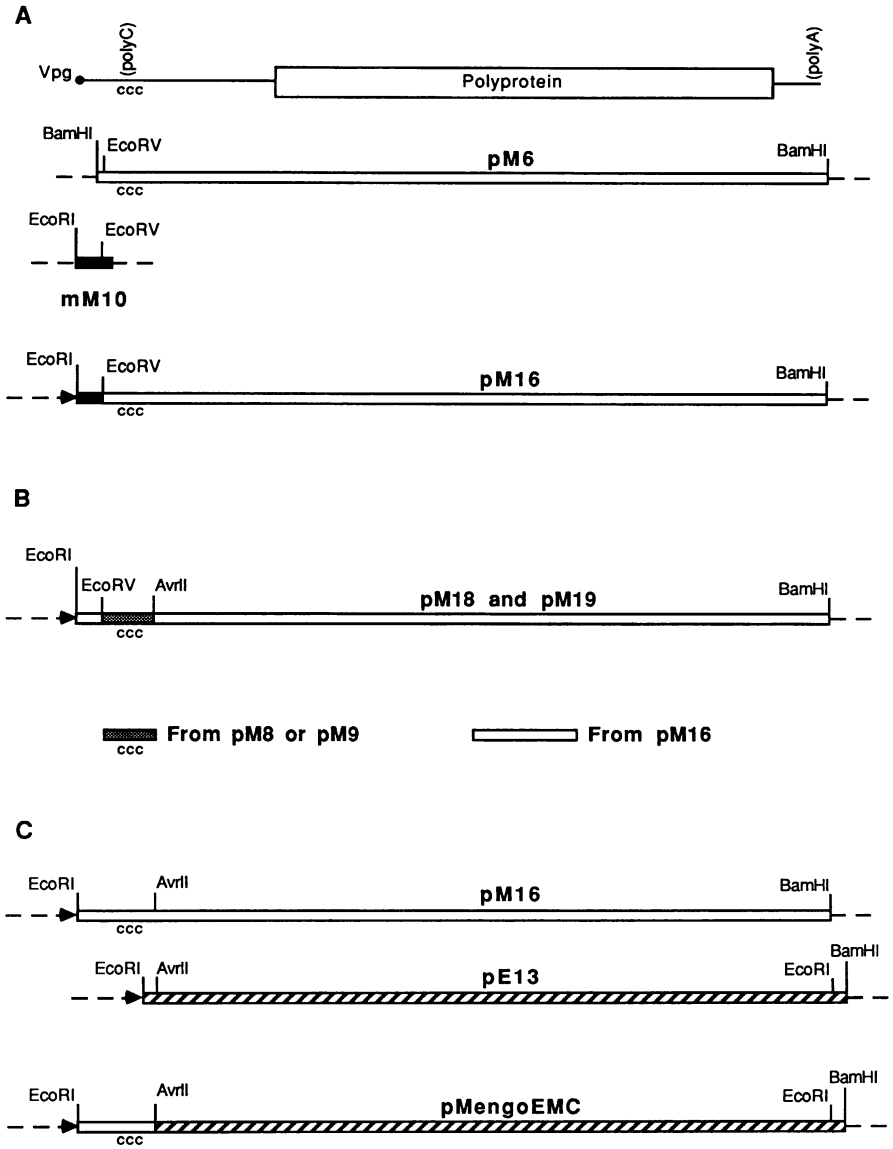

FIG. 1. Construction of pM and pMengoEMC plasmids. (A) A schematic representation of a mengovirus genome shows the relative locations of the viral segments within plasmids pM6 and mM10 which were combined to form the complete plasmid, pM16. Arrow shows the location of T7 promoter sequences within the vector (---). (B) The stippled fragment within pM16 was replaced with the analogous segment from pM8 or pM9 to form plasmids pM18 and pM19, respectively. (C) Plasmid pMengoEMC combined the indicated fragments from pM16 and pE13.

poly(C) tract than the virus from which it was derived. All of the natural poly(C)-flanking heteropolymeric viral sequences were present in the clone, but the specific tract itself ($C_{13}UC_{10}$) was 37 bases shorter than that in vRNA ($C_{50}UC_{10}$). In this regard, although pM16 contains a full-length copy of a cardiovirus genome, it does not represent the complete version of mengovirus strain M.

The transcription vector utilized for pM16 has a T7 RNA polymerase promoter adjacent to the inserted segment. RNA transcripts synthesized in vitro from *Bam*HI-linearized pM16 DNA contained mengovirus sequences of the same sense as that of vRNA. They also contained 23 nongenomic bases at their 5′ ends and 7 nongenomic bases at their 3′ ends, which resulted artifactually from our cloning procedures. Experiments with other picornavirus clones suggested that extra 5′ bases might adversely affect RNA transfection efficiencies even when the viral sequences were intact and full-length (13). But despite the shortened poly(C) tract and heterologous bases, we nevertheless tested pM16-derived RNA for infectivity in cell assays.

Figure 2 shows that transfection of HeLa monolayers with pM16 transcripts indeed induced the formation of plaques. As expected, the specific infectivity (about 10^2 PFU/μg of transcript RNA) was lower than that observed for vRNA (10^5 PFU/μg of RNA), but the ability of clone-derived RNA to form plaques was very reproducible. RNase treatment prior to transfection destroyed the infectivity of the transcripts. Plaque development could also be inhibited by the presence of anti-Maus Elberfeld (ME) virus antiserum but not by anti-poliovirus antiserum (ME virus, mengovirus, and

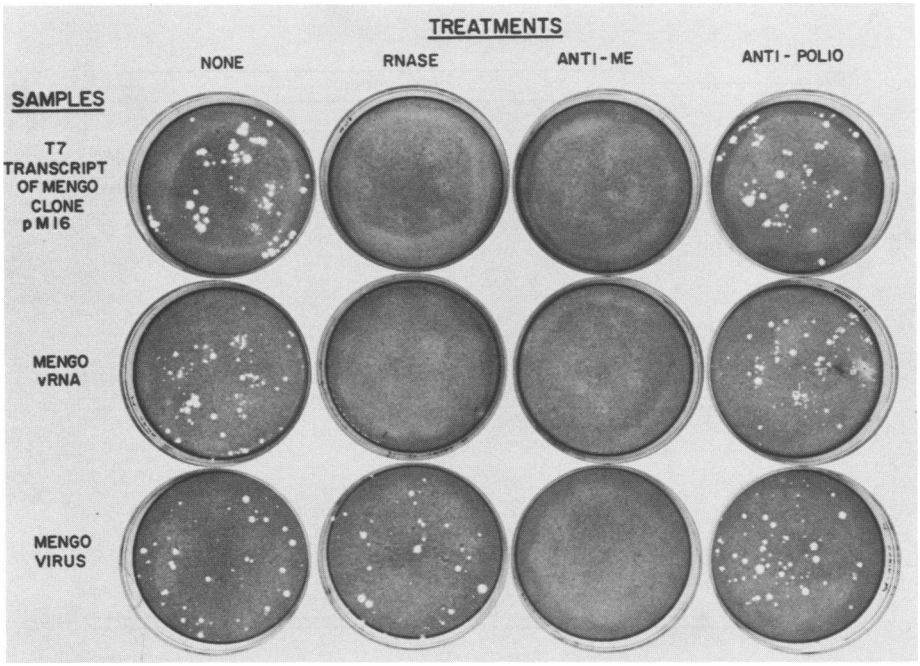

FIG. 2. HeLa cell monolayers after transfection with clone-derived RNA. Plasmid pM16 DNA was linearized with *Bam*HI, and runoff RNA transcripts were synthesized by T7 RNA polymerase (13). HeLa cell monolayers were transfected (13) with pM16 transcripts (0.5 μg of RNA per 10^6 cells) or mengovirus RNA (0.5 ng of RNA per 10^6 cells) or infected with mengovirus (50 PFU). RNase-treated samples were incubated with 10 μg of DNase-free RNase A for 30 min at 37°C prior to addition to cell monolayers. Antibody-treated samples had polyclonal rabbit antiserum to poliovirus or to ME virus added to the medium overlay before allowing plaques to develop. Plates were developed for 30 h at 37°C before staining with crystal violet for the visualization of plaques.

EMC virus have the same serotype). We are therefore confident that the observed plaques resulted from the complete cardiovirus sequences within pM16 RNA transcripts.

In the course of constructing pM16, many other mengovirus cDNA fragments were sequenced within their poly(C) regions. Although we have yet to observe cloned tracts longer than $C_{13}UC_{10}$ (pM6), two isolates, pM8 and pM9, had poly(C) regions of C_8 and C_{12}, respectively. To determine if these further reductions in poly(C) length would adversely affect infectivity to HeLa cells, the *Avr*II-*Eco*RV fragment of pM16 containing the poly(C) tract was replaced with the analogous fragment from pM8 or from pM9 (Fig. 1B). Sequence analysis of the resulting clones, pM18 and pM19, confirmed that they differed from pM16 only in their poly(C) tracts.

RNA transcribed from all three clones was used to transfect HeLa cells (Table 1). RNA transcripts from pM16, pM18, and pM19 were all infectious, and each had approximately the same specific infectivity per microgram of RNA (results not shown). As was the case for pM16 transcripts, RNase treatment of pM18 and pM19 transcripts prior to transfection destroyed their infectivity, and the plaques resulting from pM18 and pM19 transfections could be neutralized by anti-ME virus antiserum.

Plasmids containing large fragments of EMC cDNAs have also been synthesized in our laboratory by methods analogous to those described for mengovirus. Mengovirus and EMC virus are serologically indistinguishable (12), but sequence studies have shown that these genomes share less than 80% nucleotide identity throughout their lengths (in

TABLE 1. Infectivity of cardiovirus RNAs containing diverse poly(C) tracts[a]

Sample	Poly(C) tract	Treatment			
		None	RNase A	Anti-ME serum	Anti-poliovirus serum
pM16 T7 RNA transcripts	$C_{13}UC_{10}$	+	−	−	+
pM18 T7 RNA transcripts	C_8	+	−	−	+
pM19 T7 RNA transcripts	C_{12}	+	−	−	+
pMengoEMC T7 RNA transcripts	$C_{13}UC_{10}$	+	−	−	+
Mengovirus RNA	$C_{50}UC_{10}$	+	−	−	+
EMC virus RNA	$C_{115}UCUC_3UC_{10}$	+	−	−	+
Mengovirus	$C_{50}UC_{10}$	+	+	−	+
EMC virus	$C_{115}UCUC_3UC_{10}$	+	+	−	+
Transfection buffer		−	−	−	−

[a] HeLa cell monolayers were transfected (RNAs) or infected (virus) with the indicated samples as described in the text and in the legend to Fig. 2. Plaque formation (Fig. 2) was scored positive (+) or negative (−) after incubation of plates for 30 h at 37°C and staining with crystal violet.

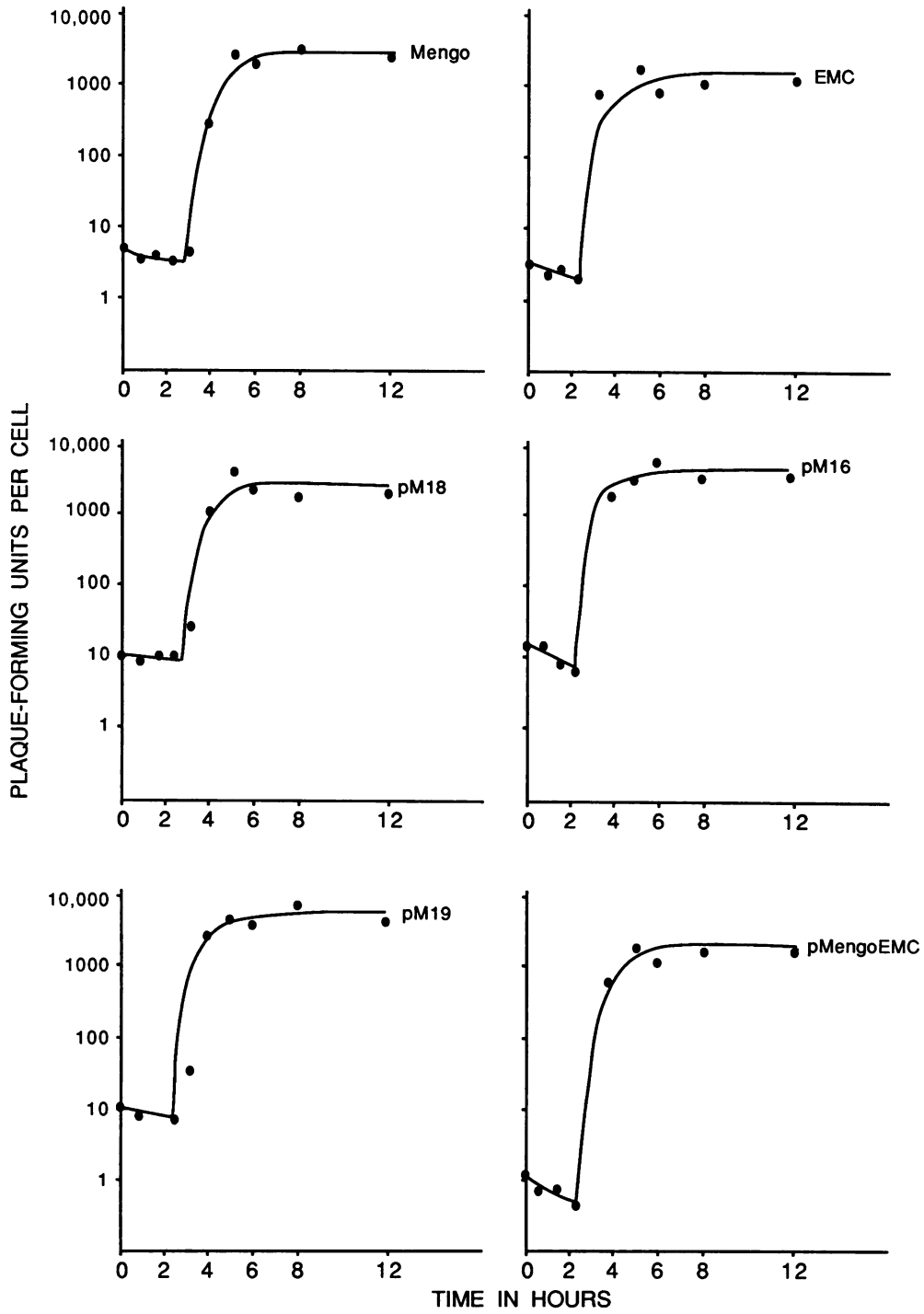

FIG. 3. One-step growth curves for parental and progeny viruses. HeLa cell suspension cultures were infected (multiplicity of infection of 50) with parental virus (mengovirus or EMC virus) or with progeny virus grown from plaques isolated after transfection with RNAs from the indicated clones. After allowing for attachment, cells were sedimented to remove unattached virus, resuspended in fresh medium, and incubated at 37°C. At various intervals thereafter, samples were removed and cells were lysed so as to release both free and membrane-bound virus. The resulting virus was titrated and expressed as PFU per cell.

preparation). Although we have yet to successfully isolate segments from the S fragment or poly(C) tract (C_{125}UC UCCCUC$_9$) from our EMC virus strain, one plasmid, pE13, did contain a cDNA representing the majority of this genome [extending from base 335 through the 3′ poly(A) tract] (10; in preparation). By using a unique restriction site conserved in analogous positions in the 5′ noncoding sequences, 7,425 contiguous base pairs of mengovirus sequence in pM16 were replaced with the corresponding EMC virus sequences from pE13 to produce the chimeric plasmid pMengoEMC (Fig. 1C). T7 RNA transcripts of pMengoEMC contained the first 299 bases of mengovirus 5′ noncoding region joined to the

last 423 bases of EMC virus 5′ noncoding region, the EMC virus polyprotein reading frame, and EMC virus 3′ noncoding region. The chimera contained the same 5′ nongenomic extra bases (23 nucleotides) as the parental mengovirus transcripts but had 26 (rather than 7) 3′ nongenomic bases because of altered restriction sites within the construction.

Transfection of HeLa cell monolayers with RNA from pMengoEMC produced plaques whose large size was characteristic of EMC virus, rather than mengovirus (data not shown). The specific infectivity of pMengoEMC RNA (about 10 PFU/μg of transcript RNA) was approximately 10-fold lower than that of transcripts synthesized from pM16, pM18, and pM19. This lower infectivity could be due to the additional 3′ end nongenomic bases or the result of reduced virus viability due to combining portions of two quite different genomes. As with the other clones, RNase treatment of pMengoEMC transcripts prior to transfection destroyed their infectivity and plaques resulting from transfection with untreated pMengoEMC transcripts could be neutralized by anti-ME virus antiserum (Table 1).

The virus derived from transfection of HeLa cells with each type of RNA transcript (progeny virus) was plaque purified for further characterization. After amplification in cells, the recovered RNAs were sequenced by primer extension reactions through their 5′ noncoding regions. All resultant viruses contained the same poly(C) tracts, mengovirus-specific bases, or EMC virus-specific bases as the original transcripts. Even after repeated passage (five cycles, multiplicity of infection estimated at 10 to 50), the short poly(C) tracts were not restored to native length or even altered by a single base. However, the 5′ ends of progeny RNAs did not contain any of the nongenomic bases that had been present in the transcripts. The heterologous bases had been removed during transfection or virus passage, making these ends identical to the sequence of mengovirus RNA (sequence data not shown). Essentially, the engineered progeny viruses differed from parental virus only in the lengths of their specific poly(C) tracts.

To determine whether the shortened tracts adversely affected the overall growth characteristics of these viruses, we carried out one-step growth experiments (Fig. 3). HeLa cell suspensions were infected with EMC virus, mengovirus, or one of the progeny viruses (pM16, pM18, pM19, or pMengoEMC). A high multiplicity of infection was chosen (i.e., 50) so that a majority of cells would be infected. Total virus production, expressed as PFU per cell, was determined for samples of the cell suspensions at various times after infection. For all tested viruses, the lag period and rise period of exponential growth were of similar duration. Both parental and progeny viruses gave comparable maximum yields, even when tested at several different temperatures (all data not shown). Thus, within experimental limitions, we conclude that the shortened poly(C) tracts in our engineered viruses did not limit their growth in HeLa cells and suggest that this tract may actually be completely dispensable for the reproductive functions of cardioviruses in tissue culture. We strongly suspect the same principle also will apply to the aphthoviruses. By deleting the poly(C) region from analogous constructions, it should now be possible to synthesize infectious clone-derived RNAs for many strains of foot-and-mouth disease virus.

Despite these engineering advances, our tissue culture experiments with EMC virus and mengovirus infectious clones have failed to clarify the biological function of the long, natural poly(C) tracts. Why are these unwieldy sequences conserved within the native viruses if they are unnessary for infectivity? To test for possible host involvement in poly(C) selection criteria, we have recently begun to inoculate mice with our cloned, progeny viruses. In contrast to the tissue culture experiments presented here, the preliminary mouse data indicate that protective host responses to cardiovirus infection may be directly mediated by the length of the poly(C) tract. We intend to use our infectious clones to fully explore this phenomenon.

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