Mutational Analysis of the Mengovirus Poly(C) Tract and Surrounding Heteropolymeric Sequences

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Previously, we described three mengovirus mutants derived from cDNA plasmids, containing shortened poly(C) tracts (C_8, C_12, and C_13UC_10), that exhibited strong attenuation for virulence in mice yet grew like wild-type virus in HeLa cells. Thirteen additional mutants have now been constructed and characterized. Five of these differ only in poly(C) length, including one with a precise deletion of the tract. The other mutants bear deletions into the regions juxtaposing poly(C). Studies with HeLa cells confirm the essential dispensability of mengovirus’s poly(C) tract but reveal a subtle, measurable correlation between poly(C) length and plaque diameter. Virulence studies with mice also reveal a strong correlation between poly(C) length and virulence. For the poly(C)-flanking mutations, the 15 bases directly 5’ of the tract proved dispensable for virus viability, whereas the 20 to 30 bases 3’ of poly(C) were critical for growth, thus implicating this region in the basal replication of the virus.

Cardiovascular-like encephalomyocarditis viruses (EMCVs) and mengovirus and also the aphthoviruses contain unusual, homopolymeric poly(C) tracts in the distal region of their 5’ untranslated regions (5’ UTRs) (Fig. 1). We previously described four cDNAs encoding infectious sequences of mengovirus. The plasmids were identical, except in their 5’ UTRs, where three had shortened poly(C) tracts of C_8, C_12, and C_13UC_10, and one had the wild-type length, C_44UC_10. All viruses were infectious to HeLa cells and had growth kinetics similar to those of natural mengovirus strain M (5, 6). However, inoculation studies demonstrated that the mengovirus poly(C) tract plays a critical role in murine virulence, because the median 50% lethal doses (LD_{50}) of the short-tract isolates were as much as 10^5-fold higher than those of mengovirus’s poly(C) tract but reveal a strong correlation between poly(C) length and plaque size is shown graphically in Fig. 2B and was determined by least-squared-fit analysis, with r^2 = 0.821.

Viruses with heteropolymeric deletions had less predictable plaque sizes. Mutant vMC15C13 gave plaques (~1.9 mm) equivalent to those of vMC12, despite its larger overall deletion. Mutant vMC15C22, missing two bases 3’ to the poly(C), likewise had a plaque size between those of vMC12 and vMC24. In contrast, all mutants with lesions extending ~10 bases 3’ of poly(C) had distinctively smaller plaques that were designated small (~1 mm) or minute (~0.5 mm). Common among these strains, but not among those with medium plaques, were deletions that impinged upon stem-loop D. Isolates vMC15C10, vMC15C14, vMC15C17, vMC15C22, and vMC15C31 each lack between 4 and 25 bases of this element. Their reduced plaque morphologies imply a stronger viral reproductive role for stem-

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loop D than for the poly(C) tract and its flanking single-stranded sequences.

The growth of mutant picornaviruses is sometimes variable at different temperatures. Representatives of the poly(C) panel were plated and allowed to grow at higher (39°C) or lower (33°C) temperatures than normal (37°C). Mengovirus strain M, vMwt, vMC15ΔC13, vMC10, vMC4, vMC17, vMC8Δ22, and vMC3Δ31 yielded equivalent plating efficiencies at all temperatures tested (data not shown). Typical of most viruses, these isolates had larger plaques at 39°C than at 37°C and smaller plaques at 33°C, but the variation was coordinate among all strains, and no mutant showed a clearly enhanced heat or cold sensitivity relative to that of mengovirus strain M or vMwt (Fig. 2A). In related thermostability experiments,
vMC_30 and vMC_24 particles proved no more thermolabile than vMwt when assayed for infectivity after incubation at 62°C (data not shown).

(iii) Tissue culture studies. For a plaque to develop, multiple rounds of infection must occur. Despite their plaque differences, the poly(C) lengths of recombinant EMCVs, mengovirus strain M, vMC_24, vMC_12, and vMC_8 have only limited influence on single-step growth in HeLa cells (6, 8). Not surprisingly, the kinetic profile of vMC_0 was also superimposable with those of vMwt and vMC_24 (Fig. 3A). All had eclipse times of about 2.25 h and maximum virus yields at 6 h. This indicates that the subtle plaque variation for viruses with poly(C) deletions required multiple rounds of infection to manifest. The growth curves of vMC_0 and vM_D15C13 were also similar (Fig. 3B) and suggest that, as with poly(C) itself, the bases 5' to the tract contribute little to virus reproduction. However, in parallel assays, the growth of isolates with deletions into stem-loop D was significantly retarded. Infection with vM_D3C0D10, vMC_8D17, vMC_4D22, or vMC_D31 required at least 3 h before progeny appeared and 8 to 12 h for maximum titer (Fig. 3B). The results are consistent with the smaller plaques for these viruses and again mark stem-loop D as functionally different from poly(C) or the flanking single-stranded regions.

(iv) Virulence in mice. Upon inoculation into animals, vMC_9, vMC_12, and vMC_24 are highly attenuated relative to their long-tract counterparts and protectively immunize recipient animals rather than kill them (5). Despite clinical use of these strains as vaccines and vaccine vectors, the tract length at which mengovirus becomes attenuated has not been carefully quantitated. Accordingly, LD_50 data were collected for the poly(C) panel after intracerebral (i.c.) (Fig. 4) or intraperitoneal (Table 1) (i.p.) inoculation. As previously reported, mengovirus strain M, vMwt, or EMCV-R killed mice at very low doses (5, 14), but killing (Swiss mice) via i.p. route typically required about 100- to 1,000-fold more virus. As expected, incremental truncation of the mengovirus poly(C) progres-
other cardioviruses in Table 1. Open symbols denote minimum values. Relative to poly(C) tract length and are presented with comparative reference to four different viral doses in groups of five mice per dose. The data are plotted BALB/c mice as described previously (7). Each determination included at least four different viral doses in groups of five mice per dose. The data are plotted relative to poly(C) tract length and are presented with comparative reference to other cardioviruses in Table 1. Open symbols denote minimum values.

FIG. 4. Lethality of recombinant mengoviruses. The LD$_{50}$ of vM isolates were measured after i.c. inoculation of 4- to 6-week-old female Swiss ICR or BALB/c mice as described previously (7). Each determination included at least four different viral doses in groups of five mice per dose. The data are plotted relative to poly(C) tract length and are presented with comparative reference to other cardioviruses in Table 1. Open symbols denote minimum values.

### Table 1. LD$_{50}$ of virus strains

<table>
<thead>
<tr>
<th>Virus</th>
<th>LD$_{50}$ (PFU)</th>
<th>BALB/c mice, i.e.</th>
<th>Swiss ICR mice, i.e.</th>
<th>i.p.</th>
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<tr>
<td>Mengovirus strain M</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>vMwt</td>
<td>1.3 × 10$^{4}$</td>
<td>9</td>
<td>1.0 × 10$^{5}$</td>
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</tr>
<tr>
<td>vMC$_{33}$</td>
<td>8.0 × 10$^{4}$</td>
<td>9</td>
<td>1.0 × 10$^{5}$</td>
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<tr>
<td>vMC$_{37}$</td>
<td>1.3 × 10$^{5}$</td>
<td>7.0 × 10$^{2}$</td>
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</tr>
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<td>vMC$_{30}$</td>
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<td>vMC$_{24}$</td>
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<td>8.0 × 10$^{4}$</td>
<td>&gt;1.0 × 10$^{16}$</td>
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<tr>
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<td>&gt;1.0 × 10$^{10}$</td>
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</tr>
<tr>
<td>vMC$_{0}$</td>
<td>&gt;2.0 × 10$^{9}$</td>
<td></td>
<td>&gt;2.0 × 10$^{9}$</td>
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<tr>
<td>vM/E</td>
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<td>5.0 × 10$^{5}$</td>
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<tr>
<td>EMCV-R</td>
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<tr>
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<td>vEC$_{e}$</td>
<td>3.0 × 10$^{3}$</td>
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* Mengovirus strain M, vM/E, and EMCV-R have poly(C) sequences of C$_{30}UC_{15}C_{30}$, C$_{27}UC_{18}$, and C$_{113}UCUC_{10}$UC$_{10}$, respectively.
* From reference 14.
* From reference 8.

sively attenuated the virus. Between tract lengths of 24 to 37 bases, the LD$_{50}$ (i.e., Swiss mice) increased by about 1 log$_{10}$ for every three C's removed from the tract. The 24-base difference between vMC$_{24}$ and vMC$_{0}$ gave an additional 3-log$_{10}$ increase, though this value was only a minimal estimate. The practical and humane ceiling on injectable volume (20 µl) for the i.c. route restricted testable doses to ~10$^{7}$ PFU. Even so, at this concentration, no 4-week-old animal was killed or even sickened by vMC$_{0}$ or vMΔ3C$_{0}$Δ10. By the i.p. route, the maximum dose was >10$^{11}$ PFU (~50 µg of virus in 100 µl) and was reached when the tract length was C$_{30}$ or smaller. Again, no animals were killed by these doses of vMC$_{24}$ of vMC$_{0}$. Shorter viruses were at least this attenuated when tested in a few animals (i.e., 5 to 10 mice), but since i.c. doses of vMC$_{0}$ and vMΔ3C$_{0}$Δ10 were completely nonvirulent, it seemed unwar-

ranted, in terms of animal use, to measure full-scale i.p. doses for these strains.

With some types of viruses, the strain of mouse can play a major role in disease development. Therefore, inbred BALB/c animals were also tested for poly(C) response. In general, these mice proved more refractory to all tested viruses than outbred Swiss animals, but again, the short poly(C) isolates were highly and progressively attenuated, with at least a 5-log$_{10}$ increase in the LD$_{50}$ when poly(C) was shortened from the length in vMwt to the length in vMC$_{0}$.

**Discussion.** The poly(C) tracts of cardio- and aphthoviruses are enigmatic sequences. The tracts are the only known homopolymeric cytidine segments within natural RNA or DNA, and their presence surely creates a significant genetic burden for such small, encapsidated viruses. Nevertheless, poly(C)s of up to 420 nucleotides, or ~5% of the total genome, are carried and passed with fidelity (7). Our previous reports (5, 8, 14), the data presented here, and related experiments with foot-and-mouth disease virus (16) agree conclusively with each other in showing that poly(C) can be deleted without notable impact on virus viability. poly(C) is functionally invisible to ribosomes (data not shown) and does not contribute to temperature-dependent growth or particle stability. vMC$_{0}$, with a precise deletion of the tract, grew identically to vMwt in single-step growth assays. To be sure, a subtle, measurable plaque reduction, dependent upon multiple rounds of infection, is reproducibly correlated with tract length for both EMCV and mengovirus, but major vegetative life cycle functions in HeLa cells are clearly not vested in this region.

Our analyses with mengovirus deletions adjacent to poly(C) now also confirm that the 15 nucleotides 5' of poly(C) are likewise dispensable for growth. Possibly, these A+G-rich sequences are functionally contiguous to poly(C) itself. Alternatively, this segment may serve as a buffer or vestigial spacer between the nearby 5' pseudoknots and the poly(C) tract. Supporting this idea is the low degree of sequence conservation among homologous cardioviruses. Within the singular stretch deleted in vMΔ15C$_{30}$, four substitutions and four insertions or deletions differentiate mengovirus strain M from EMCV-R. In contrast, the B and C pseudoknots next to stem-loop A are well conserved. Recent data for new mutations in this region seem to associate these motifs with some level of indispensable replicative activity and possibly with synthesis of viral RNA itself (10a). Thus, the knots are probably distinguished from poly(C)-linked behavior.

The mapping studies of sequences 3' of poly(C) uncovered a previously unrecognized operonic segment. All six deletions that impinged on stem-loop D resulted in retarded growth kinetics and produced viruses with small plaques. This replicative impediment was not shared by mutants with poly(C)-specific deletions or by vMC$_{18}Δ2$, which had the smallest 3' deletion. Our recombinant methods for contiguous poly(C) truncations were not designed to introduce independent deletions within this 3' segment, although this clearly now needs to be done. The D-loop phenotypes were unexpected but indicate that this region provides some additional function vital to the virus.

The object of this study, poly(C) itself, remains strongly associated with mengovirus virulence. The new cDNAs put this relationship into clearer perspective. Namely, the shorter the poly(C) tract was, the more attenuated the virus was, in an effect that was both progressive and logarithmic as measured by the LD$_{50}$. There was no specific threshold at which mengovirus suddenly became avirulent. Rather, each deletion, from C$_{32}$ to C$_{w}$, was measurably less lethal than its predecessor. To be sure, the upper limits of these data are still only estimates.
Even the highest doses of vMC0, >109 PFU, failed to kill any animals or make them sick. Nevertheless, the attenuation spans at least 6 log10 PFU in LD50 relative to that of vMwt, a scope that holds up whether assessed i.c. or i.p. in inbred or outbred mice. Without a doubt, any putatively increased genetic load of a long poly(C) tract in the natural mengovirus isolates seems overwhelmingly balanced by increased lethality.

Whether the short-tract strains are simply more effective at triggering important elements in the adult host’s defense system or whether they are defective in some critical replication step within a specific target tissue is only conjecture at this time. In mice, pigs, and primates, the defensive response to short poly(C)Tracts translates quickly into protective immunity (5, 12). Unwittingly, our cDNAs may have created sequences which, if they occurred naturally, would face strong and immediate negative selective pressure, triggering potent antiviral responses and in essence vaccinating the host instead of killing it. That vMC0 is now available and is even more attenuated than previous strains holds promise for safer live vaccines against all cardioviruses of this serotype (1, 2).

But can the mengovirus data be extrapolated to other cardio- and aphthoviruses? All mengovirus deletions, except those with the most extensive deletions (into the B, C, or D motif), were infectious to HeLa cells and to grow in a robust manner. The same is true for recombinant EMCV or foot-and-mouth disease virus isolates, in which the poly(C)Tracts have been deleted as short as C2 without affecting infectivity. However, recombinant vE20, the shortest EMCV tested, is still quite virulent (8), and shortened foot-and-mouth disease virus (C2) also retains nearly the same level of virulence for mice as its long-tract parental strain (16). The key to this paradox may soon be resolved with new poly(C)Tract chimeras. Replacement of the 5’ end of the EMCV vE20, with that from vMC24, has produced an infectious hybrid [vM/E, with a C24 poly(C) tract] with a plaque phenotype like that of EMCV but with extreme attenuation like that of vMC24 (8). Thus, buried within the 28 substitutions and 4 insertions or deletions that differentiate vE20 from vM/E must be other required bases or structural motifs that help potentiate virulence. Possibly these lie within the adjacent spacers only briefly explored by deletions described here. In a mengovirus context, a short poly(C) tract is sufficient to attenuate; in an EMCV (or foot-and-mouth disease virus?) context, a short tract will not attenuate, unless these nearby elements are also changed. Further characterization of these required sequences seems key to understanding the extremely virulent nature of these viruses.

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REFERENCES


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