JOURNAL OF VIROLOGY, Apr. 1995, p. 2697–2699 Vol. 69, No. 4
0022-538X/95/$04.00+0

Encephalomyocarditis Viruses with Short Poly(C) Tracts Are More Virulent than Their Mengovirus Counterparts

HARRY HAHN AND ANN C. PALMENBERG*

Institute for Molecular Virology and Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison, Wisconsin 53706

Received 14 November 1994/Accepted 18 January 1995

We have constructed three cDNA clones of encephalomyocarditis virus strain R (EMCV-R) with poly(C) tracts of C_4, C_9, and C_{20}. RNA transcribed from these cDNAs was infectious to HeLa cells, and the resultant viruses grew well in this system, albeit with plaque sizes that were proportional to the poly(C) length. When injected into mice, the progeny viruses were only slightly less pathogenic than EMCV-R, and the observed degree of attenuation was not nearly as dramatic as for equivalent mengoviruses with similar short poly(C)s. Short-tract poly(C)-mediated attenuation is therefore highly dependent on viral genomic context.

Encephalomyocarditis virus (EMCV) is a cardiovirus of the picornavirus family. EMCV and the related mengoviruses share with the aphthoviruses a feature not found in other RNA viruses: a homopolymeric cytidylate tract located in the 5′ untranslated region (UTR) of their genomes (1). The poly(C) tract varies in length from 60 bases in mengovirus to more than 400 bases in some isolates of foot-and-mouth disease virus (FMDV, an aphthovirus). Although the tracts are largely homopolymeric, they may occasionally contain short discontinuities, such as U residues. The poly(C) of EMCV strain R (EMCV-R) has the sequence $C_{115}UCUC_3UC_{10}$ (2).

The precise role of poly(C) in these viruses is unknown. cDNA-derived isolates of mengovirus, EMCV-D, and FMDV are viable in tissue culture even if they contain massive deletions within or around the poly(C) (4, 7, 10). The engineered strains of mengovirus, in particular, seem unfettered by such deletions, and propagated virus stocks maintain their short poly(C)s even after multiple passage in tissue culture. Studies with mengovirus in murine hosts further suggest that the poly(C) length may play an important role in viral pathogenesis because isolates with short poly(C)s are attenuated in virulence by several orders of magnitude as measured by the median lethal dose (LD_{50}) (3). Expansion of poly(C) length during passage in mice has not been observed for these isolates. With FMDV, however, the poly(C) tracts within cDNA-derived sequences tend to expand when transcript RNA is transfected into tissue culture (10). Additionally, FMDV strains with poly(C)s as short as 2 bases in length are reported to be as virulent as those with longer tracts (10). The data suggest that while FMDV and mengovirus may be closely related picornaviruses, their poly(C) tracts may potentially have different roles in tissue culture or in vivo.

We have now examined the effect of varying poly(C) length in EMCV-R, using the system previously described for the characterization of short-tract mengoviruses. The EMCV are among the most pathogenic of all cardioviruses and have only about 85% nucleotide identity with mengovirus (8). The aim of these experiments was to establish whether an EMCV with a short poly(C) would behave more like mengovirus (stable tract length and attenuated phenotype) or FMDV (unstable tract length and virulent phenotype).

Methods and results. (i) Cloning of infectious cDNA. cDNA clones of poly(C)-containing picornaviruses are difficult to construct (4, 7, 10). Reverse transcriptase and other template-dependent DNA polymerases have difficulty reproducing such tracts with fidelity. Furthermore, bacterial hosts do not faithfully maintain plasmid DNAs containing long, homopolymeric dC · dG tracts (6, 9). These technical problems have been approached in different ways. Duke et al. obtained mengovirus cDNAs with tracts of $C_{50}UC_{10}$ using avian reverse transcriptase to copy natural viral poly(C)s (3). Others have used synthetic, double-stranded oligonucleotides to recreate the poly(C) region (7, 10, 11). We now report another approach, with terminal deoxynucleotidyl transferase (TdT), a template-independent DNA polymerase, to enzymatically synthesize poly(C) tracts of varying lengths.

The construction of an infectious mengovirus-EMCV chimera (pM/E) in which all but 411 of the 5′-most bases of the genome were derived from EMCV-R has been reported (4, 5). This plasmid contains a shortened poly(C) tract of $C_{13}UC_{10}$. Our strategy was to replace the 5′ mengovirus moiety of this chimera with EMCV-R sequences. This was accomplished by use of reverse transcriptase PCR of virion RNA to produce fragments containing the sequences that flank the natural poly(C) (Fig. 1A and B). Two fragments, Sf and Lf, were independently cloned into vectors. Sf and Lf moieties were then ligated after TdT tailing of Sf with dCTP and of Lf with dGTP (Fig. 1C). This step created artificial poly(C)s of various lengths in a series of constructs named pSLf. These plasmids were sequenced to determine their precise poly(C) lengths. We selected isolates with poly(C)s of 4, 9, and 20 bases for further characterization. The requisite SLf fragments were then used to replace the mengovirus moiety of pM/E cDNA (Fig. 1E). The completed EMCV-R constructs were named pE-C_4, pE-C_9, and pE-C_{20} according to their poly(C) lengths (Fig. 1F).

(ii) Tissue culture studies. The pE plasmids were linearized at an *Sal*I site 3′ of the viral poly(A) sequence, and genomic RNA was transcribed by T7 RNA polymerase. The RNA transcripts contained two nonviral G bases at their 5′ ends and five nonviral bases, after a 17-base poly(A) tail, at their 3′ ends (Fig. 1G). RNAs from pE-C_4, pE-C_9, and pE-C_{20} were transfected into HeLa cell monolayers with DEAE-dextran, and viral plaques were produced according to previously described methods (4). The resultant cloned, progeny viruses were

* Corresponding author. Mailing address: Department of Animal Health and Biomedical Sciences, University of Wisconsin, 1655 Linden Dr., Madison, WI 53706. Phone: (608) 262-7519. Fax: (608) 262-7420. Electronic mail address: palmenberg@psl.wisc.edu.

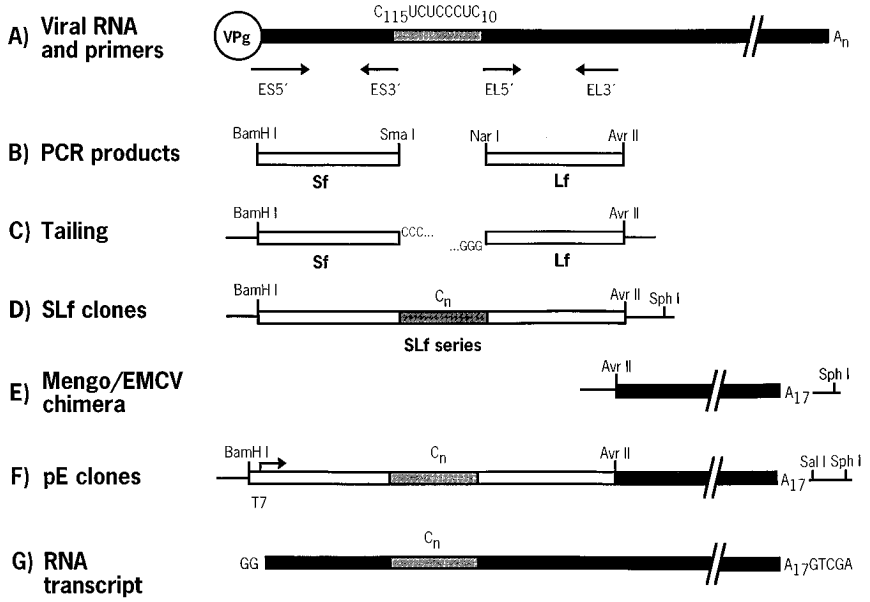

FIG. 1. Construction of EMCV-R cDNA clones with short poly(C) tracts. (A) Negative sense primers ES3′ and EL3′ primed cDNA synthesis from viral RNA. (B) The cDNA was PCR amplified with the primer pairs ES5′ plus ES3′ and EL5′ plus EL3′ to give two products, Sf and Lf. These were cloned independently. (C) Sf was cut with *Sma*I and tailed with dCTP. Lf was cut with *Nar*I, the overhang was removed with mung bean nuclease, and the resulting blunt end was tailed with dGTP. (D) Tailed Sf and Lf were ligated to give SLf. (E) The genome 3′ of the *Avr*II site was obtained from pM/E. (F) pM/E was cleaved with *Avr*II and *Sph*I, and the viral fragment replaced the equivalent fragment in the SLf construct. The final construct was called pE. (G) RNA transcripts were synthesized by T7 RNA polymerase from its promoter on the DNA template linearized downstream of the viral sequence at a *Sal*I site. These transcripts have 2 nonviral residues at the 5′ end and 5 at the 3′ end.

named vE-C_4, vE-C_9, and vE-C_{20}. The specific infectivity of these transcripts was 10^2 to 10^3 PFU/μg of RNA (Table 1). These values were about 10-fold lower than those for RNA extracted from EMCV virions but equivalent to those for cDNA-derived RNA from the parental plasmid, pM/E. The lower specific infectivity of transcript RNA relative to virion RNA may have been caused by the presence of the nonviral bases at the 5′ or 3′ ends, or the lack of the 5′ protein (VPg) that normally caps the 5′ termini of virion RNA, or the truncated nature of the transcript poly(C) tracts.

When the recombinant viruses were compared with EMCV-R in a single-step growth study (Fig. 2), all strains showed an eclipse phase of about 2 h, after which the titer grew exponentially to a peak of about 10^3 PFU per cell. We saw no measurable difference in the growth profiles of the cDNA-derived viruses and wild-type EMCV, confirming that long poly(C)s are not required by EMCV for efficient growth in tissue culture.

However, in 28-h plaque assays, the vE viruses consistently showed morphologies that were different from that of the wild type (Table 1). In these assays, EMCV-R gave a large plaque of about 4 mm. Mengovirus gave plaques that were 60 to 70% of this size. The diameters of vM/E and vE-C_{20} plaques were measured at 80 to 90% of those of EMCV-R, while diameters

TABLE 1. Characteristics of EMCV and mengovirus isolates

RNA source[a]	Poly(C) tract	Specific infectivity[b]	Plaque size[c]	LD_{50}[d]
EMCV-R	$C_{115}UCUC_3UC_{10}$	2.7×10^4	+++++	$<10^2$
pM/E	$C_{13}UC_{10}$	1.6×10^3	++++	5×10^7
pE-C_4	C_4	3.5×10^3	+++	3×10^3
pE-C_9	C_9	2.1×10^2	+++	1
pE-C_{20}	C_{20}	1.1×10^3	++++	10
pMwt	$C_{50}UC_{10}$		++	10^e
pM-C_{24}	$C_{13}UC_{10}$		++[e]	8×10^{6e}

[a] EMCV RNA was extracted from virions; RNAs of all others were transcribed from plasmid DNA. wt, wild type.

[b] RNA was transfected into HeLa cell monolayers with DEAE-dextran. Specific infectivity is expressed in PFU per microgram of RNA.

[c] The diameters of 30 to 50 plaques were measured with a microscope eyepiece micrometer. These were averaged and compared against EMCV plaque diameters, defined as 100% (five pluses). Standard deviations were on the order of 15 to 20%.

[d] Expressed in PFU. Three to five 4- to 6-week-old female Swiss mice were injected intracerebrally in 4 to 5 dosage groups.

[e] From Duke and Palmenberg (4) and Duke et al. (3). pM-C_{24} and pMwt had similar plaque sizes in those experiments.

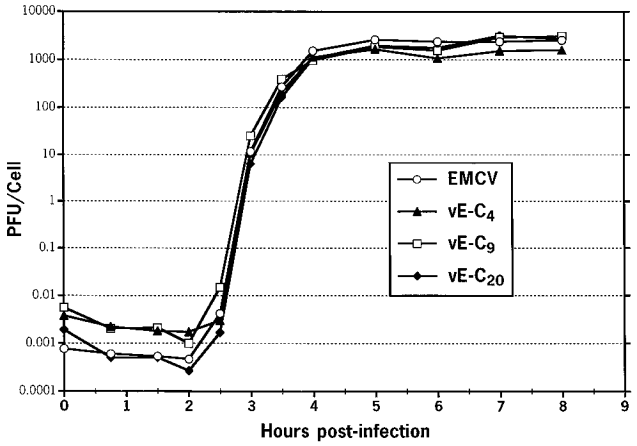

FIG. 2. Single-step growth curves for parental and cDNA-derived viruses. HeLa cell suspension cultures were infected with virus at a multiplicity of 10. Following a 30-min attachment period, unattached virus was removed by sedimenting the cells and resuspending them in fresh medium. The cells were then incubated at 37°C, and samples were taken at various intervals. Samples were lysed, titrated, and expressed as PFU per cell.

of plaques of vE-C_4 and vE-C_9 were about 70 to 80% of those of EMCV-R. A correlation therefore exists between poly(C) length and plaque size, with the short-tract EMCV having slightly smaller plaques.

These results contrast with results for mengovirus, in which poly(C) tract length does not contribute to plaque size (4, 8). In the assays under discussion, the tested mengovirus strains gave similar, medium-sized plaques that were smaller than those of vE-C_4, despite their longer poly(C)s. Moreover, the vM/E chimera with the same $C_{13}UC_{10}$ poly(C) sequence as that of mengovirus vM-C_{24} gave plaques more similar to those of vE-C_{20}. The data suggest that poly(C) length and the polyprotein context (EMCV versus mengovirus) must both contribute to plaque size, because a short tract combined with an EMCV polyprotein always gave plaques that were larger than the same tract combined with a mengovirus polyprotein.

The genetic stability of the vE poly(C) tracts was examined by sequencing RNA extracted from virions that had undergone multiple rounds of HeLa cell infection. Two separate isolates of each virus were examined. The RNA served as template for reverse transcriptase PCR, and the products were sequenced. For all vE isolates, the poly(C) length was that of the parental cDNA, indicating that HeLa cells did not select for altered poly(C)s (data not shown).

(iii) In vivo studies. The in vivo phenotype of the vE viruses was examined in an LD_{50} study. Three to five 4- to 6-week-old female Swiss mice per dosage group were injected intracerebrally with EMCV-R, vM/E, vE-C_4, vE-C_9, or vE-C_{20} (four or five groups per virus) and then observed for 2 weeks as described elsewhere (3, 8). The LD_{50}s of the vE viruses ranged from 1 PFU (vE-C_9) to 3×10^3 PFU (vE-C_4), compared with about 100 PFU for EMCV-R (Table 1). Given the expected variance within small groups of animals (about 25 total animals per virus) and at very high dilutions of virus, a difference in these assays of 1 log unit is probably not significant, and the short-tract vE viruses can all be characterized as highly virulent. vE-C_4, with the shortest poly(C) tract, was attenuated by a factor of only about 10 relative to EMCV-R. The equivalent short-tract mengoviruses, with poly(C)s between C_8 and C_{24}, have LD_{50}s greater than 10^6 PFU (3, 8). The chimeric vM/E virus, with a short poly(C) and a mengovirus UTR, was also highly attenuated, with an LD_{50} of 5×10^7 PFU, and behaved in mice much more like a short-tract mengovirus than its predominantly EMCV genome might have predicted.

Virus recovered from the brains of mice that were killed by vE-C_4, vE-C_9, or vE-C_{20} were sequenced to determine if expansion of the poly(C) tract was responsible for mortality. Two separate isolates of each virus were examined. The sequenced isolates had poly(C) lengths of the injected virus (data not shown). Lethality of these strains, then, was not attributable to expansion of the poly(C) tracts.

Discussion. We have constructed three short-poly(C)-tract cDNA clones of EMCV-R and characterized the resultant virus behavior in tissue culture and mice. Whereas analogous short-tract mengoviruses do not show alterations of plaque morphology in HeLa cells, the new EMC viruses gave plaques that varied with poly(C) tract length. A recombinant mengovirus-EMCV chimeric virus with a tract of $C_{13}UC_{10}$ behaved more like EMCV (vE-C_{20}) in these cells than like mengovirus with an identical poly(C), clearly implicating the viral polyprotein, in addition to poly(C) length, in the plaque size phenotype. The genetic stability of recombinant EMCVs was the same as that described for mengovirus, rather than FMDV, as the short poly(C) tracts passed with fidelity in tissue culture and in mice.

In view of the marked effects of shortened poly(C) tracts on mengovirus virulence in mice (3, 8), it was surprising to find that short-poly(C) EMCV were attenuated only slightly, if at all, unless the poly(C) was situated within the chimeric mengovirus UTR (vM/E). The results again implicate the genomic context in the modulation of poly(C) activity and specifically target the 5′ UTR in the observation of an attenuated phenotype. Short-tract mengoviruses and EMCVs with mengovirus 5′ ends were attenuated, while full-length EMCVs, regardless of their poly(C) length, were always more virulent. The EMCV behavior resembles that described for recombinant FMDV and invites speculation that an appropriate FMDV 5′ UTR combined with a short poly(C) might also permit attenuation of this virus.

The genomic sequences of the vM/E chimera and the full-length pE viruses differ only by 28 substitutions and 4 1-base insertions or deletions within the 400-base segment that surrounds the poly(C) tract. Somehow, these few localized changes allow the respective short-tract viruses to vary in LD_{50} by 5 to 6 orders of magnitude. Definition of required elements and determination of how they work in conjunction with poly(C) seem to be the key to poly(C)-mediated pathogenesis.

H.H. thanks S. Connolly, H. Duque, M. Goldsby-Hill, M. Hoffman, L. Martin, and J. Osorio for invaluable technical assistance.

This work was supported by National Institutes of Health grants AI-17331 and AI-30566 to A.C.P. and by NIH training grant GM-07215 to H.H.

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