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Construction and Characterization of Two Infectious Molecular Clones of Encephalomyocarditis Virus

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We constructed and characterized two infectious molecular clones of encephalomyocarditis (EMC) virus. Both constructs, pD_L and pD_A, were assembled from five overlapping cDNA clones derived from the diabetogenic variant of EMC virus (EMC-D) and from two synthetic oligonucleotide cartridges. pD_A contained a single point mutation at position 1720 within the "puff" region of capsid protein 1AB that was derived from the nondiabetogenic variant of EMC virus (EMC-B). This point mutation resulted in an amino acid substitution of arginine (EMC-B) for lysine (EMC-D). Our construction illustrates two novel findings: (i) that the problem of stably cloning long poly(C) tracts of EMC virus can be circumvented by the use of a shortened, synthetic, poly(dC-dG) oligonucleotide cartridge, and (ii) that a single point mutation in the puff region of the capsid protein 1AB leads to change in its electrophoretic mobility and to a change in the plaque size of recombinant virus.

The B and D variants of encephalomyocarditis (EMC) virus (EMC-B and EMC-D, respectively) were plaque purified in 1980 by Yoon et al. (16) from the M strain of EMC virus (4) and selected solely for their ability to produce diabetes (EMC-D) or benign infection (EMC-B) in SJL mice. Subsequently, it was discovered that virulence was associated with the ability of EMC-D to escape or disrupt interferon induction in mice (17) and in infected cells (2). We have previously cloned and sequenced the Davis variants of EMC-D and EMC-B (3, 8) as a means of identifying genetic differences that may contribute to pathogenesis. We now described the construction and characterization of two infectious molecular clones of EMC virus, assembled from five overlapping cDNA clones (3, 7, 8) and two oligonucleotide cartridges (Fig. 1). These constructs represent the first infectious nonhybrid molecular clones of an EMC virus, although the cloned virus did not display the diabetogenic phenotype of its parent, EMC-D. Our construction illustrates two novel findings: (i) that the problem of stably cloning long poly(C) tracts of cardioviruses (6, 12) can be circumvented by using a shortened, synthetic, poly(dC-dG) oligonucleotide cartridge; and (ii) that a single point mutation in the "puff" region of capsid protein 1AB alters both the mobility of this protein in Laemmli sodium dodecyl sulfate-polyacrylamide gels (9) and the plaque size of the resulting recombinant virus.

EMC-D and EMC-B viruses were obtained from Ji-Won Yoon (16). Both viruses were grown in L929 cells and purified by polyethylene glycol precipitation and cesium chloride gradient centrifugation (14). The diabetogenic properties of each virus were confirmed in male SJL/J mice before viral RNA was prepared for use in subsequent cloning experiments (3, 8). Based on earlier work that demonstrated a difference in the electrophoretic mobilities of the 1AB proteins of the Davis variants of EMC-D and EMC-B (R. K. Naviaux, S. H. Cohen, K. M. Vanden Brink, and G. W. Jordan, Abstr. ICN-UCI Int. Conf. Virol., Newport Beach, Calif., 1988, p. 22) and on sequencing data that showed a point mutation at position 1720 that mapped to the puff region (10) of the capsid protein 1B (3), we constructed two molecular clones of EMC virus, pD_L and pD_A, that differed precisely by this mutation (Fig. 1) to explore its phenotypic effects. Recombinant clone pD_L contained the EMC-D sequence AAA (lysine) at codon 156 within the 1B gene, whereas pD_A contained the EMC-B sequence AGA (arginine) at this location. By analogy to the crystallographic structure of mengovirus, this amino acid substitution mapped to an extended loop of the viral capsid known as the

TABLE 1. Specific infectivity of cloned EMC virus nucleic acid

Sample	Nucleic acid[a]	Treatment[b]	PFU/μg
pD_L	cRNA	None	$2.3 \times 10^3 \pm 0.4 \times 10^3$ ($n = 5$)
	cRNA	DNase	2×10^3
	cRNA	RNase	<1
	cRNA	Anti-coxsackievirus B4	2×10^3
	cRNA	Anti-EMC	<1
	cDNA	None	<0.1
pD_A	cRNA	None	$6.2 \times 10^3 \pm 1.0 \times 10^3$ ($n = 5$)
	cRNA	DNase	5×10^3
	cRNA	RNase	<1
	cRNA	Anti-coxsackievirus B4	4×10^3
	cRNA	Anti-EMC	<1
	cDNA	None	<0.1
EMC-B	vRNA	None	9.0×10^4
EMC-D	vRNA	None	5.6×10^5

[a] cRNA, Positive-sense transcript RNA; vRNA, EMC RNA extracted from virions.

[b] DNase treatment was at 4 U/ml for 15 min at 37°C, RNase treatment of 1 μg cRNA was at 40 μg/ml for 15 min at 37°C, anti-coxsackievirus B4 and anti-EMC antiserum were used at a concentration of 1% in a 1.2% methylcellulose overlay; no plaques were observed in monolayers transfected with 10 μg of supercoiled cDNA.

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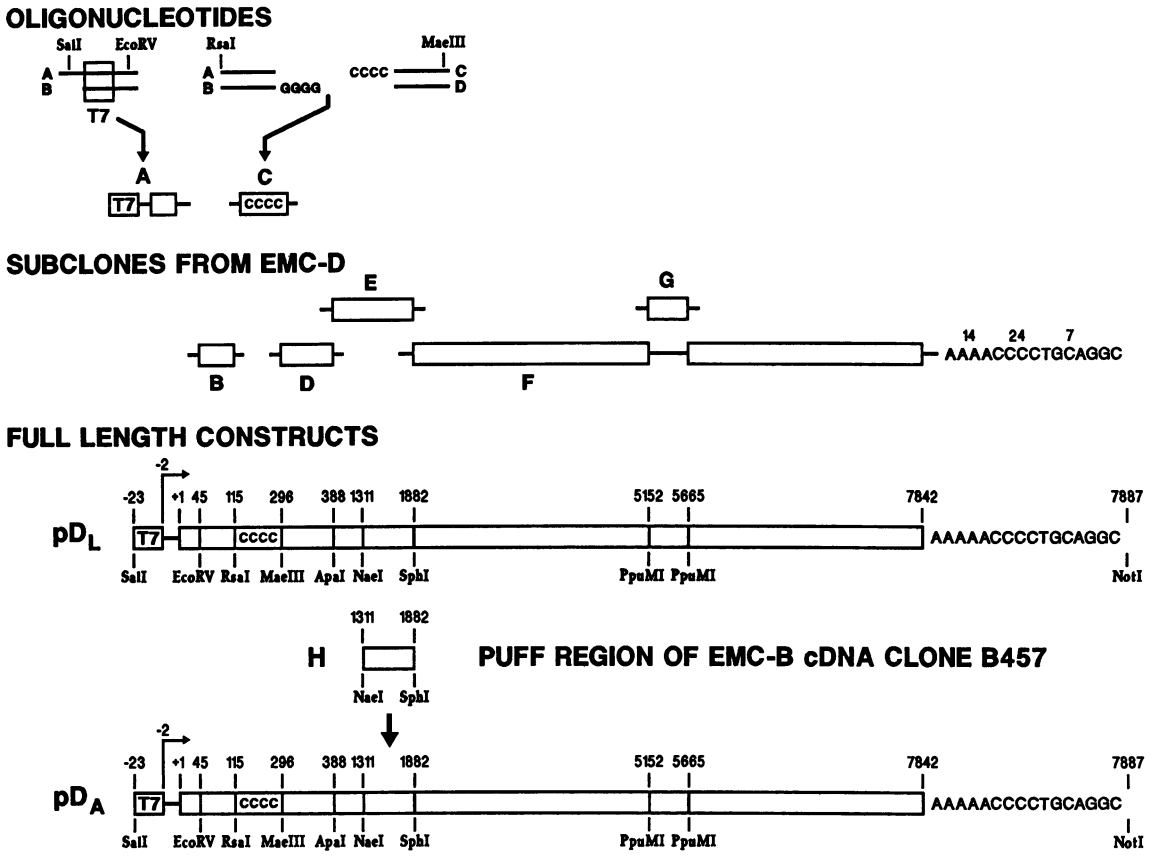

FIG. 1. Construction of a synthetic cardioviral poly(C) tract cartridge and two infectious molecular clones of EMC virus. Four oligonucleotides (A, B, C, and D) were designed to produce upon annealing a 106-base-pair cartridge containing a 34-base poly(dG-dC) tract. pD_L and pD_A were assembled from five overlapping cDNA clones of EMC-D (B, S107; D, S139; E, D4127; F, D41; G, D88) and two synthetic oligonucleotide cartridges (A, T7H#26; C, MC24). In addition, pD_A was constructed by subcloning the *Nae*I-*Sph*I fragment of EMC-B clone H (B457) into the corresponding region of pD_L. This 571-base-pair fragment is identical to that of EMC-D except for a single point mutation at position 1720 that results in a lysine-to-arginine substitution in protein 1B. The bases contributed by each are positions −2 to 44 (A), 45 to 114 (B), 115 to 295 (C), 296 to 387 (D), 388 to 1881 (E), 1882 to 7842 (F), 5152 to 5664 (G), and 1311 to 1882 (H). The *Ppu*MI-*Ppu*MI fragment of G was subcloned into the corresponding region of F to correct a stop mutation discovered at position 5199.

puff region and thought to represent one of the three immunodominant epitopes of the cardiovirus capsid (10).

Attempts to obtain a cDNA clone that traversed the 144-base poly(C) tract of EMC-D viral RNA were unsuccessful. We therefore designed a synthetic poly(C) tract cartridge comprised of four oligonucleotides that allowed the introduction of a 34-base C tract into an otherwise full-length clone of EMC-D through the use of flanking restriction sites (Fig. 1). After adding a T7 promoter cartridge (15) to the 5′ end of this clone (Fig. 1), we obtained a final construct that could be linearized with *Not*I and transcribed to yield infectious transcripts (Fig. 2, Table 1). These transcripts had a deduced length of 7,779 bases and contained 2 nongenomic guanosine residues at their 5′ ends and 31 nongenomic bases after a 14-base poly(A) tail at their 3′ ends.

In vitro translation of pD_L and pD_A transcripts yielded viral proteins that reproduced the electrophoretic mobility differences of wild-type EMC-D and EMC-B 1AB proteins (Fig. 3). Because the cRNA transcripts of pD_L and pD_A differed only by a single point mutation at position 1720, these in vitro translation experiments confirmed that a single point mutation at codon 156 of protein 1B (an A-to-G transition, resulting in a lysine-to-arginine substitution) was sufficient to alter the electrophoretic mobility of the 1AB protein of EMC virus and provided a convenient phenotypic marker for differentiating the viral proteins of the Davis variants of EMC-D and EMC-B. This mobility difference was eliminated by the addition of 2 M urea to the standard Laemmli gels (data not shown), suggesting that incomplete denaturation and a point-mutation-associated conformational change were responsible for the observed differences in electrophoretic mobility. The functional significance of this point mutation remains unclear.

The Davis and Calgary variants of EMC-D and EMC-B were derived from a common parental stock in 1981. Both geographic variants have preserved the appropriate diabetogenic and interferon-inducing phenotypes of the original plaque-purified isolates derived by Yoon et al. but now differ by at least 28 sites in their 7,842-base genomes (1, 3). This divergence has been neutral with regard to the parental diabetogenic and interferon-inducing phenotypes. Unlike the Davis variants of EMC virus, the 1B proteins of the Calgary variants of EMC-D and EMC-B are identical (1) and therefore cannot be differentiated by protein electrophoresis (13). They share 100% identity with the 1B protein of EMC-D (Davis), encoding a lysine at codon 156 (3). These sequence comparisons suggest that the lysine-to-arginine substitution in protein 1B may be incidental to the diabetogenic and

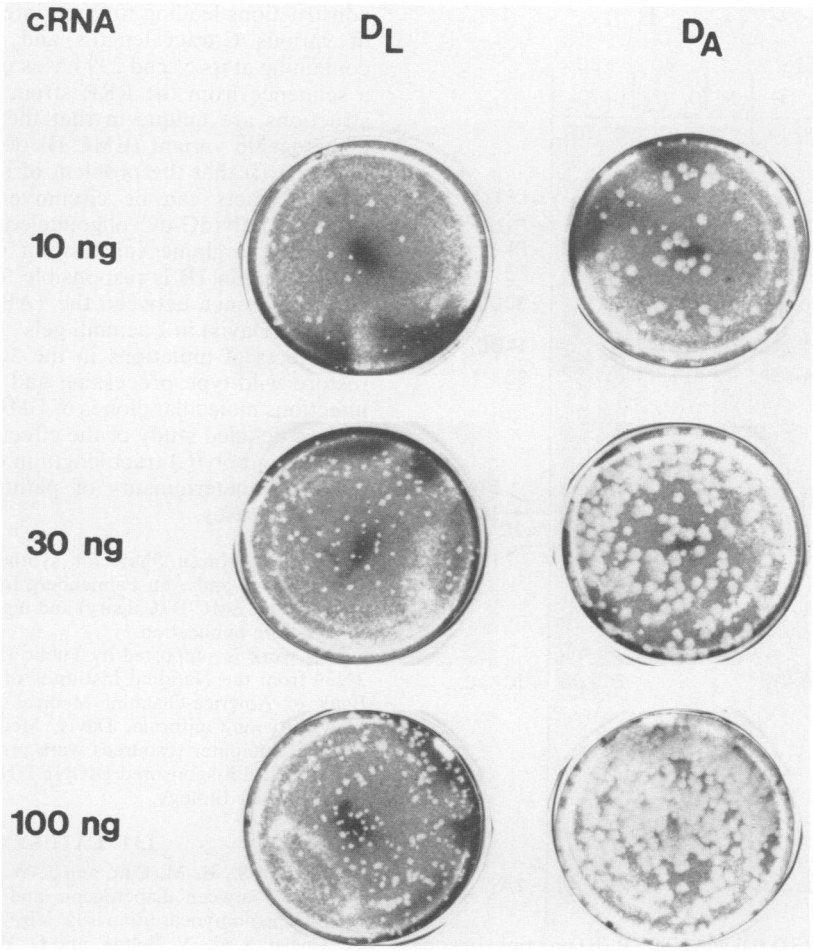

FIG. 2. Transfection of L929 cell monolayers. cRNA was transcribed from *Not*I-linearized pD_L (D_L) and pD_A (D_A), and the indicated amounts of RNA were transfected into L929 cells by DEAE-dextran facilitation (5). Plates were fixed and stained 3 days posttransfection.

interferon-inducing phenotypes of the parental EMC viruses (2, 16, 17).

The plaques of recombinant pD_A virus were twice the size of pD_L plaques on L929 cell monolayers (0.8 mm versus 0.4 mm) (Fig. 3). The cRNA transcripts of pD_A were also three times more infectious than those of pD_L (Table 1). Supercoiled cDNA was not infectious. RNase treatment destroyed infectivity, whereas DNase did not (Table 1). pD_L and pD_A viruses were neutralized by anti-EMC serum but not by anti-coxsackievirus B4 serum (Table 1). Sequence analysis of progeny virus recovered from transfected cells confirmed the presence of a wild-type 5′ end structure (5′ UU without any residual bases contained in the cRNA used in transfection) and the conservation of the 34 base poly(C) tract used in our original constructions.

pD_L and pD_A transcripts had identical translational efficiencies in rabbit reticulocyte lysates, and recombinant viruses did not differ significantly in one-step growth experiments in L929 cells (data not shown). However, recombinant pD_L and pD_A viruses were non-wild-type variants of their EMC-D progenitor. Wild-type virus grew to a titer of 10^6 to 10^7 PFU/ml on L929 cell monolayers, whereas pD_L and pD_A grew to 10^3 PFU/ml. The eclipse phase of wild-type EMC-D and EMC-B virus was 3 to 4 h, whereas that of recombinant virus was 7 to 8 h (data not shown). These differences may be explained in part by the slow processing phenotypes of recombinant EMC viruses pD_L and pD_A described below.

EMC virus RNA is translated as a polyprotein that is subsequently cleaved into mature viral proteins through the action of the virally encoded 3C protease and its precursor 3CD (11). The wild-type EMC-D and EMC-B virus precursor protein LP12A appeared within 15 to 30 min during in vitro translation and was processed to mature viral protein in 1 to 2 h (Fig. 3A). Processing of recombinant pD_L and pD_A polyprotein took over 4 h (Fig. 3B). However, when unlabeled, wild-type EMC proteins (including 3C and 3CD proteases) were added in *trans*, recombinant pD_L and pD_A polyproteins were completely processed to mature viral proteins that could be immunoprecipitated with anti-EMC serum (Fig. 3B). These experiments demonstrated (i) that pD_L and pD_A polyproteins served as legitimate substrates for wild-type EMC 3C and 3CD proteases added in *trans* and (ii) that the increased electrophoretic mobility of the 1AB protein of EMC-B (Fig. 3A) was faithfully reproduced by pD_A (Fig. 3B) and was due to an arginine substitution at codon 156 in the puff region of 1B, which is the only difference between our recombinant clones of EMC virus, pD_A and pD_L (Fig. 1 and 3B).

We next studied the biology of wild-type and recombinant viruses in SJL/J mice. All 10 mice infected with 10^4 PFU of EMC-D became diabetic (blood glucose level, 448 ± 90

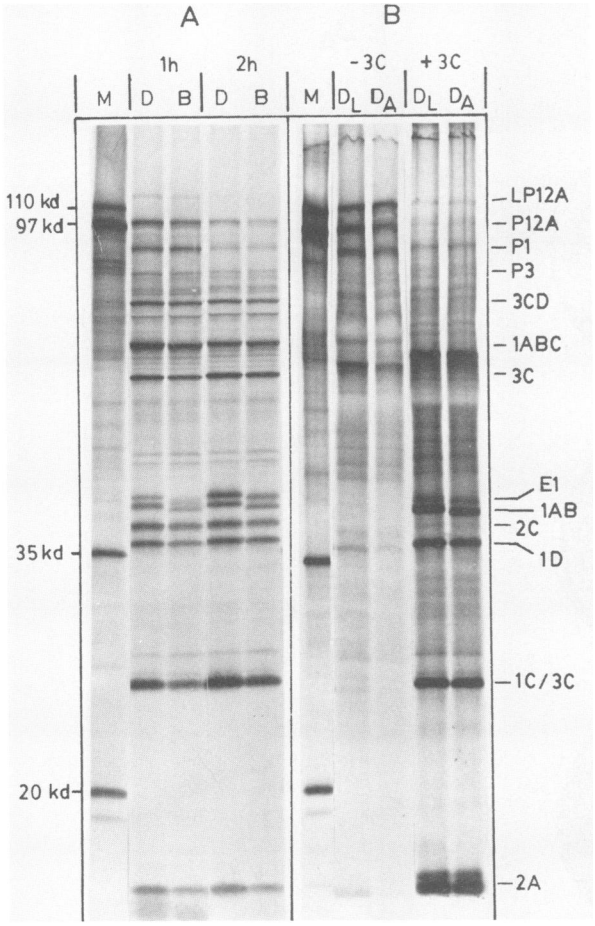

FIG. 3. Wild-type EMC-D (D) and EMC-B (B) proteins. In vitro translation products of EMC-D and EMC-B viral RNA in rabbit reticulocyte lysates were labeled with [^{35}S]methionine, programmed for 1 and 2 h, analyzed in a sodium dodecyl sulfate–polyacrylamide gel system (9), and autoradiographed. The stacking gel was 5% polyacrylamide; the separating gel was 12% (11.68% acrylamide–0.32% bisacrylamide) polyacrylamide. Molecular weight markers (M) are brome mosaic virus proteins. (B) Cross-processing of recombinant viral proteins. cRNA transcripts of recombinant EMC viruses pD_L (D_L) and pD_A (D_A) were used to program in vitro translations for 4 h at 30°C without (−3C) or with (+3C) unlabeled wild-type EMC viral proteins added in *trans*, immunoprecipitated with anti-EMC serum, and analyzed as in panel A.

mg/dl), whereas none of the 10 mice infected with EMC-B developed diabetes (blood glucose, 185 ± 24 mg/dl). Recombinant virus failed to produce diabetes. None of 10 animals infected with 10^4 PFU of pD_A virus developed diabetes (blood glucose, 177 ± 4 mg/dl); 9 of 10 animals infected with 10^4 PFU of pD_L virus also failed to develop diabetes (blood glucose, 197 ± 13 mg/dl). Osorio et al. (J. Osorio, G. Duke, and A. C. Palmenberg, Abstr. Annu. Meet. Am. Soc. Virol., 1989, Session 11, p. 14) have recently shown that by increasing the poly(C) tract length of infectious clones of mengovirus from 8 to 60 bases, virulence in mice is markedly increased. The shortened 34-base poly(C) tracts of our infectious EMC clones may also contribute to their reduced virulence in mice.

pD_L and pD_A represent the first infectious, nonhybrid, molecular clones of EMC virus. In a related study, Duke and Palmenberg (6) have recently published an elegant series of constructions leading to three infectious mengovirus clones of various C-tract lengths and one mengo-EMC hybrid containing at its 5′ end 299 bases of mengovirus followed by a sequence from the RRR strain of EMC virus. Our constructions are unique in that they were derived from the diabetogenic variant (EMC-D) of EMC virus. Our studies illustrate (i) that the problem of stably cloning EMC virus poly(C) tracts can be circumvented through the use of synthetic poly(dG-dC) oligonucleotide cartridges and (ii) that a lysine-to-arginine substitution within the puff region of capsid protein 1B is responsible for an electrophoretic mobility difference between the 1AB proteins of EMC-D and EMC-B (Davis) in Laemmli gels. We are currently studying the effects of mutations in the 3CD region in an effort to restore wild-type processing and growth properties to our infectious molecular clones of EMC virus. This will facilitate a more detailed study of the effects of puff replacement and changes in poly(C) tract length in our ongoing studies of the molecular determinants of pathogenesis by EMC-D and EMC-B viruses.

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