Attenuating Mutations in Coxsackievirus B3 Map to a Conformational Epitope That Comprises the Puff Region of VP2 and the Knob of VP3

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Ten antibody escape mutants of coxsackievirus B3 (CVB3) were used to identify nucleotide substitutions that determine viral virulence for the heart and pancreas. The P1 region, encoding the structural genes of each mutant, was sequenced to identify mutations associated with the lack of neutralization. Eight mutants were found to have a lysine-to arginine mutation in the puff region of VP2, while two had a glutamate-to-glycine substitution in the knob of VP3. Two mutants, EM1 and EM10, representing each of these mutations, were further analyzed, initially by determining their entire sequence. In addition to the mutations in P1, EM1 was found to have two mutations in the 3D polymerase, while EM10 had a mutation in stem-loop II of the 5' nontranslated region (5'NTR). The pathogenesis of the mutants relative to that of CVB3 strain RK [CVB3(RK)] then was examined in A/J mice. Both mutants were found to be less cardiotropic than the parental strain, with a 40-fold (EM1) or a 100- to 1,000-fold (EM10) reduction in viral titers in the heart relative to the titers of CVB3(RK). The mutations in VP2, VP3, and the 5'NTR were introduced independently into the RK infectious clone, and the phenotypes of the progeny viruses were determined. The results substantiated that the VP2 and VP3 mutations reduced cardiovirulence, while the 5'NTR mutation in EM10 was associated with a more virulent phenotype when expressed on its own. Stereographic imaging of the two mutations in the capsomer showed that they lie in close proximity on either side of a narrow cleft between the puff and the knob, forming a conformational epitope that is part of the putative binding site for coreceptor DAF.

Group B coxsackieviruses (CVB1 to CVB6) are important human pathogens associated with a variety of diseases, which range from mild respiratory infections to severe and occasionally fatal infections of the central nervous system (meningitis and encephalitis), heart (myocarditis), and pancreas (pancreatitis and diabetes mellitus). Different serotypes within this group, as well as variants of a single serotype, vary in virulence and the pattern of disease that they produce (31). For example, in animal models, CVB4 has a stronger association with diabetes, while CVB3 is thought to be the most cardiovirulent. The degree of genetic variation between strains has not allowed these pathogenic differences between serotypes to be explained at the molecular level; however, variants of each serotype have enabled mapping of mutations associated with defined phenotypic characteristics in a number of instances. For example, in a laboratory-adapted strain of CVB3, a myocarditis phenotype has been associated with a C-to-U mutation at nucleotide (nt) 234 in the 5' nontranslated region [5'NTR] (40). In addition, a study of clinical isolates has identified a number of mutations in the 5'NTR that appear to be associated with cardiovirulence; these mutations are localized in stem-loop II of the predicted secondary structure of this region (9).

While the evidence that the 5'NTR plays an important role in determining CVB3 cardiovirulence is believed to be well

documented, the role of the structural proteins has not been examined in detail. In only one study has a mutation in the P1 region, which encodes the CVB3 structural proteins, been shown to affect viral damage to the heart—specifically, a mutation associated with an asparagine-to-aspartate substitution in the EF loop or puff region of VP2 (17). However, tropism for the pancreas and liver was recently shown to map to the P1 structural gene region, although a precise definition of the determinants involved was not investigated (11). For other enterovirus systems, there is also strong evidence that the structural genes affect both tissue tropism and virulence. For example, mutations in both the VP1 and the VP4 structural genes have been shown to modulate the virulence of CVB4 for pancreatic tissue (6, 33, 46), while poliovirus attenuation is associated with mutations in VP1, VP3, and VP4 as well as in the 5'NTR (5, 26, 29, 39).

In the study reported here, we present further evidence that mutations in the CVB3 structural genes can influence viral virulence and pathogenicity for the myocardium. We identified a second mutation in the EF surface loop of VP2 as well as a mutation in the knob of VP3; both of these mutations are highly attenuating for heart tissue. As our approach involved the characterization of mutants able to escape from a highly neutralizing monoclonal antibody, we also determined that these two regions comprise a conformational epitope on the virus surface which we believe forms part of the binding site for coreceptor DAF.

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MATERIALS AND METHODS

Cell lines and virus stocks. Vero cells (American Type Culture Collection) were cultured in Dulbecco modified Eagle medium (DMEM)–F-12 medium

containing 10% fetal bovine serum and 1% gentamicin. The cells were incubated at 37°C in an atmosphere of 5% CO₂.

CVB3 strain RK [CVB3(RK)] (from R. Kandolf, University of Tübingen, Tübingen, Germany) was originally derived from an infectious clone of CVB3, pCVB3(T7) (GenBank accession number M33854). The virus had been passaged in mice, and virus derived from heart tissue was used to produce stocks of CVB3(RK) in HeLa cells. In our laboratory, viral stocks were prepared in Vero cells infected at 0.1 PFU/cell and harvested at 24 or 48 h, when the cytopathic effect (CPE) reached 100%. After three freeze-thaw cycles, the viral supernatant was stored in aliquots at -70°C and titrated in Vero cells by standard techniques.

Isolation of antibody EMs. The parental wild-type virus stock, CVB3(RK), was pretreated with a neutralizing MAb, 948 (immunoglobulin G2B; Chemicon). The residual nonneutralized fraction was allowed to infect Vero cells cultured in DMEM-F-12 medium containing 5% fetal bovine serum and supplemented with a 1:100 dilution of MAb 948. Following the development of CPE, a plaque assay was carried out on the supernatant, and the virus in individual plaques was isolated and used to infect Vero cells. The cultures were incubated in the presence of a 1:100 dilution of MAb 948, and the resulting virus preparations were designated escape mutants (EMs) (EM1 to EM10, respectively).

Temperature sensitivity and viral decay curves. Temperature sensitivity was determined by carrying out three sets of plaque assays (duplicate samples); both adsorption and incubation for 48 h were performed at 37 and 39°C. Decay rates for EM1 and CVB3(RK) were determined after incubation of each virus strain at 37 or 39°C in a 5% CO₂ humidified incubator for 1 week. An aliquot was removed each day for viral titration, and the decay rates for each virus strain were compared by plotting the viral titer against the length of incubation.

Viral infection of mice. Five-week-old male A/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and were allowed to acclimatize for 1 week before injection. They were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996). The mice were injected intraperitoneally with phosphate-buffered saline (control) or 10⁵ PFU of CVB3(RK) or one of the mutants. At the time of harvest, three animals from each group were sacrificed and 0.5 ml of nonheparinized blood was obtained by cardiac puncture from each animal for the isolation of serum. In addition, the heart, spleen, pancreas, and liver were removed aseptically, and each tissue was sectioned transversely into two pieces. One part was fixed with 4% paraformaldehyde for histopathological analysis and in situ hybridization (ISH), and the other was snap-frozen in liquid nitrogen for plaque titration.

ISH. (i) Preparation of probes. RNA probes to detect genomic RNA were prepared from plasmid pCVB3(R1) (provided by R. Kandolf) by use of SP6 RNA polymerase as described previously (15). In vitro transcription by use of SP6 was carried out with SalI-linearized plasmid and digoxigenin-labeled UTP in the nucleoside triphosphate mixture (Boehringer Mannheim). The resulting digoxigenin (DIG)-labeled probes were precipitated with 3 M sodium acetate and ethanol and were stored in 10 mM Tris-1 mM EDTA buffer (pH 8.0).

(ii) ISH procedure. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm sections, which were placed on silane-treated glass slides. The sections were baked overnight at 60°C, deparaffinized with xylene, and rehydrated in graded alcohols. The tissues were permeabilized with 0.2 N HCl-2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-20 mM Tris-2 mM calcium chloride containing 1 μg of proteinase K/ml and then quenched in 0.25% acetic anhydride containing 0.1 M triethanolamine. The slides were dehydrated in graded alcohols. Hybridization solution containing 100 ng of DIG-labeled probe/ml was added to each section, which then was covered with a glass coverslip and kept in a sealed humidified dish at 42°C overnight. Posthybridization washes were performed with 50% formamide-10 mM Tris-1 mM EDTA-600 mM NaCl overnight in a 56°C rocking water bath, followed by several washes with 2× SSC. The slides were equilibrated in buffer 1 (0.15 M NaCl, 0.1 M Tris-HCl) and blocked with 2% lamb serum in buffer 1. Anti-DIG antibody linked to alkaline phosphatase was added to each section and incubated for 45 min at room temperature in a humidified chamber. The slides were washed with buffer 1 and equilibrated to pH 9.5 in 0.1 M NaCl-0.1 M Tris-HCl-0.05 M MgCl₂. The bound alkaline phosphatase-linked antibody was detected by incubation with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate substrate (Sigma fast tablets) for 24 h at room temperature. The slides were counterstained with eosin and examined with a light microscope for a positive reaction indicated by a blue-black color.

Viral RNA isolation and cDNA sequencing. RNA was prepared from viral supernatant (strain RK and each mutant) partially purified by centrifugation through sucrose. This process involved layering 15 ml of viral supernatant onto 3.6 ml of 30% sucrose in phosphate-buffered saline in Beckman ultraclear centrifuge tubes and centrifuging at 20,000 rpm for 3 h at 4°C in a Beckman L8-80 centrifuge. The viral pellet was extracted with Trizol reagent (Canadian Life

Technologies) according to the manufacturer's instructions. After precipitation with isopropanol for 10 min at room temperature, the RNA pellet was washed with 75% ethanol, air dried, and dissolved in diethyl pyrocarbonate-treated water. First-strand synthesis was carried out on the viral RNA by use of SuperScript II (Invitrogen) and random hexanucleotides [Pharmacia pd(N)₆] as primers. Reaction mixtures contained 5 μl of 5× first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 250 mM MgCl₂ [pH 8.3]), 2 μl of 0.1 M dithiothreitol, 1 μl of 250 pmol of pd(N)₆, 1 μl of 25 mM deoxynucleoside triphosphates, 2 to 4 μl of viral RNA, and sufficient diethyl pyrocarbonate-treated water to yield a final volume of 25 μl. The mixtures were denatured at 95°C for 2 min and cooled on ice for 1 min. Next, 1 μl of RNase inhibitor and 1 μl of SuperScript II reverse transcriptase were added. The tubes were incubated for 1 h at 42°C.

PCR was carried out with *Taq* DNA polymerase (New England Biolabs) and primers designed to yield overlapping cDNA fragments complementary to the 5'NTR and the P1, P2, and P3 regions. For the 5'NTR, two primers, C1, 5'-TTA AAA CAG CCT GTG GGT TGA-3' (nt 1 to 21), and C2, 5'-CCG GGT CTT GAG TGA AAT CCT GC-3' (complementary to nt 892 to 870), were used. For the P1 region, the primers were P1L, 5'-AAC TCA GCC AAT CGG CAG GAT-3' (nt 856 to 876), and P1R, 5'-GGT CCT TCA AAC GAA ATT GGG-3' (complementary to nt 3530 to 3510). For P2, the primers were P2L, 5'-TGG ATA CCT AGA CCA CCT AGA CT-3' (nt 3184 to 3206), and P2R, 5'-GCC CAG CAT GGT AAA CTC GCC-3' (complementary to nt 5448 to 5428). For P3, the primers were P3L, 5'-AAG CCC AGA GTG CCT ACC C-3' (nt 5323 to 5341), and P3R, 5'-CAC GTG ATC TTG GGT GTT CTT-3' (complementary to nt 7155 to 7135). Finally, for the 3' nontranslated region (3'NTR), the primers were 3NTL, 5'-ACA CCA GCA GAT AAG GG-3' (nt 6976 to 6992), and 3NTR, 5'-(T)₇CCG CAC-3' [complementary to part of the poly(A) tail and 3' end from nt 7399 to 7394].

The amplification parameters were as follows: 5'NTR—95°C for 2 min for 35 cycles and then 72°C for 1 min; for P1 (2.5 kb) and P2 (2.2 kb)—95°C for 2 min for 35 cycles and then 60°C for 3 min; for P3 (1.8 kb)—95°C for 2 min, 95°C for 30 s and 59°C for 30 s for 35 cycles, and then 72°C for 2 min; and for 3'NTR (450 bp)—95°C for 2 min, 95°C for 30 s and 50°C for 30 s for 35 cycles, and then 72°C for 40 s. The PCR products were analyzed on 0.7% agarose gels. Samples were purified by use of Qiagen Quick-Spin purification columns and then sequenced by use of ABI AmpliTaq dye terminator cycle sequencing chemistry at the Nucleic Acid and Protein Services unit at the University of British Columbia.

Construction of KR, EG, and CT mutant viruses. An infectious clone of CVB3, pCVB3(T7), was used to produce the KR and EG mutants by restriction fragment exchange with cDNA prepared from EM1 and EM10. To construct the KR mutation, EM1 cDNA was subjected to PCR with primers C1 (nt 1 to 21; see above) and C5, 5'-CTT TAT GCT GCC TGA CCA ATG-3' (complementary to nt 2082 to 2062). The PCR parameters were 2 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 57°C, and 2 min at 72°C. PCR was carried out with a 50-μl volume containing 5 μl of ThermoPol reaction buffer [10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8), 2 mM Mg₂SO₄, 0.1% Triton X-100, 250 μM deoxynucleoside triphosphates, 10 ng of template cDNA, 0.6 μM each primer, 2.5 U of Deep Vent DNA polymerase] (New England Biolabs). The PCR product was purified by use of a Qiagen PCR clean-up kit and digested with BglII and BstBI. The sample was cloned into plasmid pCVB3 that had also been digested with BglII (nt 2042) and BstBI (nt 249) by use of T4 ligase prior to electroporation into electrocompetent *Escherichia coli* DH5α cells. Colonies grown on Luria-Bertani agar plates with ampicillin were used to prepare plasmids that were screened by size and then sequenced to ensure that the correct mutation was present. The EG mutation was prepared in the same way, except that EM10 cDNA was used as the starting material.

For the CT mutation, the cloning strategy is outlined in Fig. 1. Briefly, cDNA from EM10 was subjected to PCR with primers C1 (nt 1 to 21; see above) and C5 (complementary to nt 2082 to 2062; see above). The PCR product was digested with AgeI and KpnI to yield a fragment of about 1.35 kb containing the CT mutation. This fragment was gel purified and then inserted into subcloning vector pCVB3/HIII [derived from pCVB3(T7) that had been digested with HindIII and religated]. pCVB3/HIII was digested with AgeI and KpnI, and the 1.35-kb fragment was inserted. The sample then was incorporated into pCVB3 that had been partially digested with HindIII to yield full-length pCVB3 containing the 5' CT mutation at nt 119.

All of the mutants were sequenced through the 5'NTR and the P1 region after construction to ensure that the mutations had been inserted correctly and that no other mutations had been introduced. To produce the corresponding mutant virus, each plasmid construct containing the required mutation was linearized with SalI. Viral RNA was produced by in vitro transcription with T7 and transfected into Vero cells. After 48 or 72 h of incubation at 37°C, when the CPE reached 100%, viral supernatant was harvested and stored at -70°C.

FIG. 1. Construction of the CT mutant. The diagram shows the strategy used to insert the CT mutation at nt 119 in the 5'NTR of the infectious clone pCVB3(T7).

RESULTS

Isolation and characterization of antibody EMs. Ten antibody EMs, EM1 to EM10, were selected from a stock of CVB3(RK) by use of a highly neutralizing MAb, 948, that restricted the growth of the parental strain (see Materials and Methods for details). After plaque purification, the mutants were propagated in Vero cells in the presence of MAb 948 to prevent reversion and were shown to have titers similar to those of parental strain RK, although both produced smaller plaques. To identify the mutations acquired by EM1 to EM10, the P1 structural gene region of each was sequenced following RT-PCR of purified viral RNA with primers based on the 5'NTR and the 5' end of the contiguous P2 region. Only two mutations were identified in the structural genes of the 10

mutants. Eight of the EMs contained an A-to-G transition at nt 1421 that produces a lysine-to-arginine mutation at amino acid 158 of the VP2 protein. This amino acid lies in the EF loop of the VP2 protein that is referred to as the puff region (27). The remaining two EMs (EM7 and EM10) contained a mutation at nt 1916 of the P1 region. An A-to-G transition at this locus results in a glutamate-to-glycine substitution at amino acid 60 of the VP3 protein (Fig. 2b). Amino acid residues 58 to 69 of VP3 form a major protrusion on the capsid surface that is referred to as the knob region (27). Like the puff of VP2, the knob of VP3 contains a major neutralization or immunogenic site for both poliovirus 1 and human rhinovirus 14 (30, 35, 36). As this is the only amino acid substitution in the structural proteins of

FIG. 2. Stereographic images of coxsackievirus VP2 and VP3 proteins. The locations of the lysine-to-arginine mutation at amino acid 158 in the VP2 capsid protein of EM1 (a) and the glutamate-to-glycine mutation at amino acid 60 of VP3 (b) are indicated. The alpha-carbon stereographic images were constructed with the help of Michael Murphy, Department of Microbiology and Immunology, University of British Columbia, by using previously described methods (18, 25).

EM7 and EM10, this mutation can be inferred to be responsible for their antibody escape phenotype.

Two mutants (EM1 and EM10) representing the capsid mutations identified were chosen for further study. Initially, the entire sequence of each was determined and compared to that of CVB3(RK). For EM1, a total of 6 nt substitutions were identified, but none was in the 5'NTR or the 3'NTR, and only three resulted in amino acid substitutions. An A-to-G transition at nt 6826 produced an isoleucine-to-valine mutation at amino acid 309 of the 3D polymerase gene product. Also, a T-to-C transition at nt 7025 in the cDNA caused a valine-to-alanine mutation at amino acid 375 of the 3D polymerase. The third mutation in EM1 was the lysine-to-arginine mutation at amino acid 158 of the VP2 protein described above and shown in Fig. 2a.

For EM10, four mutations were identified throughout the genome, but two of these were silent mutations, leaving two

TABLE 1. Neutralization of EMs and strain RK by MAb 948

Virus strain	Titer (PFU/ml) of:	
	Virus	Virus + MAb
CVB3(RK)	2.1×10^8	2.5×10^4
EM1	5.3×10^7	5.1×10^7
EM10	7.4×10^7	6.7×10^7

that might affect virulence. The first was a C-to-T nucleotide transition at position 119 of the 5'NTR cDNA. nt 119 lies within a bulge in stem-loop II of the 5'NTR, according to the secondary structure reported previously (9, 47). The second was the E-to-G substitution in VP3 shown in Fig. 2b.

Do mutations in EM1 and EM10 affect temperature sensitivity or capsid stability? The titers of both EM1 and EM10 were shown to be unaffected by MAb 948 (Table 1), while the infectivity of strain RK was reduced over 1,000-fold. To assess whether the mutations affected temperature sensitivity, the yields of CVB3(RK), EM1, and EM10 at 37 and 39°C were determined (Fig. 3a). Parental strain CVB3(RK) was found to be slightly temperature sensitive, with about a three- to fivefold reduction in titers at 39°C relative to 37°C. In comparison, the titers of EM1 were reduced almost 10-fold at 39°C, while EM10 showed no reduction in titers at the higher temperature,

FIG. 3. (a) Temperature sensitivity of strains EM1 and EM10. The results of plaque assays performed at 37 and 39°C with parental strain RK and both of the mutants are shown. Both strains RK and EM1 were found to display reductions in growth—about 10- and 50-fold, respectively—at the higher temperature, while the yields of strain EM10 were the same at both temperatures. Error bars indicate standard deviations. (b) Viral decay curves for EM1 and EM10. The titers of virus remaining after prolonged incubation at 39°C are shown. EM1 and RK showed very similar decay curves, while EM10 was extremely stable, with measurable infectivity after 10 days of incubation at 39°C.

indicating that this mutant is more resistant to elevated temperatures than its parent. All three mutations in EM1 may contribute to its temperature sensitivity at 39°C and its small-plaque phenotype. For the mutations in the polymerase gene, evidence from other picornaviruses supports the possibility that they can affect temperature sensitivity. For example, it is known that a major determinant of temperature sensitivity in the poliovirus type I Sabin vaccine strain is located in the 3D polymerase gene (5, 32).

To determine relative capsid stability, the infectivity of the mutants following prolonged incubation at 39°C was also examined. The decay curves for infectivity at 39°C over a 10-day period are shown in Fig. 3b. The surviving infectivity of strain CVB3(RK) was almost 1,000-fold lower after 24 h of incubation at 39°C. After 3 days of incubation, the titers of strain CVB3(RK) were reduced a further 100-fold to 10^2 PFU/ml, and by day 5 postinfection, this strain was barely detectable. The capsid stability of the EM1 mutant was very similar to that of CVB3(RK) at 39°C, while the EM10 mutant was remarkably heat stable, its titers decreasing only 10-fold after 24 h and between 50- and 100-fold after 3 days at both temperatures. Even after 7 days of incubation, the titers of this mutant were only a further 10-fold lower (10^3 to 10^4 PFU/ml), while strain CVB3(RK) and the EM1 mutant were undetectable. As the only mutation in the structural genes of EM10 is the glutamate-to-glycine substitution in the knob of VP3, it may be inferred that this is responsible for the increased capsid stability. In view of the marked attenuation of this strain (see below), the stability of the EM10 capsid may hinder uncoating and A-particle formation.

Determination of pathogenesis of EM1 and EM10 in A/J mice. To determine whether the mutations in EM1 and EM10 were associated with alterations in virulence, the pathogenesis of each mutant was compared with that of cardiovirulent strain CVB3(RK) in mice. Five-week-old male A/J mice were injected intraperitoneally with 10^5 PFU of CVB3(RK), EM1, or EM10 and sacrificed on days 1 through 5 postinfection. The serum, heart, pancreas, and spleen were removed from each animal for viral quantitation by plaque assay, and a portion of tissue was fixed in 4% paraformaldehyde for histological analysis and ISH to detect the viral genome.

A comparison of the titers of each virus in the tissues tested is shown in Fig. 4. The mean serum titers of strain CVB3(RK) on days 1 and 2 were 5.6×10^3 and 1.6×10^3 PFU/ml, respectively, very similar to the titers of EM1, whereas the titers of EM10 were about fourfold lower on each day (1.3×10^3 and 4.3×10^2 , respectively). These results indicate that there were no substantive differences in the amounts of virus circulating and available to infect the tissues. In the spleen (data not shown), all three viruses were detected at high levels (10^5 to 10^6 PFU/g) on day 3, with no significant difference between the mutants and strain CVB3(RK), and no virus was found on day 5. The pancreas was extremely susceptible to infection with all three viruses, but both mutants were detected at slightly lower titers than CVB3(RK). For example, on day 2, the mean titer of CVB3(RK) was at its highest, at 9.8×10^6 PFU/g, while for EM1 it was 3.17×10^6 and for EM10 it was 1.3×10^6 (Fig. 4b). These modest differences in the titers of each virus were associated with slight but consistent differences in the degree of cytopathology seen in pancreatic sections

stained with Masson's trichrome stain (Table 1 and Fig. 5) and the level of the viral genome detected by ISH, although the majority of the exocrine tissue was affected in each case (Fig. 6). Moreover, by day 10 postinfection, the pancreas in EM10-infected mice had partially regenerated, such that about 30 to 40% of acinar tissue was normal, a significant increase from the 10% of tissue still intact on day 3, the peak of infection in the pancreas.

Heart tissue displayed the greatest difference in susceptibility to the three strains. Strain CVB3(RK) showed steadily increasing levels of virus over the 5 days; the titers were 50- to >100-fold higher than those for the mutants (Fig. 4c). The replication of EM10 was the most restricted in the heart, with maximal titers (mean and standard deviation) of $(7.9 \pm 0.22) \times 10^3$ PFU/g detected on day 2 postinfection; for CVB3(RK), the maximal titers were $(2.76 \pm 0.35) \times 10^5$ PFU/g. Thereafter, the titers of EM10 decreased so that on day 5, the difference in titers between CVB3(RK) and EM10 in the heart was 1,000-fold. EM1 showed an intermediate phenotype, with maximal titers on day 4 $[(3.8 \pm 0.18) \times 10^4]$ PFU/g that were about 40-fold lower than those of CVB3(RK) $[(1.03 \pm 0.3) \times 10^6]$ PFU/g. The restricted growth of EM1 and EM10 in the heart was also reflected in the degree of histopathology on day 5 postinfection found in sections stained with Masson's trichrome stain (Fig. 7) and revealed by ISH (Fig. 6a to d). In Fig. 6b, the CVB3(RK) genome was seen to be expressed throughout the section, resulting in the widespread damage to myocytes seen on the section stained with Masson's trichrome stain. In contrast, for both mutants, only a few individual cells containing the viral genome were scattered over the section.

Which mutations in EM1 and EM10 confer the noncardio-virulent phenotype? While mutations in the structural genes can be assumed to confer the antibody escape phenotype, the lack of cardiovirulence could be due to mutations identified in the 5'NTR of EM10 or to EM1 mutations in the 3D polymerase gene. Previous research by others identified the 5'NTR as being important in determining virulence, both in clinical isolates (9) and in a laboratory strain (40), suggesting that the CT transition at nt 119 might play an important role in the marked attenuation of EM10. nt 119 lies within a bulge in stem-loop C of the 5'NTR (44), also identified as stem-loop II of the 5'NTR (9) (Fig. 8). While a substitution at this nucleotide would not be predicted to cause a major change in the stem-loop structure, the mutation could affect the binding of proteins to this region.

Prior to examination of the role of individual mutations in the viral phenotype, the sequence of CVB3(RK), from which EM1 and EM10 were derived, was compared with that of virus derived directly from infectious clone pCVB3(T7), which was used to generate the KR and EG mutants. After plaque purification and multiple passages in vitro, CVB3(RK) contained 14 mutations relative to CVB3(T7), but only 2 of these resulted in an amino acid substitution or were in the nontranslated regions. Both of these were C-to-T transitions; the first was at position 609 in the 5'NTR and corrected a mismatch in the predicted structure of stem-loop H, as determined by MFOLD (Fig. 8), and the second was at position 7025 in P3 and caused an alanine-to-valine substitution in the 3D polymerase. The virulence of CVB3(T7) in vivo was determined and shown to be comparable to that of CVB3(RK), which has been passaged

FIG. 4. Titers of strains EM1 and EM10 relative to those of parental strain RK in tissues of A/J mice. The titers of each virus in the serum, pancreas, and heart were determined on days 1 to 5 postinfection. The heart tissue in particular showed a marked difference in susceptibility to the two mutants; 100- to 1,000-fold less virus was detected on day 5 postinfection.

both in vitro and in vivo (16). The relative titers of each in the heart, pancreas, and spleen are shown in Fig. 9. Also, CVB3(T7) was found to cause similar destruction of the exocrine pancreas (Table 2 and Fig. 5) and cardiovirulence. These two mutations in CVB3(RK) therefore were not found to significantly affect virulence.

The EM1 lysine-to-arginine mutation in VP2, the EM10 glutamate-to-glycine mutation in VP3, and the C-to-T transition at position 119 in the 5'NTR of EM10 were incorporated separately into the infectious clone as described in Materials and Methods. The resulting KR, EG, and CT viruses were plaque purified and sequenced through the 5'NTR and P1 region to confirm that they contained the correct mutations. Initially, the pathogenesis of the KR and EG mutants was

compared with that of CVB3(T7), CVB3(RK), EM1, and EM10. The CT mutant was subsequently tested together with CVB3(T7) and EM10. In each experiment, 10^5 PFU were injected into 5-week-old A/J mice. Animals were sacrificed on days 3, 5, and 10 postinfection, and the serum, spleen, pancreas, and heart were removed for analysis.

The results of plaque assays conducted on the tissues are shown in Fig. 9. As before, the EM1 and EM10 mutants had lower titers than parental strain CVB3(RK). For example, on day 5 postinfection, when the titers (mean and standard deviation) of CVB3(RK) in the heart were maximal [$(5.2 \pm 0.35) \times 10^5$ PFU/g], the titers of EM1 and of the KR mutant were $(1.3 \pm 0.25) \times 10^4$ and $(1.0 \pm 0.21) \times 10^4$ PFU/g, respectively, in each instance a reduction of nearly 50-fold. In the pancreas,

FIG. 5. Histopathological analysis of a pancreas infected with the KR and EG single mutants, EM1, and EM10. The sections were stained with Masson's trichrome stain to accentuate tissue damage. Complete necrosis of acinar tissue (NAc) but preservation of islets (I) was seen with strain T7 (b and c), as previously found with strain RK (data not shown). There was also widespread acinar tissue (Ac) damage on day 3 with both EM1 (d) and the KR single mutant (g). This effect was followed by massive lymphocytic infiltration (LI) into the exocrine tissue on day 5 (e and h) but very limited tissue regeneration by day 10 (f and i). For EM10, early acinar cell damage (j) was followed by extensive lymphocytic infiltration on day 5 and partial recovery of the tissue by day 10 (l). Tubular structures (tub) of ductal tissue that may represent sites of regeneration are indicated. For the EG mutant, there was only limited damage to the pancreas (m and n) and almost complete recovery of the tissue by day 10 (o). Magnification, $\times 63$. (a) Control pancreas. (b) T7, day 3 postinfection (p.i.). (c) T7, day 10 p.i. (d) EM1, day 3 p.i. (e) EM1, day 6 p.i. (f) EM1, day 10 p.i. (g) KR, day 3 p.i. (h) KR, day 6 p.i. (i) KR, day 10 p.i. (j) EM10, day 3 p.i. (k) EM10, day 6 p.i. (l) EM10, day 10 p.i. (m) EG, day 3 p.i. (n) EG, day 6 p.i. (o) EG, day 10 p.i.

FIG. 6. Detection of viral genomes in the heart (a to d) and the pancreas (e to h) by ISH. In the heart, the RK genome was found to be widespread in large areas of infected tissues (arrows) (b), while only single infected cells or small foci of three or four infected cells (arrows) were seen in tissues infected with EM1 (c) or EM10 (d). In the pancreas, the entire exocrine tissue was found to contain high levels of genomic RNAs of all three strains, while the islets and ductal and vascular tissues were largely preserved.

the reduction was only about fourfold; the titers were $(1.05 \pm 0.4) \times 10^7$ PFU/g for CVB3(RK), $(2.5 \pm 0.25) \times 10^6$ PFU/g for EM1, and $(1.6 \pm 0.32) \times 10^6$ PFU/g for the KR mutant on day 3 postinfection. Therefore, as before, EM1 displayed reduced

cardiovirulence but was not notably less pancreatropic, while the titers of the KR mutant matched those of EM1 closely, indicating that the attenuation of EM1 can be explained by this mutation in the EF loop of VP2. The two mutations found in

FIG. 7. Histopathological analysis of heart tissue infected with strains RK, EM1, and EM10. Viral damage to the heart was assessed on day 5 postinfection in sections stained with Masson's trichrome stain. Areas of tissue damage were seen scattered throughout heart tissue infected with RK (arrows) (b), while only individual dead or dying myocytes, together with some lymphocytic infiltration, could be seen in the EM1 section (arrows) (c). A portion of the EM1 section showing a dying myocyte (arrowhead) and some infiltrating lymphocytes (arrow) is shown at a higher magnification ($\times 380$) in panel e. The EM10 section (d) could not be distinguished from the control section (a). Magnification in panels a to d, $\times 190$.

the 3D polymerase gene of EM1 therefore are unlikely to have a strong influence on cardiovirulence, although they may contribute to the slight temperature sensitivity of EM1. The titer of EM10 was 20-fold lower than that of CVB3(RK) in the pancreas on day 3 postinfection [$(5.0 \pm 0.35) \times 10^5$ and $(1.05 \pm 0.4) \times 10^7$ PFU/g, respectively] and 1,000-fold lower in the heart on day 5 postinfection [$(4.8 \pm 0.3) \times 10^2$ and $(5.2 \pm 0.35) \times 10^5$ PFU/g, respectively]. Interestingly, the EG single mutant appeared to be more attenuated than EM10, with 5- to 10-fold-lower titers in the pancreas on day 3 postinfection [$(6.3 \pm$

$0.32) \times 10^4$ and $(5.0 \pm 0.35) \times 10^5$ PFU/g, respectively] and lower titers in the heart [only $(5.0 \pm 0.15) \times 10^2$ PFU/g on day 3, decreasing to <50 PFU/g by day 5, in each case about 10-fold lower than the titers of EM10 and 1,000- to 10,000-fold lower than the titers of CVB3(RK)]. This increased attenuation was later explained when the CT mutation in the 5'NTR of EM10 was tested and found to enhance virulence when incorporated into the T7 background (Fig. 9 and 10). The titers of the CT mutant in the tissues were about fourfold higher than those of T7 [$(8.0 \pm 0.22) \times 10^6$ and $(1.3 \pm 0.3) \times 10^6$

FIG. 8. Predicted secondary structure of the 5'NTR near the CT transitions at nt 119 and 609. MFOLD was used to predict the RNA folding of the regions of the 5'NTR encompassing the CT transitions at nt 119 and 609 (47). (Left panel) nt 119 is located in a large bulge in the stem of stem-loop C (44). (Right panel) The substitution of T corrects a mismatch at nt 609 in stem-loop H (44).

PFU/g, respectively, on day 3 and $(6.3 \pm 0.55) \times 10^6$ and $(1.51 \pm 0.4) \times 10^6$ PFU/g, respectively, on day 7 in the heart]. These differences in titers were reflected in the levels of the viral genome detected in the heart and pancreas (Fig. 10) as well as in the tissue damage seen (Fig. 5).

The high degree of attenuation of the EG mutant was confirmed by histopathological examination of the tissues, which showed large areas of surviving exocrine tissue in the pancreas (involving over 30% of each section on day 3 and increasing to over 70% on day 10). In contrast, with CVB3(T7), EM1, and the KR mutant, the exocrine pancreas was totally ablated by day 5 and never recovered (Fig. 5). Some surviving acinar tissue was also seen in EM10-infected mice (about 10% of each section on day 3 and increasing to 30 to 40% on day 10), but there was much more damage to the pancreas than with the EG mutant. With all of the mutants, tubular structures of ductal tissue that have been proposed to represent sites of pancreatic regeneration (4) were seen on day 5.

In the heart, only a small amount of cytopathology of myocytes was seen with EM1 and the KR mutant, together with a few infiltrating lymphocytes, on day 7 postinfection. The highly restricted growth of EM10 in the heart (Fig. 4 and 6) was again seen, with only one or two individual infected myocytes observed by histopathological analysis or indicated by ISH for the viral genome, while no infected myocytes were detected in the EG mutant-infected heart (Fig. 10j). This mutant is the most attenuated that we have observed and is the only one that displays significant growth restriction in the exocrine pancreas, a tissue that is extraordinarily permissive to CVBs (Fig. 5m to

o and Fig. 10e). The lower titers and lower levels of tissue damage seen on day 3 allowed regeneration of the acinar tissue in the EG mutant-infected pancreas by day 10, while the CVB3(T7)- and KR mutant-infected exocrine tissues were totally necrotic (compare Fig. 5o with Fig. 5c and i). Interestingly, with all of the viruses, surviving islets were seen, even when they were surrounded by highly infected acinar tissue; this result demonstrates the high resistance of islets to CVB3 infection, as recently shown with CVB4 as well (45).

To determine whether increasing the inoculum of EM1, the KR mutant, EM10, or the EG mutant gave them a wild-type phenotype, the amounts of each virus injected were increased by 10- and 50-fold (the maximum possible without concentrating virus through sucrose, which might alter its properties). Mice injected with larger amounts of CVB3(RK) died between days 5 and 7, while those injected with any of the mutant viruses survived. Some increases in viral titers were seen, but even when 50-fold more virus was injected, all of the mutants caused only small foci of damage to the heart. Also, the pancreatic tissue in EG mutant-infected animals still regenerated (data not shown).

Mapping of the KR and EG mutations in three-dimensional structures of EM1 and EM10. The fact that the mutations in EM1 and EM10 were originally derived by escape from neutralization by a single MAb indicated that they must lie close to each other, comprising a tertiary conformational epitope in the three-dimensional structure of the virus capsid that encompasses both VP2 and VP3. To confirm this possibility, the locations of the mutations on a computer-generated image of

FIG. 9. Titers of KR and EG single mutants and of EM1, EM10, RK, and T7 viruses. (a to c) A/J mice were sacrificed on days 3, 5, and 10 following injection with the KR and EG single mutants and the EM1 and EM10 original escape mutants. The titers of the mutants were compared with those of strain RK, the parental strain for EM1 and EM10, and T7 virus, derived directly from the infectious clone pCVB3(RK), from which KR and EG were made. RK and T7 had very similar titers in each of the tissues tested. EM1 and the KR single mutant also had similar titers in each of the tissues, while the replication of the EG single mutant was even more restricted than that of EM10 in both the heart and the pancreas. (d to f) Titers of the CT mutant, T7, and EM10 in tissues of A/J mice sacrificed on days 3 and 7 postinfection. The titers of the CT mutant were found to be three- to sixfold higher in both the pancreas and the heart, indicating that this mutation provides a slight growth advantage to highly attenuated EM10. Error bars indicate standard deviations.

alpha carbons in each constituent protein of a single protomer, based on the crystallographic structure of CVB3 (27), were determined and are shown in Fig. 11. A view of the protomer displaying the major groove is shown in Fig. 11A, and both mutations can be seen to be distant from the binding site for receptor CAR. When the image was rotated to bring the mutations into view, they can be seen to be located on the either side of a narrow cleft that separates the puff of VP2 from the knob of VP3. Both of these regions have been identified as forming part of the footprint of DAF binding in the closely related echovirus 7 (13) (Fig. 11B).

DISCUSSION

The use of neutralizing antibodies to select viral variants that can be exploited in studies of viral pathogenesis is one of the classical techniques in virology. The advent of monoclonal antibodies greatly strengthened the power of the technique as it allowed the selection of variants with mutations localized within the epitope of the MAb used. This approach has enabled the mapping of neutralization sites on viruses (2, 35) and also the generation of variants that are sufficiently similar to the parental virus to enable precise mapping of nucleotide or

TABLE 2. Summary of results of histological analysis

Strain	Organ	Result on the following day:		
		3	5	10
Control	Heart Pancreas	Normal Normal	Normal Normal	Normal Normal
RK	Heart	Small foci of damaged myocytes scattered throughout sections	Large foci of tissue damage; notable lymphocytic infiltration	Areas of fibrosis covering 15–25% of each section
	Pancreas	Large areas of acinar necrosis; islets spared	Acinar tissue totally necrotic; islets condensed	No recovery of acinar tissue; condensed and enlarged islets present
T7	Heart Pancreas	Similar to effects of RK Similar to effects of RK	Similar to effects of RK Similar to effects of RK	Similar to effects of RK
EM1	Heart	Occasional myocyte displaying vacuolation	Tiny foci of damaged tissue; some infiltrating immune cells	Small areas of fibrosis encompassing less than 2% of tissue
	Pancreas	Widespread necrosis of acinar tissue; islets intact	Many tubular structures seen in acinar tissue; large numbers of infiltrating lymphocytes	Acinar tissue still largely necrotic; condensed, enlarged islets present
KR	Heart Pancreas	Similar to effects of EM1 Similar to effects of EM1	Similar to effects of EM1 Similar to effects of EM1	Similar to effects of EM1 Similar to effects of EM1
EM10	Heart	No obvious damage seen	Rare clusters of damaged myocytes	One or two tiny foci of fibrosis seen per section (<1% of tissue)
	Pancreas	Extensive damage but ca. 10% of acinar tissue intact; islets spared	Many tubular structures present throughout; large numbers of infiltrating lymphocytes	Considerable regeneration of acinar tissue comprising 30–40% of each section; many islets present
EG	Heart	No damage seen	Slightly increased numbers of infiltrating lymphocytes but no definitive damage seen	No damage seen
	Pancreas	Necrosis of ca. 70% of acinar tissue but large pockets of surviving tissue	Large numbers of infiltrating lymphocytes; many intact islets	Approximately 70–80% of acinar tissue intact; many normal islets

amino acid substitutions that affect pathogenesis and tissue tropism (19, 24). In this study, a combination of this classical technique of isolating antibody EMs and recombinant DNA technology has enabled us to (i) identify a conformational neutralizing epitope that encompasses both the puff region of VP2 and the knob of VP3 and (ii) show that two mutations within this epitope, a lysine-to-arginine mutation at amino acid 158 of the VP2 protein and a glutamate-to-glycine substitution at amino acid 60 of the VP3 protein, can each significantly reduce the virulence of the parental strain.

Amino acid 158 lies in the EF loop (Fig. 2a), a large and highly variable surface loop of VP2 proteins of all enteroviruses, referred to as the puff region. It comprises amino acid residues 129 to 180 in CVB3, which is shorter than the puff region described for poliovirus I (Mahoney strain) but slightly longer than that of human rhinovirus 14 (3) or echovirus 7 (13). The puff region is known to be a major neutralization site in both the polioviruses and rhinoviruses and contains many amino acid substitutions between the different serotypes of

each (30, 35). This region is also variable between intertypic and intratypic variants of group B coxsackieviruses (34), and at least one amino acid substitution at this site, an asparagine-to-aspartate mutation at VP2 amino acid 165 in a nonmyocarditis strain, was previously linked to cardiocirulence (17). In a further study on clinical isolates of CVB3, several mutations in the puff region were identified in myocarditis as opposed to nonmyocarditis strains. However, mutations in the 5'NTR were found to be the principal determinants of cardiocirulence in two of these strains by the construction of chimeric viruses (10). Site-directed mutagenesis was not performed in this study; therefore, it is possible that the VP2 mutations also played a role in the loss of cardiocirulence.

The second mutation that allowed escape from neutralization, at amino acid 60 of VP3, was identified as being part of a major protrusion on the capsid surface; referred to as the knob region, which encompasses amino acids 58 to 69 of VP3 (27). Like the puff region of VP2, the knob of VP3 is known to contain a major neutralization site for both poliovirus I (Ma-

FIG. 10. Detection of viral genomic RNAs of the single mutants, EM1, and EM10 in the pancreas (a to e) and the heart (f to j) by ISH. High levels of the viral genome were found throughout the pancreas for strain T7 (b) and the CT single mutant (c) containing the 5'NTR substitution identified in EM10. For the KR (d) and EG (e) single mutants, widespread distribution of the viral genome was seen in the pancreas, but the staining was less intense and there was some preservation of the exocrine tissue (seen also in Fig. 5). In the heart, the viral genome was also found to be widely distributed throughout the tissue for strains T7 (g) and CT (h), while only small foci were seen with strain KR (i) and no genome was detected in any of the sections with strain EG (j). Con, control.

A. Stereographic imaging of the mutations in VP2 and VP3

B. Footprint of DAF binding in Echovirus and comparison with the position of the puff region of VP2 and the “knob” of VP3 in the protomer structure

FIG. 11. (A) Stereographic image of a coxsackievirus protomer. The predicted protomer structure is shown containing one of each of the viral capsid proteins VP1 (blue), VP2 (green), VP3 (red), and VP4 (yellow). Two orientations are shown: (Left) the major groove is visible; (ii) the image has been rotated to show the positions of the mutations more clearly. The EM1 VP2 lysine-to-arginine mutation at amino acid 158 and the EM10 VP3 glutamate-to-glycine mutation at amino acid 60 are indicated. The two mutation sites are in close proximity, and the distance spanning the gap between them ranges from 6 to 12 Å, depending upon the rotation of the functional groups of the amino acid residues. This image was generated by using Program O with data from Muckelbauer et al. (27) and with the help of Michael Murphy, Department of Microbiology and Immunology, University of British Columbia. (B) Diagram showing the footprint of DAF binding of echovirus 7, adapted from the data of He et al. (13).

honey) and HRV14 (30, 35, 36). However, the finding reported here that residues in both the knob and the puff region comprise a highly neutralizing conformational epitope was not previously reported. Interestingly, both of these mutations are in a region of the protomer structure that has been shown to be part of the footprint of DAF binding for echovirus 7 (13) (Fig. 11B, regions A, B, and C). As the echoviruses are structurally very closely related to the coxsackieviruses, it is likely that DAF binds similarly to each virus. By alignment of the amino acid sequences of the puff region and the knob of each, the VP2 KR mutation at amino acid 158 was predicted to be equivalent to glutamine 156 of echovirus 7, which forms part of a large contact region with DAF (Fig. 11B, region A). This region is located outside the south rim of the canyon and beyond the footprint of CAR binding defined by He et al. (12); the latter region is shown in Fig. 11B as an area enclosed by a light-blue line. On the other hand, the EG mutation at amino acid 60 of VP3, in the sequence VGEKV, is equivalent to NNIVK in echovirus 7, where amino acids I, K, and V form part of binding region B shown in Fig. 11B. By extrapolation from the echovirus data, the mutations are likely to be part of the binding site for coreceptor DAF, a possibility that is currently being investigated.

Previously, it was shown that the relative abilities of CVB3 variants to bind to DAF correlates with the amount of heart damage they cause (22). The fact that both the KR and EG mutations cause such a significant reduction in the cardiovirulence of EM1 and EM10 strains would also point to the importance of binding to a secondary receptor in promoting infection of the heart. This would be DAF in humans but possibly a substitute molecule in mice, as there is a report that CVBs do not bind murine DAF (38). Possible alternative coreceptors include other members of the regulators of complement activation (RCA) family, such as murine Crry, MCP-1, CD46, and CR2, which have a similar structures of four or five extracellular short consensus repeat domains, several of which are used by other viruses as attachment sites for entry (21, 28). Whatever the identity of this secondary receptor in mice, it is apparent that a molecule binding to a similar site on the capsid as DAF is important in pathogenesis, both from our data and from the finding that soluble human DAF injected into A/J mice significantly reduces the extent of myocardial lesions (42).

We interpret the requirement for a coreceptor to suggest that in cultured cells, such as HeLa cells, where CAR is readily accessible, the requirement for a secondary receptor such as DAF may be low, explaining why EM1 and EM10 replicate at only slightly lower titers than wild-type strain RK in both HeLa and Vero cells. However, *in vivo*, where it is thought that CAR may be localized in tight junctions (7), the virus may require binding to a more accessible molecule, such as DAF, first and then secondarily to CAR, allowing A-particle formation and viral entry. The evidence that DAF facilitates CVB infection of polarized epithelial cells *in vitro* supports such a role for DAF (37). Thus, the attenuation of EM1 and EM10 for the heart may be due to the inaccessibility of CAR in cardiomyocytes in the intact tissue. In contrast, cultured cardiomyocytes, which we have found to be as permissive to EM1 as to strain RK (38a), do not grow to a density that allows the formation of monolayers; therefore, tight junctions do not form.

While the puff region of CVB3 was previously implicated in

cardiovirulence (17), the knob of VP3 was not. In our study, the EG mutation at amino acid 60 in VP3 is far more attenuating than the KR mutation in VP2. Moreover, a double mutant containing both the KR and EG mutations was recently found to be viable but highly attenuated for both the pancreas and the heart, further emphasizing the importance of this conformational epitope in promoting infection. The second mutation that was identified in EM10 was in the 5'NTR. The 5'NTR comprises the first 741 nt and has been predicted to contain multiple stem-loop structures that are critical for viral replication (44). The 5'-terminal cloverleaf is believed to play a role in RNA replication, while the internal ribosome entry site for CVB3 (between nt 450 and 650) (20) is a region that includes several stem-loop structures as well as the polypyrimidine tract, where the 40S ribosomal subunit is thought to bind (43). The mutation in EM10 lies in the major bulge within stem-loop II (nt 105 to 180) (9), also known as stem-loop C in the structure predicted by MFOLD (44). In this position, the CT transition does not affect the predicted secondary structure but could potentially alter the binding of proteins, such as eIF2 α , which is thought to bind to stem-loop II (8). Mutations in this region have been identified as major determinants of cardiovirulence in natural isolates of CVB3 (10), and we had expected to find that the CT transition at nt 119 would be at least partially responsible for the highly attenuated nature of EM10. However, this mutation was in fact found to increase the virulence of parental strain RK when introduced independently of the EG mutation. It appeared to give a slight growth advantage to the highly attenuated EG mutant in both the pancreas and the heart, a finding that further substantiates the role of the 5'NTR in modulating virulence and that highlights the fact that the overall phenotype of a virus is a composite of both attenuating and enhancing determinants.

In conclusion, while mutations in the 5'NTR were previously documented to affect cardiovirulence, our studies have shown that mutations in the structural proteins can also play a significant role. Intuitively, one would expect structural proteins to be major determinants of tissue tropism, through their binding affinity for the cell receptor. It would seem likely that mutations affecting virulence will be found throughout the genome, as previously shown for polioviruses (1, 23, 41), with structural gene mutations being responsible for capsid stability and tissue tropism. In addition, nucleotide substitutions in the 5'NTR as well perhaps as in nonstructural genes may play key roles in the ability of a virus to propagate in different cell types.

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