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Molecular Determinants for Virulence in Coxsackievirus B1 Infection

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Studies demonstrated that a strain derived from an infectious clone of coxsackievirus B1 (CVB1N) (N. Iizuka, H. Yonekawa, and A. Nomoto, J. Virol. 65:4867–4873, 1991) was 3 to 4 $\log_{10}$ less virulent than the myotropic Tucson strain of CVB1 (CVB1T) following intraperitoneal inoculation of newborn mice. Replacement of nucleotides (nt) 69 to 804 from the 5′ untranslated region (5′ UTR) and 1A coding region of CVB1N or nt 117 to 161 from the 5′ UTR with the corresponding part from CVB1T restored greater than 90% of the virulence. Sequencing of the 5′ UTR of CVB1T demonstrated areas with a greater similarity to particular echoviruses than to CVB1N, suggesting that recombination events might have occurred, perhaps influencing the virulence phenotype.

Coxsackievirus B1 (CVB1), a member of the *Enterovirus* genus of the *Picornaviridae* family, is one of six CVB serotypes. Other members of this genus include coxsackievirus A, poliovirus, and echovirus serotypes. All enteroviruses have a similar genetic organization and sequence, with a positive-sense RNA genome of approximately 7,500 nucleotides (nt) attached at the 5′ end to the protein VPg and with a polyadenylated tract at the 3′ end. The viral genome is translated as a polyprotein in one long open reading frame.

The entire nucleotide sequence of CVB1 has been determined (10), and an infectious cDNA clone has been prepared from a clinical isolate (11). The RNA is 7,389 nt long, with an open reading frame that extends from nt 742 to 7287. The first 650 nt of the genome, in the 5′ untranslated region (UTR), are believed to play a role in viral replication and translation.

CVB1 has been implicated in human cases of pleurodynia, aseptic meningitis, meningoencephalitis, and myocarditis (19). In various strains of mice, CVB1 has been used to experimentally induce diabetes mellitus (29), myocarditis, hepatitis, pancreatitis, and encephalitis (20).

In 1979, Ray et al. reported the isolation of a CVB1 strain which causes myositis in certain mouse strains (22); this isolate has become known as the Tucson strain of CVB1 (CVB1T). CVB1T has been used by several laboratories in order to investigate an experimental model of enterovirus-induced myositis (26). Our studies demonstrated that CVB1T was more virulent following intraperitoneal (i.p.) inoculation than virus produced from an infectious cDNA clone of CVB1 (CVB1N). To identify the molecular determinant(s) for the difference in virulence between these two viruses, we prepared chimeras between the cDNAs and investigated the 50% lethal dose (LD_{50}) of the recombinant viruses. We focused on recombinant viruses with a chimeric 5′ UTR, since studies involving poliovirus have identified this region as being important in neurovirulence (6, 18). Our experiments demonstrated that a region spanning nt 117 to 161 was important in virulence. Sequencing studies comparing the 5′ UTR of CVB1T and that of CVB1N demonstrated areas of substantial similarity between CVB1T and echovirus. These regions may represent recombination events and may have influenced the virulence phenotype.

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MATERIALS AND METHODS

Cells. HeLa cells provided by B. Semler, University of California, Irvine, were used for all transfections, virus propagation, and plaque assays.

Preparation of cDNA from CVB1T. CVB1T was provided to us at passage number 10 by P. Tam, University of Minnesota, Minneapolis. The virus was plaque purified three times on HeLa cells, and a virus stock was prepared. In order to obtain RNA from partially purified virus, infected cells were lysed in a final concentration of 10% Nonidet P-40 (Sigma, St. Louis, Mo.), and the lysate was incubated in a final concentration of 0.75% sodium dodecyl sulfate (J. T. Baker, Phillipsburg, N.J.) for 30 min at room temperature. Virus was then pelleted overnight at 100,000 × *g* in a Beckman (Palo Alto, Calif.) SW28 rotor at 18°C through a 30% sucrose cushion. The viral pellet was resuspended in 20 mM Tris (pH 7.4) and phenol-chloroform extracted twice, and the viral RNA was ethanol precipitated and then resuspended in 50 μl of diethyl pyrocarbonate-treated distilled water. Two microliters of the resuspended CVB1T RNA was used as a template in a reverse transcriptase (RT)-PCR with 2 μl of 10× GeneAmp PCR buffer (Perkin-Elmer, Norwalk, Conn.), 4 μl of 10 mM deoxynucleoside triphosphates (dNTPs) (Pharmacia, Piscataway, N.J.), 9.5 μl of water, 0.5 μl of RNasin (28 U/μl; Promega, Madison, Wis.), and 1 μl (10 pmol/μl) of a reverse primer (CVB1N, nt 814 to 794). Following incubation at 72°C for 1 min and on ice for 1 min, 1 μl of Moloney murine leukemia virus RT (200 U/μl; Gibco/BRL, Gaithersburg, Md.) was added. The mixture was then incubated at room temperature for 15 min, 42°C for 15 min, and 95°C for 10 min. The reverse-transcribed product was phenol-chloroform extracted, ethanol precipitated, and resuspended in 10 μl of distilled water. Two microliters of this resuspended cDNA was added to 10 μl of 10× GeneAmp PCR buffer, 2 μl of 10 mM dNTPs, 83 μl of water, and 1 μl of the reverse primer described above as well as a forward primer (CVB1N, nt 58 to 78; 10 pmol/μl). The mixture was placed in a thermal cycler (Ericomp, San Diego, Calif.) at 95°C for 1 min, at which time 1 μl of AmpliTaq DNA polymerase (5 U/μl; Perkin-Elmer) was added, followed by a further incubation at 95°C for 2 min. The cycle continued for 30 cycles, with each cycle consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The resulting DNA was phenol-chloroform extracted, ethanol precipitated, and resuspended in 5 μl of distilled water. One microliter of this product was ligated to the TA cloning vector pCR (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions. After overnight incubation at 16°C, the ligation mixture was used to transform XL-1 Blue bacteria (Stratagene, La Jolla, Calif.). Transformants were screened by restriction enzyme digestion for the presence of the desired insert (CVB1T nt 58-814). The CVB1T nt 58-814 cDNA insert, or segments of it derived by restriction enzyme digestion, were used to construct chimeric cDNAs and/or as a template for sequencing the CVB1T 5′ UTR (see below).

Construction of chimeric cDNAs. A full-length infectious cDNA clone of CVB1N called pN14 (11) was generously provided to us by A. Nomoto (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). This cDNA was generated from the RNA of a Conn-5 strain which was isolated from the stool of a patient with meningitis and then passed several times in tissue culture. A series of chimeras was prepared by replacing part of pN14 with the corresponding CVB1T cDNA fragment, prepared as described above. Chimeras which replaced a segment of CVB1N with the corresponding part of CVB1T were named by listing first the 5′ and 3′ boundaries of the CVB1T fragment (using the CVB1N nucleotide numbering), followed by a slash, and then the designation for the CVB1N infectious clone (N14). The following chimeric cDNAs were con-

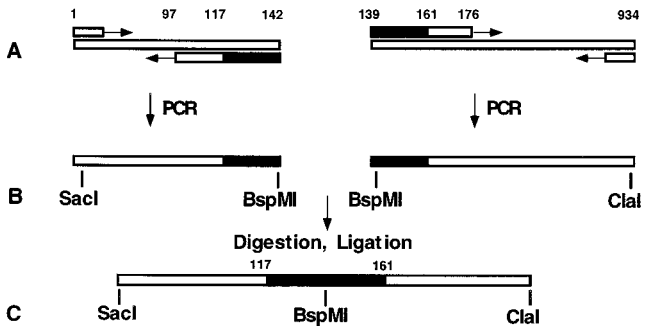

FIG. 1. Diagram showing the construction of pCVB1T 117-161/N14. In order to prepare pCVB1T 117-161/N14, the pCVB1T nt 117-161 insert was first made in two separate pieces by incorporating one of two separate parts of the 45-nt segment into a forward or reverse PCR primer for two separate PCRs using CVB1N (pN14) as a template (A). Each of the two products from these PCRs was cloned separately (B) and then digested with *Sac*I/*Bsp*MI or *Bsp*MI/*Cla*I and ligated (C) into the 9,243-bp fragment of *Sac*I- and *Cla*I-digested pN14 DNA to generate pCVB1T 117-161/N14.

structed by digestion of the CVB1T nt 58-814 cDNA with the appropriate restriction enzyme followed by ligation to digested pN14. pCB1T 69-226/N14 contains a CVB1T fragment from the *Kpn*I site at nt 69 to the *Xmn*I site at nt 226. pCB1T 227-804/N14 contains a CVB1T fragment from the *Xma*I site at nt 227 to the *Eco*RI site at nt 804. pCB1T 69-804/N14 contains a CVB1T fragment from the *Kpn*I site at nt 69 to the *Eco*RI site at nt 804. pCVB1T 117-161/N14 contains a CVB1T fragment from nt 117 to 161 and was constructed as described below.

In order to prepare pCVB1T 117-161/N14, the pCVB1T nt 117-161 insert was first separately cloned in two separate pieces which were then ligated as shown in Fig. 1. This chimeric clone and the resultant recombinant virus did not have a T7 promoter because of details related to its construction. In order to insert the T7 promoter, a fragment of pCVBT 117-161/N14 from the *Nhe*I site at nt 48 to the *Eco*RV site at nt 3441 was ligated to two fragments of pN14 (an *Eco*RV/*Nde*I fragment from nt 3442 to 7749 and an *Nde*I/*Nhe*I fragment which contained the rest of the clone, including the T7 promoter). The junctions of the chimeric fragments were sequenced to confirm the presence of the correct insertion.

Sequencing studies of the CVB1T 5′ UTR. The sequence of the CVB1T 5′ UTR was performed by using Gibco/BRL double-stranded DNA cycle sequencing. Primers used for sequencing included CVB1N nt 814-794 and CVB1N nt 58-78, the two primers noted above, as well as two universal primers for enteroviruses (3), E1 (reverse, nt 640 to 623) and E2 (forward, nt 446 to 460). The sequence of the first 100 nt of CVB1T was obtained by sequencing an RT-PCR product of CVB1T viral RNA that had been obtained by using the E1 primer as the reverse primer and CVB1T nt 1-20 as the forward primer. This uncloned PCR product was sequenced with a reverse primer which extended from CVB1T nt 159 to 136. The UWGCG BestFit and Gap programs (Genetics Computer Group, Madison, Wis.) were used for comparisons between CVB1T and CVB1N, and the UWGCG FastA program (Genetics Computer Group) was used for searches.

In vitro transcription and transfection. Prior to transcription, pN14 (the parental CVB1N clone) or the CVB1T-CVB1N chimeric cDNAs were linearized with *Xba*I, which digests 3′ to the viral coding region. A transcription reaction mixture consisting of 1 μg of the linearized DNA, 5× transcription buffer (Stratagene), 1 μl of 10 mM recombinant NTPs (Pharmacia), 1 μl of 0.75 M dithiothreitol, 1 μl of RNasin (28 U/μl; Promega), and T7 RNA polymerase (50 U/μl; Stratagene) in a final volume of 25 μl was incubated at 37°C for 40 min. HeLa cells were transfected with the in vitro-transcribed RNA, and the transfection-derived virus was plaque purified as described for another picornavirus (24). In the case of pCVB1T 117-161/N14, virus was prepared by transfection into HeLa cells of either cDNA from a clone that did not contain the T7 promoter or RNA derived from a clone with the T7 promoter; in both cases, cytopathic effect appeared within a few days, and a virus stock was prepared. In order to decrease the possibility that the phenotype of the recombinant virus was influenced by mutations which occurred outside the region of genetic manipulation, studies were generally performed with at least two separate recombinant viruses which were generated from each of two separately derived, putatively identical chimeric cDNA clones. RT-PCR was used for sequencing junctions of the chimeric fragments with a Gibco/BRL double-stranded DNA cycle sequencing kit to confirm the presence of the correct insertion into the genome of the recombinant viruses.

Virus infectivity assays. Virus was assayed by a plaque assay on HeLa cells as previously described (18).

One-step growth curve. A one-step growth curve for CVB1N and CVB1T on HeLa cells was performed with a multiplicity of infection of 10 for each virus as previously described (17). The monolayer was scraped, and virus titers were determined on petri dishes in duplicate for each time interval.

Animal inoculations. Newborn (<24-h-old) CD-1 mice (Charles River) were i.p. inoculated with 50 μl of 10-fold dilutions of virus. For the LD_{50} experiments (23), at least 10 mice were used per dilution, the mice were observed for 3 weeks, and most experiments were at least performed in duplicate. Newborn CD-1 mice were inoculated with 1 LD_{50} of each virus. Heart, liver, brain, pancreas, and skeletal muscle tissues were generally collected from 5 to 8 mice 3 to 7 days postinoculation (p.i.) and from a similar number of mice 14 to 21 days p.i. with each of the two parental viruses and each recombinant virus and then fixed in 10% neutral buffered formalin. Samples were embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin for histopathological examination.

RESULTS

Virulence of parental and recombinant viruses. The virulences of CVB1T and CVB1N were compared following i.p. inoculation of newborn CD-1 mice. CVB1T had an LD_{50} which was approximately $10^{3.7}$ PFU/ml lower than that of CVB1N, indicating that it was more virulent (Fig. 2). Similar results were found with an inbred strain of mice, BALB/c (data not shown). There was rare mortality in 3- to 4-week-old CD-1 mice inoculated with the parental and recombinant CVB1 strains (data not shown), supporting an age-dependent susceptibility of enteroviruses.

The most common pathology seen from 3 to 7 days p.i. in newborn mice inoculated with the different viruses was present in the skeletal muscle. At least 50% of animals sacrificed with each parental and recombinant virus showed severe inflammation and necrosis of this tissue. Prominent inflammation and necrosis of the acinar pancreas was also seen; however, this occurred more variably in animals inoculated with the different viruses, as follows: 40% of mice (following infection with CVB1N), 17% (CVB1T), 50% (CVB1T 69-804/N14), 40% (CVB1T 69-226/N14), 83% (CVB1T 227-804/N14), and 50% (CVB1T 117-161/N14). Small necrotic areas with mild inflammation were also observed in the heart, but this occurred in less than 25% of mice inoculated with any of the viruses. At least half of the animals that survived from 14 to 21 days p.i. with each of the viruses continued to show pathology in the skeletal muscle, usually with regeneration and ongoing inflammation. Adipose replacement and mild inflammation were not infrequently seen in the pancreas, ranging from 20% of animals inoculated with CVB1T 69-804/N14 to 83% of animals inoculated with CVB1T 117-161/N14. The heart occasionally demonstrated a few fibrotic lesions with little if any inflammation, ranging from 0% of animals (0 of 3 mice inoculated with CVB1T 69-804/N14) to 31% (4 of 13 mice inoculated with CVB1T 117-161/N14). No pathology was seen in the brain or liver at any time. Our conclusions from these studies were that the pathology of the two parental strains and that of the recombinant viruses were similar and that the most likely reason for death was related to acute severe myositis and acinar pancreatitis.

The difference in virulence of the parental strains and recombinant viruses was not a result of a global difference in virus growth, since one-step growth curves of all the viruses in HeLa cells were similar (Fig. 3). This result indicated that there are tissue-specific factors that regulate the LD_{50}s of the different viruses.

Determinants of virulence. To begin to identify the determinants responsible for this increased virulence, we constructed a variety of recombinant viruses in which most or part of the 5′ UTR and the upstream part of the P1 capsid coding region of CVB1N (nt 69 to 804) was replaced with that from CVB1T (Fig. 2). CVB1T 69-804/N14 virus had an LD_{50} that was approximately $10^{1.4}$ PFU/ml lower than that of CVB1N (Fig. 2). This result indicates that at least 90% of the virulence is restored when most of the CVB1N 5′ UTR and the upstream

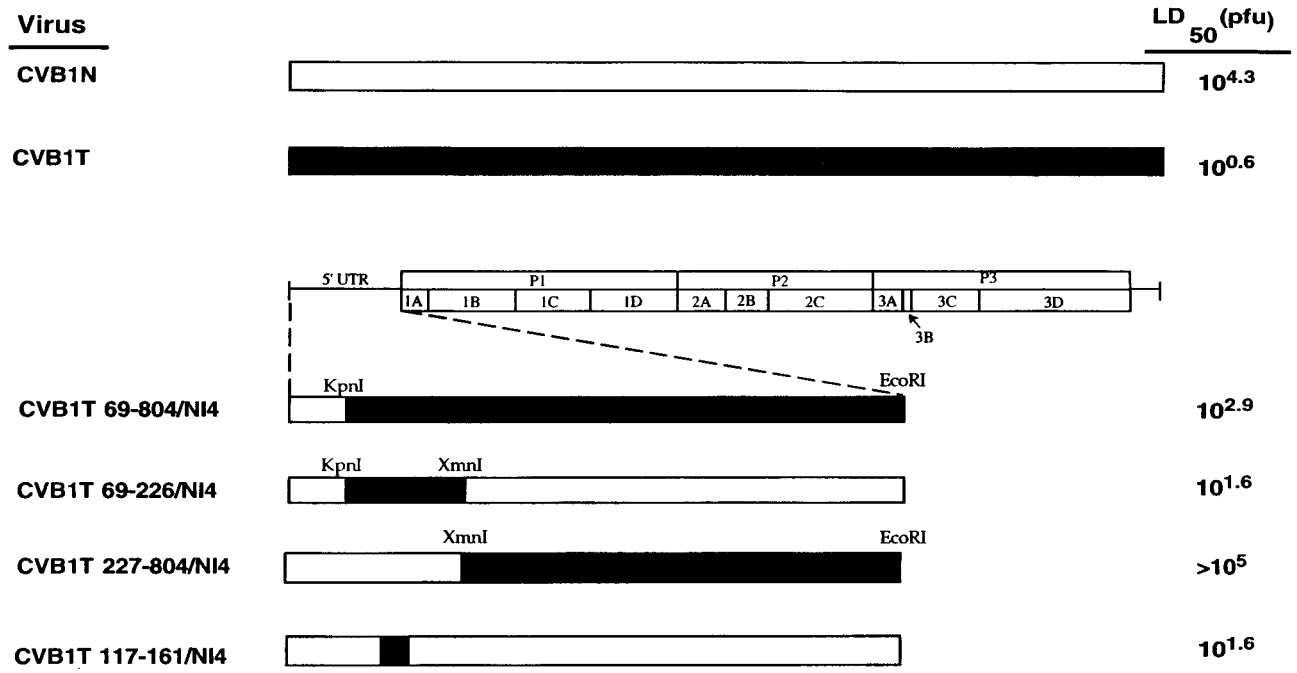

FIG. 2. CVB1 recombinant viruses and their LD_{50}s (in PFU per milliliter) following i.p. inoculation of newborn CD-1 mice. The 5′ UTR and upstream part of the 1A coding region, which contains substitutions of the CVB1T genome for the CVB1N genome, are shown enlarged. Restriction sites used to generate the constructs are noted at the margins of the chimeric segment, except in the case of CVB1T 117-161/N14 virus, where RT-PCR was used as described in Materials and Methods. CVB1N segments are shown as open bars, while CVB1T segments are shown as black bars.

part of the VP4 coding region (1A) is replaced with that of CVB1T.

To further delineate the CVB1T determinant(s) which contributes to the increased virulence, we generated recombinant viruses that had the above-described segment of CVB1T divided into two fragments. CVB1T 227-804/N14 virus had an average LD_{50} of more than 10^5 PFU/ml, which is even greater than that of CVB1N (Fig. 2), suggesting that the CVB1T nt 227-804 segment has an attenuating effect. In contrast, a virulence determinant was present in CVB1T nt 69-226, since CVB1T 69-226/N14 virus had an LD_{50} that was approximately $10^{2.7}$ PFU/ml lower than that of CVB1N (Fig. 2). In order to further define the virulence determinant in CVB1T nt 69-226, we next constructed CVB1T 117-161/N14 virus, partly because this region had a significant difference in sequence when we compared CVB1T and CVB1N nt 69-804 (see below). CVB1T 117-161/N14 virus had an average LD_{50} of $10^{1.6}$ PFU/ml, which is similar to the LD_{50} of CVB1T 69-226/N14. These results demonstrate that a major virulence determinant is present within CVB1T nt 117-161.

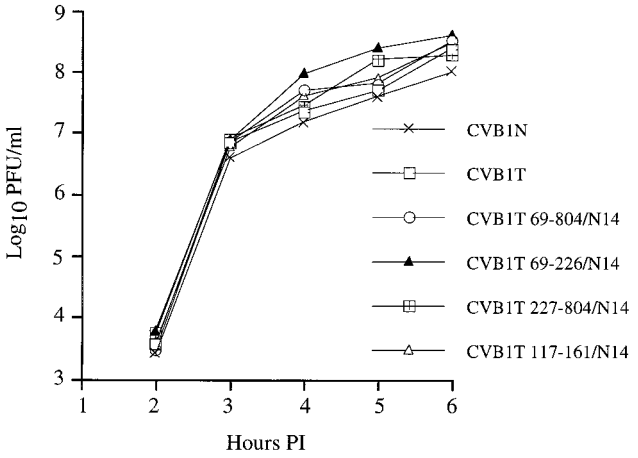

FIG. 3. One-step growth curve of parental and recombinant viruses in HeLa cells. PI, postinoculation.

Sequence comparisons. Since the recombinant virus studies demonstrated that the 5′ UTR contained significant determinants for virulence, we sequenced this region of the CVB1T genome and compared it with that of CVB1N. The 5′ UTRs of CVB1T and CVB1N have an overall sequence identity of 82% (Fig. 4). Interestingly, there is an overall sequence identity that is even greater than this but that does not exceed 86%, when the CVB1T 5′ UTR is compared with the 5′ UTRs of a number of other enteroviruses or related viruses: echoviruses 6, 9, 12, and 25 and swine vesicular disease virus (which bears sequence similarity to CVB5) (31). This result confirms the remarkable similarity in sequence of many of the enteroviruses with respect to the 5′ UTR. Two areas of the CVB1T 5′ UTR (nt 117 to 161 and 487 to 511), however, stand out because they have a much lower identity (52%) to the corresponding regions of CVB1N. Sequence comparisons showed that these regions have a much greater sequence identity to echoviruses (Fig. 4). Specifically, CVB1T nt 117-161 shares 78% identity with the corresponding region of echovirus 12; the only other picornavirus besides echovirus 12 that shares a greater sequence identity than CVB1N to CVB1T nt 117-161 is enterovirus 71 (76%). CVB1T nt 487-511 has 88% identity with the corresponding region of echovirus 6; this is the only picornavirus with a greater sequence identity than CVB1N to CVB1T nt 487-511.

The results described above raised questions as to whether recombination between CVB1 and echovirus strains might

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CVB1T XXXXXXXXXXXXXXXXGUUGUXCCCXCCCGCAGGGCCCAXU.GGCGUUAGC      50
CVB1N UUAAAACAGCCUGUGG     U   A   A    C  AUUG.    C        50

CVB1T ACUCUGGUAUCACGGUACCCUUGUGCGCCUGUUUUAUACAUCCCACCCCA      100
CVB1N                     U              ..        CU   C      98

CVB1T AA...GUAACUUAGAAGUAUAUUACAAAUGAUCAAUAGUCAGCUCAACAC      147
CVB1N   AUU    U         U CAC  . CC    U   CA   GUGG         147
EV12                     UC  C                 U        A

CVB1T GCCAGUUGAGUACCGAUCAAGCACUUCUGUUACCCCGGACUGAGUAUCAA      197
CVB1N A    CCAU  UUU                                          197
EV12  A       CCUA

CVB1T UAAGCUGCUAACGCGGCUGAAGGAGAAAACGUUCGUUAUCCGACCAACUA      247
CVB1N   GA C          U                       C   G           247

CVB1T CUUCGGGAAGCCUAGUACCACCAUGAAAGUUGCGCACGGUUUCGUUCCGC      297
CVB1N      AA  A       A       G        G GU      C  A       297

CVB1T ACAGCCCCAGUGUAGAUCAGGCCGAUGAGUCACCGCG.UCCCCGCAGGCG      346
CVB1N   UA                  U                U     A G        347

CVB1T ACUGUGGCGGUGGCUGCGUUGGCGGCCUGCCCAUGGGGUGACCCAUGGGA      396
CVB1N   C                                 U C    AA    G A      397

CVB1T CGCUUCAAUACUGACAUGGCGCGAAGAGUCUACUGAGCUAGCUGGUGGUC      446
CVB1N     CU     A       U             U        U    AA       447

CVB1T CUCCGGCCCCUGAAUGCGGCUAAUCCUAACUGCGGAGCAGGCAAUCACAA      496
CVB1N                                          CAU CC U       497
EV6                                               UGC

CVB1T UCCAGUGGGUGGCCUGUCGUAACGGGCAACUCCGCAGCGGAACCGACUAC      546
CVB1N A   G   CA UG                   U                .      546
EV6

CVB1T CUUUGGGUGUCCGUGUUUCUUUUUAUUCUUACAUUGGCUGCUUAUGGUGA      596
CVB1N                     A        C  U C                     596

CVB1T CAAUUACAGAAUUGUUACCAUAUAGCUAUUGGAUUGGCCAUCCAGUGACU      646
CVB1N      GACAGG              U                  G           646

CVB1T AACAGAGCAAUCGUGUACCUUUUUGUUGGAUUUGUACCACUAAACAUCAC      696
CVB1N            UA A  U  C        G   A       U G U G A      696

CVB1T GAAUUUUACAACACUAAACUACAUUCUAUUAUUAAACUCAGCAAA           741
CVB1N AG GG   A       C UCU   CA UAA C    UA  A               741
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FIG. 4. Nucleotide sequence of the 5′ UTRs of CVB1T and CVB1N and selected regions of echovirus 12 (EV12) and echovirus 6 (EV6). Only nucleotides that differ from the presented CVB1T sequence are shown. The numbers on the right refer to the nucleotide numbers of the CVB1T and CVB1N virus sequences. CVB1N nt 117-161 and CVB1N nt 487-511 and the corresponding nucleotides of CVB1T, EV12, or EV6 are shown against a gray background. Deleted nucleotides are shown by a dot. X, nucleotides at the 5′ terminus for which the sequence was not determined.

have occurred in these segments of the 5′ UTR and whether there were any features of the secondary structure of the CVB1T 5′ UTR that might make such an event more likely. Both of these segments are located in stem-loops of a secondary structure of the enterovirus 5′ UTR predicted from computer analysis and biochemical studies (12). The region involving nt 117 to 161 includes loop II (as well a region just upstream of it), and the region involving nt 487 to 511 is the most distal part of loop IV, a loop which contains over 100 nt (Fig. 5).

DISCUSSION

The availability of full-length infectious cDNA clones of picornaviruses has made possible the delineation of determinants for virulence and other disease-related phenotypes. We investigated the difference between CVB1N and CVB1T viruses with respect to virulence following i.p. inoculation of neonatal mice. Substituting nt 69 to 804 of CVB1T into CVB1N increased the virulence of CVB1N virus by more than 90%. Narrowing this segment down by only substituting a region from CVB1T nt 69-226 increased the virulence still further, to an LD_{50} similar to that seen with CVB1T, suggesting that a major determinant for virulence lies within this area. The virulence determinant was further delineated to a region that included CVB1T nt 117-161.

Our data suggested that there also may be attenuating determinants in the CVB1 5′ UTR: substitution of CVB1T nt 227-804 into the CVB1N 5′ UTR led to further attenuation of the less virulent CVB1N virus. In addition, this CVB1T seg-

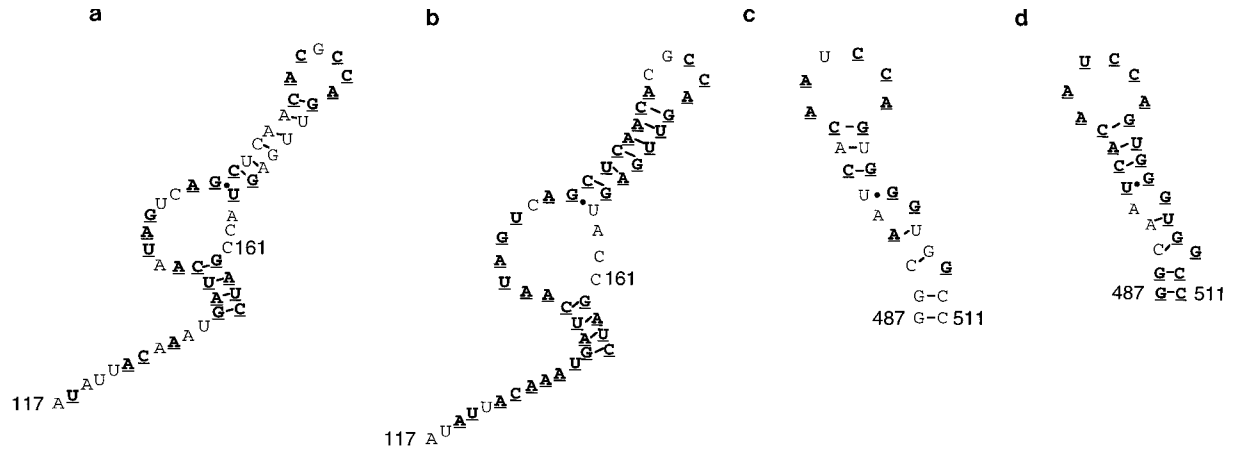

FIG. 5. Secondary structure prediction, based on the model of Jackson and Kaminski (12), of CVB1T 5′ UTR in regions of nt 117 to 161 (a and b) and nt 487 to 511 (c and d). In all cases, the sequence presented is CVB1T. Identical nucleotides are shown by underlined boldfaced letters. (a) CVB1T and CVB1N; (b) CVB1T and echovirus 12; (c) CVB1T and CVB1N; (d) CVB1T and echovirus 6.

ment appears to have a dominant attenuating effect over the virulence determinant in CVB1T nt 69-226, since CVB1T 69-804/N14 virus, which contains CVB1T nt 227-804 along with CVB1T nt 69-226, was less virulent than a recombinant virus of CVB1N that contained only CVB1T nt 69-226. The varying effect of a chimeric insertion depending on the context of the remainder of the genome has been described before (13) and highlights the shortcomings of experiments using chimeric cDNAs as well as the difficulties in interpreting the results.

The finding that a major determinant for virulence maps to the 5′ UTR is not unexpected, since this is also the case with other enteroviruses. For example, a poliovirus neurovirulence determinant for type 1 maps to nt 480 (15), for type 2 it maps to nt 481 (18), and for type 3 it maps to nt 472 (6). In addition, cardiovirulence of CVB3 maps to a 5′ UTR determinant at position 234 (30).

The 5′ UTR of enteroviruses is believed to be important for virulence because of its roles in allowing ribosomal binding to an internal ribosome entry site that is located in this region and in regulating the efficiency of translational initiation at an AUG downstream from the internal ribosome entry site (14, 21). A 21-base sequence in the downstream part of the CVB1N 5′ UTR, nt 546 to 566, has been found to be essential for internal initiation of translation (11), but more extensive regions of the 5′ UTR are also likely to be critically important for these activities. The importance of nt 117 to 161 to virulence may relate to differences in tissue-specific protein binding to this region in CVB1T versus CVB1N and the effect on translation. A number of host cell proteins bind the 5′ UTR of picornaviruses and regulate translation (reviewed in reference 5); these proteins may also have a critical role in virulence, as is believed to be the case with poliovirus (27).

We found that the 5′ UTRs of CVB1T and CVB1N had an overall similarity of 82%, demonstrating that different strains of the same enterovirus can have a substantial degree of sequence variation. A recent report similarly notes that the nucleotide sequence of the CVB1N 2A coding region, which encodes a protein with a protease activity, has as much as 25% diversity compared to many CVB1 isolates (32).

A close relationship between the sequence of CVB viruses and echoviruses, including the sequence of the 5′ UTR (4), has been previously noted (1). Recent sequence comparisons involving approximately 630 bases from the 5′ UTR found a nucleotide identity of over 90% between CVB1N and CVB3, as well as similarities to a number of echoviruses (9). Interestingly, we found a greater overall sequence similarity of the CVB1T 5′ UTR with the 5′ UTRs of members of the echovirus group and of other CVB serotypes than with that of CVB1N. There was a substantial sequence identity of two regions of the CVB1T 5′ UTR with the corresponding regions of two echovirus serotypes.

Our finding of a similarity in the sequence of most of the 5′ UTR of CVB1N and CVB1T with that of several other enteroviruses, as well as the presence of two regions of relative sequence divergence between CVB1T vis-a-vis CVB1N, raise the possibility that recombination events may have occurred between echovirus strains and CVB1T (especially two regions of CVB1T). Intertypic poliovirus recombinant viruses have been previously identified during human vaccination (2, 7, 8), and viruses with recombination of the 5′ UTR of poliovirus with the 5′ UTR of CVB3 (25) as well as with the 5′ UTR of CVB1 (11) have been experimentally produced; however, there are no reports of recombination occurring in nature between two different enteroviruses. The prediction that nt 117 to 161 and 487 to 511 are located at the end of a stem-loop seems well suited for a recombination event, since recombination hot spots are predicted to occur in regions of increased secondary structure (28), perhaps because of the greater ease of "copy choice" occurring under these circumstances (16).

Recombination is a valuable strategy by which a virus strain can enhance its virulence and promote its growth and may be a more efficient means for evolution of a viral genome than mutation alone (7). It may be that the CVB1T genome is a product of both recombinational events as well as mutations, since the sequences of nt 117 to 161 and 487 to 511 are not identical to the sequences of the closest echovirus match. Selective pressure may have led to maintenance of the recombined former segment because of an enhancement of virulence. A recent report presents data describing two areas of recombination (with one in the 5′ UTR) in a vaccine-nonvaccine poliovirus type 2 found in nature in which the recombination event may have conferred a selective advantage over the vaccine parent because of enhanced neurovirulence and pathogenicity (8).

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