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Identification of a Single Amino Acid Residue in the Capsid Protein VP1 of Coxsackievirus B4 That Determines the Virulent Phenotype

MICHELE CAGGANA, PERRY CHAN, AND ARLENE RAMSINGH*

Wadsworth Center for Laboratories and Research, New York State Department of Health, Empire State Plaza, P.O. Box 509, Albany, New York 12201-0509

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To identify the molecular determinants of virulence for coxsackievirus B4, a panel of recombinant, chimeric viruses were constructed from cDNA clones of a virulent virus, CB4-V, and a nonvirulent virus, CB4-P. Initial studies mapped a major determinant of virulence to the 5′ end of the viral genome, which contained the 5′ untranslated and the P1 regions (A. Ramsingh, A. Hixson, B. Duceman, and J. Slack, J. Virol. 64:3078–3081, 1990). To determine whether the 5′ untranslated region contributed to the virulent phenotype, a chimeric virus (vCB403) containing this region of the virulent virus on an avirulent background was tested in mice. The vCB403 construct displayed a phenotype similar to that of CB4-P, suggesting that the 5′ untranslated region did not significantly contribute to virulence. Analysis of the sequence data of the P1 regions of both CB4-V and CB4-P revealed five mutations that resulted in amino acid substitutions in VP1, VP2, and VP4 (A. Ramsingh, H. Araki, S. Bryant, and A. Hixson, Virus Res. 23:281–292, 1992). Analysis of individual mutations in both VP1 and VP2 revealed that a single residue (Thr-129 of VP1) determined the virulent phenotype.

Coxsackieviruses, members of the family *Picornaviridae*, possess a single-stranded RNA genome of positive polarity surrounded by a protein coat comprising four capsid proteins, VP1, VP2, VP3, and VP4 (17). Although there is a great deal of information on the biochemical, biophysical, and genetic characteristics of picornaviruses, the mechanisms by which these RNA viruses cause disease are poorly understood. Coxsackieviruses of the B group have been implicated in diseases such as pancreatitis, type I insulin-dependent diabetes mellitus, myocarditis, and myositis (4, 9). The existence of variants within a single serotype contributes to the variability in the pathogenesis of coxsackievirus infections.

A powerful tool in the study of the genetic basis of virulence of picornaviruses has been the use of recombinant, chimeric viruses derived from cDNA clones of virulent and nonvirulent viruses. Early studies demonstrated that the cloned cDNA of poliovirus type 1 was infectious (12). More recently, in vitro-derived RNA transcripts of full-length picornavirus cDNAs have also been shown to be infectious (22). Using a virulent, pancreatropic variant of coxsackievirus B4 (CB4-V) (15) and the nonvirulent, prototypical JVB strain of coxsackievirus B4 (CB4-P), we have constructed a panel of recombinant, chimeric viruses. Our previous studies mapped a major determinant of virulence to the 5′ end of the genome, which encompasses both the 5′ untranslated region (UTR) and the P1 region, which encodes the four capsid proteins (14). Comparison of the sequence data in this region of CB4-P and CB4-V allowed the identification of candidate determinants of virulence in the 5′ UTR and the capsid proteins VP1, VP2, and VP4 (13). This study shows that one residue, Thr-129 of VP1, is a major determinant of virulence for coxsackievirus B4. In addition, this study also shows that the 5′ UTR of coxsackievirus B4 does not significantly contribute to virulence.

* Corresponding author.

MATERIALS AND METHODS

Cells and viruses. The passage histories of the two viruses, CB4-P and CB4-V, have been previously described (15). Large-scale stock preparations of these viruses were prepared in LLC-MK2(D) cells, and viral infectivity was determined by using a plaque assay (10).

Construction of recombinant viruses. By using RNA extracted from purified preparations of either CB4-P or CB4-V, cDNA libraries were prepared by standard methods (19, 23). To clone the extreme 5′ end of the viral RNA, the technique of reverse transcription coupled with the polymerase chain reaction was used as previously described (14). All cDNAs were cloned into the phagemid pBSKS+ (Stratagene). An *Xba*I and a *Sac*I site were introduced at the extreme 5′ and 3′ ends, respectively, of the viral cDNA. Four subclones were used for the construction of the recombinant full-length cDNA clones. One pair of clones contained the 5′ UTRs and the P1 regions of CB4-P and CB4-V as *Xba*I-*Hin*dIII fragments. A convenient *Hin*cII site at position 758 was used to split the 5′ UTR from the P1 region. Another pair of clones contained the P2 and P3 regions plus the 3′ UTR of either CB4-P or CB4-V as *Hin*dIII-*Sac*I fragments. Recombinant full-length cDNAs of CB4-P and CB4-V were generated from these subclones as *Xba*I-*Sac*I fragments (Fig. 1). Each of the recombinant, chimeric cDNAs was made by swapping the appropriate restricted fragment on either a CB4-P or a CB4-V cDNA background (Fig. 1). Two approaches were used to generate the chimeric cDNAs. The first approach involved using unique restriction enzyme sites that flanked the nucleotide substitution in CB4-V. For example, nucleotide 2833 of the gene encoding VP1 (Thr-129) of CB4-V is flanked by a *Bgl*II site (position 2655) and a *Bss*HII site (position 3108). The 453-bp fragment was then subcloned into a *Bgl*II-*Bss*HII-digested clone containing CB4-P cDNA to yield pCB420. The second, more commonly used approach involved generating subfragments from the viral subclones. For example, to construct clone pCB421, various cDNA clones of CB4-P and CB4-V were used to obtain the

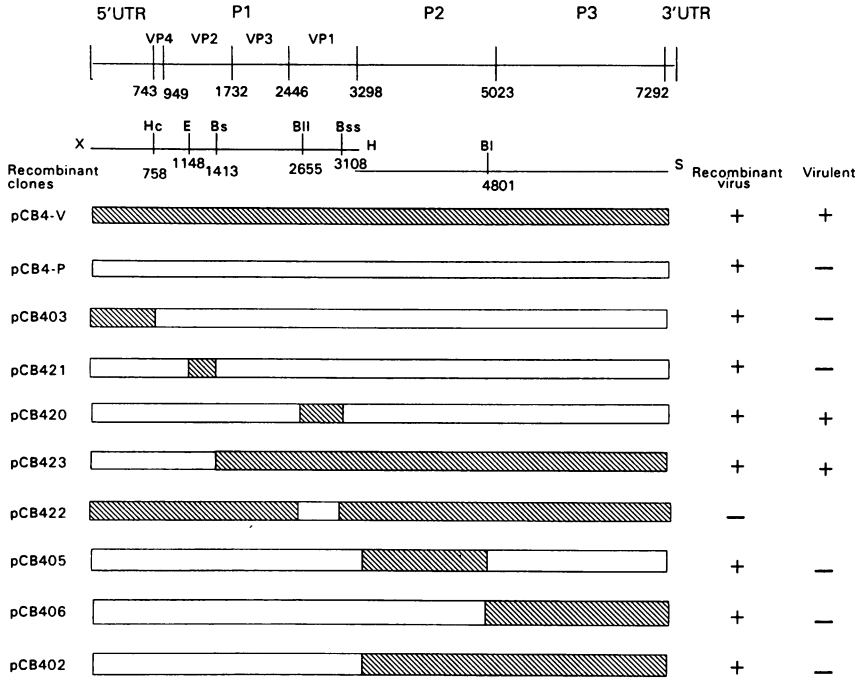

FIG. 1. Genotypes of chimeric coxsackievirus B4 cDNA clones. The top line depicts the structural organization of the coxsackievirus B4 (JVB strain) genome (6). A partial restriction map of the subclones used for the construction of the chimeric cDNAs is shown. One pair of subclones contained the 5' UTRs and the P1 regions of CB4-P and CB4-V as *Xba*I-*Hin*dIII fragments. Another pair contained the P2 and P3 regions and the 3' UTRs of CB4-P and CB4-V as *Hin*dIII-*Sac*I inserts. X, *Xba*I; Hc, *Hin*cII; E, *Eco*RI; Bs, *Bst*BI; BII, *Bgl*II; Bss, *Bss*HII; H, *Hin*dIII; BI, *Bgl*I; S, *Sac*I. Virulence was initially scored as moribundity (+) or the lack of it (−) in mice 2 weeks p.i.

*Xba*I-*Eco*RI (CB4-P), *Eco*RI-*Bst*BI (CB4-V), *Bst*BI-*Hin*dIII (CB4-P), and *Hin*dIII-*Sac*I (CB4-P) fragments. These fragments were then ligated simultaneously into the vector, pBSKS+, which had been digested with *Xba*I and *Sac*I. Designations for cloned viral cDNAs begin with "pCB4." The clones containing the full-length cDNAs of CB4-P and CB4-V are designated pCB4-P and pCB4-V, respectively.

Recombinant viruses were obtained by transfecting LLC-MK2(D) cells with in vitro-derived RNA transcripts. Briefly, T3 RNA polymerase was used to make plus-sense RNA transcripts. RNA transcripts were routinely assayed by gel electrophoresis after denaturation in glyoxal and dimethyl sulfoxide (8). Transfections were carried out by using DEAE-dextran as the facilitator (19). After cell suspensions were incubated with RNA and 0.8 mg of DEAE-dextran per ml at 37°C for 2 h, T25 flasks were seeded with cells. The medium was replaced 24 h later. Virus was harvested when cells exhibited 80 to 100% cytopathic effect. Designations for recombinant viruses begin with "vCB4." Cloned CB4-P and CB4-V are referred to as vCB4-P and vCB4-V, respectively. The titers of the recombinant viruses obtained after transfection were determined by plaque assay, and these viruses were used directly to infect mice.

Infection of mice. Previous studies had shown that of the B10 *H-2* congenic mice tested, B10.T(6R) mice were very susceptible to infection with CB4-V (15). These mice were bred in our animal facility. Mice used in these experiments were 4 to 6 weeks old and were maintained three per cage. Mice were allowed to eat and drink ad libitum. Groups of three to six B10.T(6R) or B10.Q mice were injected intraperitoneally with 10^4 PFU of virus diluted in phosphate-buffered saline (PBS). Control mice were injected intraperitoneally with PBS. All experiments were done at least twice. All injected mice were monitored daily. Animals found to be moribund were euthanized immediately by CO_2 overdose. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center for Laboratories and Research.

Glucose and amylase assays. Nonfasting mice were bled from the tail vein at various days postinfection (p.i.). All assays were performed with pooled serum samples. Glucose concentrations were determined by the glucose oxidase test (11). Serum amylase activity was assayed by using a modification of the *p*-nitrophenol-D-maltoheptaoside chromogenic method described by Barger and Craighead (1).

DNA and RNA sequencing. To determine whether the recombinant cDNAs contained the desired mutations at nucleotide positions 1353 and 2833, limited DNA sequence analyses were performed. The cDNAs were sequenced in both the forward and reverse orientations by the dideoxy chain-termination method (20) by using coxsackievirus-specific primers and Sequenase (U.S. Biochemicals).

To determine whether the recombinant viruses contained the appropriate mutations at nucleotide positions 1353 and 2833, limited sequence analyses of viral genomic RNAs were carried out. Virus, obtained after transfection, was used to infect LLC-MK2(D) cells at a multiplicity of infection of 0.1 PFU per cell. Total RNA was extracted by using RNAzol (Cinna/Biotecx) at 24 h p.i. After reverse transcription using random primers, specific regions of the viral cDNA were amplified by the polymerase chain reaction (18) and sequenced by the dideoxy chain-termination method (20). The cDNAs were sequenced in both the forward and the reverse orientations. RNA from infected cells was sequenced at least twice.

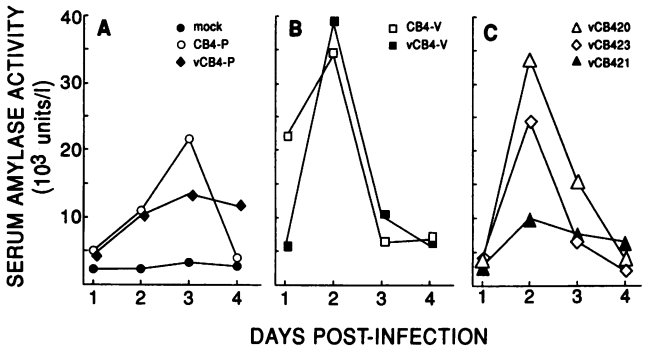

FIG. 2. Serum amylase activity of mice infected with coxsackievirus B4. B10.T(6R) mice were infected with parental stock viruses, viruses derived from cDNA clones, or recombinant, chimeric viruses and bled from the tail vein at various times p.i. The amylase activity of pooled serum samples was determined.

RESULTS

Characterization of the disease syndrome associated with CB4-V infection. We had previously shown that, in our mouse model system, CB4-V induced a disease syndrome characterized by pancreatitis with accompanying hypoglycemia (15). Pancreatitis was assessed histologically. In this report, the disease syndrome is characterized more extensively. A quantitative assessment of pancreatitis was made by measuring serum amylase. Groups of four to six mice were infected with either CB4-V or CB4-P, and blood samples were obtained from the tail vein at various times p.i. The serum amylase activity in pooled blood samples was determined. Experiments were done at least twice, and results from a representative experiment are shown in Fig. 2. In CB4-V-infected mice, serum amylase activity peaked at 2 days p.i. and represented at least a sevenfold increase above the average activity seen in mock-infected control mice. These findings were supported by histological assessment of pancreatic tissues (Fig. 3). A severe acinar pancreatitis characterized by a generalized degranulation of the acinar cells and partial loss of exocrine secretory units was observed. Infected mice began to appear moribund at 4 days p.i., and these mice were sacrificed. Since the sample size decreased rapidly after 4 days, data from after this time point were used. In CB4-P-infected mice, serum amylase activity at 3 days p.i. represented a three- to fourfold increase over the background level. Histological assessment of pancreatic tissue also revealed a milder pancreatitis in infected mice (Fig. 3). These data suggest that CB4-V infection induced a greater amount of damage in the exocrine pancreas than CB4-P infection did. As previously reported (15), CB4-V also induced a marked hypoglycemia, while CB4-P-infected mice remained normoglycemic. To determine whether additional factors contributed to the hypoglycemia observed in CB4-V-infected mice, serum insulin concentrations were evaluated. In CB4-V-infected mice, serum insulin concentrations were within the normal range and were similar to those of mock-infected mice (data not shown).

This study extended our previous observations and showed that CB4-V infection was associated with hyperamylasemia, severe pancreatitis, hypoglycemia, normal serum insulin concentrations, histological and ultrastructural changes in the liver, and histological changes in the heart (data not shown). Mice infected with CB4-P developed a mild pancreatitis, maintained normal serum glucose and insulin concentrations, and did not show any histological alterations of the liver and heart. CB4-V-infected mice, unlike CB4-P-infected mice, succumbed to infection.

Generation of recombinant viruses. To determine whether the four cDNA constructs, pCB420 to pCB423 (Fig. 1), contained the desired mutations at nucleotide positions 1353 and 2833, a partial DNA sequence was obtained. The observed nucleotides at positions 1353 and 2833 (Table 1) corresponded to the predicted ones (13).

Recombinant viruses were then generated by transfecting LLC-MK2(D) cells with in vitro-derived RNA transcripts of the cloned, full-length cDNAs. The RNA transcripts were routinely assayed by gel electrophoresis after denaturation in glyoxal and dimethyl sulfoxide (8). All of the constructs yielded RNA transcripts of the appropriate size. Virus was generally harvested from transfected cells 4 to 7 days later. However, for one construct, pCB422, transfected cells appeared to undergo a crisis at 3 to 4 days, followed by recovery. Although several attempts were made, no infectious virus was harvested from these cultures, suggesting that the pCB422 construct was not viable in LLC-MK2(D) cells.

To identify the nucleotides at positions 1353 and 2833, genomic RNAs from all recombinant viruses were partially sequenced (Fig. 4). The only nucleotide substitutions observed were at those specific positions. No additional mutations were observed within the surrounding regions of at least 100 nucleotides.

Infection of mice with either the parental stock virus or the cloned recombinant virus. B10.T(6R) mice infected with either CB4-P or virus derived from the cloned cDNA were indistinguishable. A mild pancreatitis, similar to that seen in CB4-P-infected mice, was observed in vCB4-P-infected mice (Fig. 3). Again, a threefold increase in serum amylase activity was observed at 3 days p.i. (Fig. 2). Serum glucose concentrations were within the normal range (Fig. 5), and mice did not succumb to the infection.

B10.T(6R) mice infected with virus derived from the cloned cDNA of CB4-V displayed signs similar to those observed in mice infected with stock CB4-V. Both viruses were virulent in mice, virulence being defined as the ability to induce severe pancreatitis with accompanying hypoglycemia. In mice that possess the K^q allele, such as B10.T(6R), infection is also lethal. Serum amylase activity in vCB4-V-infected mice peaked at 2 days p.i. and represented an eightfold increase over the activity in mock-infected mice (Fig. 2). Upon histological examination, pancreatic tissue from mice infected with the recombinant virus showed a severe pancreatitis similar to that observed in CB4-V-infected mice (Fig. 3). Serum glucose concentrations decreased markedly at 4 days p.i. (Fig. 5), and mice began to appear moribund. These data suggest that virus derived from the cloned cDNA was very similar to the parental stock virus, CB4-V.

Mapping of the virulent phenotype to the capsid protein VP1 of CB4-V. Initially, virulence was scored as the percentage of infected mice that became moribund within 2 weeks p.i. Mice infected with CB4-P or vCB4-P appeared healthy and did not succumb to the infection. All mice infected with CB4-V or vCB4-V became moribund within 2 weeks. Since mice infected with vCB403, which contained the 5′ UTR of the virulent virus on a nonvirulent background, were healthy and did not succumb to the infection, it appears that the 5′ UTR does not significantly affect the virulent phenotype. Mice infected with vCB405 or vCB406, which contained the P2 or the P3 region plus the 3′ UTR, respectively, of the

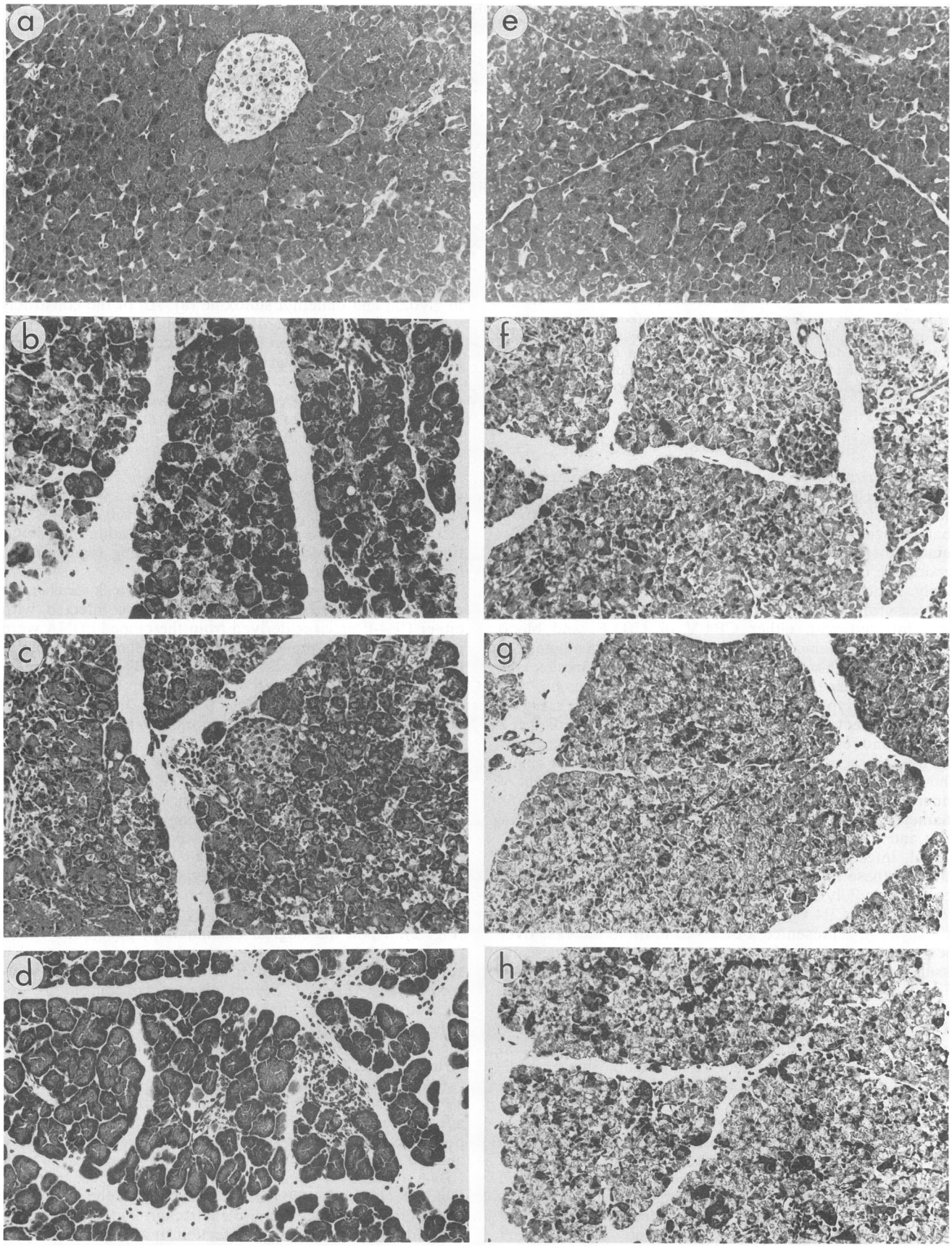

FIG. 3. Histopathology of pancreatic tissue from B10.T(6R) mice. Mice were injected intraperitoneally with 10^4 PFU of coxsackievirus B4, as follows: parental stock virus CB4-P (b), recombinant virus vCB4-P (c), chimeric virus vCB421 (d), parental stock virus CB4-V (f), recombinant virus vCB4-V (g), and chimeric virus vCB420 (h). Control mice were mock infected with PBS (a and e). Magnification, ×116.

TABLE 1. Summary of DNA sequence data for nucleotide positions 1353 and 2833 of recombinant coxsackievirus B4 cDNA clones

Recombinant plasmid	Nucleotide at position 1353	Corresponding amino acid at VP2-135	Nucleotide at position 2833	Corresponding amino acid at VP1-129
pCB4-P	A	Thr	T	Met
pCB4-V	G	Ala	C	Thr
pCB420	A	Thr	C	Thr
pCB423	A	Thr	C	Thr
pCB421	G	Ala	T	Met
pCB422	G	Ala	T	Met

virulent virus on an avirulent background, also appeared healthy during the 2-week follow-up period. Furthermore, mice infected with an additional recombinant virus, vCB402, containing the P2 and P3 regions and the 3′ UTR of the virulent virus on a nonvirulent background also behaved similarly to vCB4-P-infected mice. These data suggest that the P2 and P3 regions and the 3′ UTR of the viral genome, in addition to the 5′ UTR, do not significantly affect the virulent phenotype of coxsackievirus B4.

A more detailed study of the mice infected with the recombinant viruses that contained an altered VP1 or VP2 capsid protein (i.e., vCB420, vCB421, and vCB423) was then undertaken. The serum amylase activity in mice infected with vCB420 or vCB423 was similar to that in mice infected with vCB4-V, peaking at 2 days p.i. (Fig. 2). The serum amylase activity of mice infected with vCB421 was similar to that of mice infected with vCB4-P. Histological studies supported these findings (Fig. 3). Pancreatic tissue from vCB420-infected mice showed a severe pancreatitis, while tissue from vCB421-infected mice showed a mild pancreatitis. Mice infected with vCB421 were also normoglycemic (Fig. 5). However, serum glucose concentrations decreased sharply at 4 days p.i. in both vCB420- and vCB423-infected mice. All of the mice infected with vCB420 and vCB423 became moribund (Fig. 1). The recombinant viruses, vCB420, vCB423, and vCB4-V, all had a threonine at position 129 of the VP1 capsid protein and were virulent in mice.

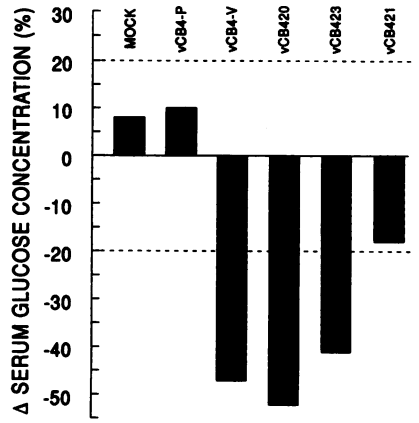

FIG. 5. Change in serum glucose concentrations in mice infected with recombinant coxsackieviruses. Mice were injected intraperitoneally with recombinant viruses and bled from the tail vein at various times p.i. Control mice were mock infected with PBS. The glucose concentrations of pooled serum samples were determined. Dashed lines indicate the range in normal values. Δ Serum glucose concentration = {[glucose(4 days p.i.)] − [glucose(2 days p.i.)]}/[glucose(2 days p.i.)] × 100.

DISCUSSION

To elucidate the molecular basis of virulence for coxsackievirus B4, a panel of recombinant, chimeric viruses was constructed from a nonvirulent and a virulent virus. Infection with the virulent virus, CB4-V, was associated with hyperamylasemia, severe pancreatitis, and hypoglycemia. Serum amylase activity peaked at 2 days p.i. B10.T(6R) mice succumbed to infection with CB4-V. Infection with CB4-P resulted in a mild pancreatitis with serum amylase activity peaking at 3 days p.i. Mice did not succumb to infection with CB4-P.

Previous studies identified the 5′ end of the viral genome, which encompasses the 5′ UTR and the P1 region, as containing a major determinant of virulence (14). The present study showed that the 5′ UTR of coxsackievirus B4 did not significantly contribute to the virulent phenotype since the chimeric virus vCB403, which contained the 5′ UTR of the virulent virus, was avirulent in mice. This is in

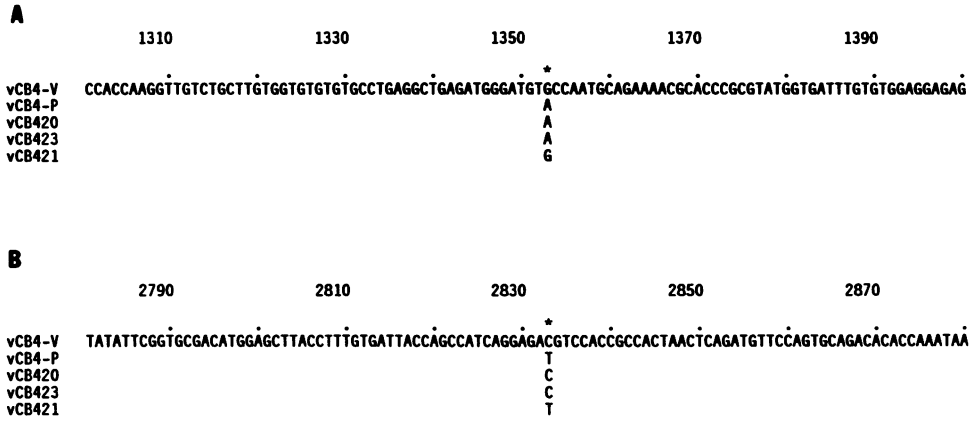

FIG. 4. Partial genomic sequences of the regions surrounding nucleotides 1353 (A) and 2833 (B) of recombinant coxsackieviruses.

contrast to results obtained with other picornaviruses, such as poliovirus, Theiler's murine encephalomyelitis virus, and mengovirus, in which determinants of attenuation have been mapped to the 5′ UTR. For poliovirus, the nucleotides U-472, G-480, and A-481 have been identified as determinants of attenuation for P3/Sabin, P1/Sabin, and P2/P712 (similar to P2/Sabin), respectively (7, 16, 24). For Theiler's murine encephalomyelitis virus, the 5′ UTR can affect neurovirulence, although the neurovirulent phenotype is multigenic (3). For mengovirus, truncation of the poly(C) tract in the 5′ UTR dramatically attenuates the pathogenicity of the virus in mice (2).

Previous studies had identified five amino acid substitutions within the VP1, VP2, and VP4 capsid proteins of the virulent virus CB4-V (13). One of these substitutions, Ala-201 of VP2, represented a conservative amino acid change and is not expected to affect virulence. The remaining four substitutions are nonconservative and represent potential determinants of virulence. Two mutations, Arg-20 and Arg-16, occurred at the amino termini of VP1 and VP4, respectively, and are expected to lie within the interior of the virion. The remaining two mutations, Ala-135 and Thr-129 of VP2 and VP1, respectively, are predicted to be on the surface of the virion. This prediction was based on a sequence and structure alignment (13). Initially, the amino acid sequences of VP1 and VP2 of CB4-V and poliovirus type 2 (P712) (21) were aligned. By using data from the three-dimensional structure of poliovirus type 1 (Mahoney strain) (5), the sequence alignment allowed an approximation of the region of the molecule where the substituted amino acids in CB4-V mapped. By this comparison, Thr-129 of VP1 would map to the loop connecting beta strands D and E, while Ala-135 of VP2 would map to the large loop that connects beta strand E with the radial helix on the back surface of the eight-stranded antiparallel beta barrel.

These studies show that Thr-129 of VP1, but not Ala-135 of VP2, is a major determinant of virulence. The VP2 capsid protein did not contribute to the virulent phenotype, since vCB421, which contained Ala-135 of VP2 on a nonvirulent background, was not lethal in mice. A recombinant virus, vCB420, containing a single-amino-acid substitution, Thr-129 of VP1, on a nonvirulent background, behaved similarly to CB4-V and was lethal in B10.T(6R) mice. Since the virulent phenotype was reconstituted by a single-amino-acid substitution in the VP1 capsid protein, it is likely that, in addition to the 5′ UTR, the P2 and P3 regions and the 3′ UTR of the viral genome do not significantly contribute to virulence. This conclusion is also supported by our finding that the recombinant viruses vCB405 and vCB406, which contained the P2 region or the P3 region plus the 3′ UTR, respectively, of the virulent virus on a nonvirulent background, were avirulent in mice. An additional construct, vCB402, containing the P2 and P3 regions and the 3′ UTR of CB4-V on a CB4-P background, was also avirulent in mice, further indicating that the major determinant of virulence maps to the region encoding the structural proteins. Several attempts to construct an additional recombinant virus (vCB422) that contained Met-129 of VP1 on a virulent background were made to determine whether this single-amino-acid substitution would attenuate the virus. However, after repeated attempts to transfect LLC-MK2(D) cells with in vitro-derived RNA transcripts from pCB422, infectious virus was not made. This suggests that a methionine at position 129 of VP1 may be inconsistent with an arginine at position 16 of VP4 and/or an arginine at position 20 of VP1.

Interestingly, the sequence and structure alignment positions Thr-129 on the DE loop of VP1 next to Ile-143 of poliovirus, an amino acid which has been shown to be a major determinant of attenuation (16). Although the pathogeneses of poliovirus and coxsackievirus infections are very different, these data suggest that the molecular mechanisms underlying virulence in these viruses share some features mediated by the DE loop of the VP1 capsid proteins.

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