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A Point Mutation in the VP4 Coding Sequence of Coxsackievirus B4 Influences Virulence

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In analyzing the molecular determinants of virulence of coxsackievirus B4, chimeric viruses were constructed from avirulent and virulent viruses. The vCB424 recombinant contained a single nucleotide substitution on an avirulent genetic background, resulting in replacement of Ser-16 of VP4 with Arg-16. Mice infected with vCB424 displayed an intermediate phenotype.

Coxsackievirus B4, one of six viruses in the B serogroup of enteroviruses, is a member of the *Picornaviridae*. The virus genome consists of a single-stranded RNA of positive polarity. Excluding the poly(A) tract, the RNA genome consists of 7,395 nucleotides and is composed of a 5′ untranslated region (UTR) of 743 nucleotides, a 3′ UTR of 105 nucleotides, and an open reading frame encoding a polyprotein of 2,183 amino acids, which is proteolytically cleaved (8). The open reading frame is divided into three regions, P1, P2, and P3. The four capsid proteins, VP1 through VP4, are encoded within the P1 region, while the nonstructural proteins that are involved in virus replication are encoded within the P2 and P3 regions. The group B viruses have been implicated in a variety of diseases such as pancreatitis, type I insulin-dependent diabetes mellitus, myocarditis, and myositis (6, 12). The existence of variants within a single serotype further complicates the pathogenesis of coxsackievirus infections. 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. A powerful tool in the study of the genetic basis of virulence of picornaviruses is the use of recombinant chimeric viruses derived from cDNA clones of virulent and avirulent viruses. Using this approach, several groups have identified determinants of attenuation or virulence for several picornaviruses. Of the three Sabin poliovirus strains, determinants of attenuation have been mapped to the 5′ UTR (9, 19, 21) and to the VP1 (11, 19, 20) and VP3 (21) gene sequences. In the mouse model, neurovirulence is determined by single-amino-acid substitutions in the VP1 and VP2 capsid proteins (5) and by multiple mutations in regions encoding both viral proteinase $2A^{pro}$ and capsid protein VP1 (10). For coxsackievirus B3, Zhang et al. (22) have shown that a transversion at nucleotide position 690 in the 5′ UTR of a chimeric virus is not attenuating. Recent comparisons between the noncardiovirulent strain CVB3/0 and two cardiovirulent strains have identified two nucleotides in the 5′ and 3′ UTRs and eight amino acids throughout the polyprotein that are potential determinants of virulence (3).

In our mouse model system, CB4-V is virulent, inducing a disease syndrome characterized by pancreatitis, hyperamylasemia, and hypoglycemia. Infection is lethal in some strains of mice (18). The prototypical JVB strain of coxsackievirus B4 (CB4-P) in our system is nonvirulent. Previous studies mapped a major determinant of CB4-V virulence to the 5′ end of the genome, which encompasses both the 5′ UTR and the P1 region (17). Comparison of the sequence data in this region of CB4-P and CB4-V allowed the identification of candidate determinants of virulence (16). Thr-129 of VP1 was shown to be a major determinant of virulence (2). In this report, we show that Arg-16 of VP4 is also a determinant of virulence, but to a lesser extent than Thr-129 of VP1.

Construction of recombinant viruses. The construction of recombinant viruses has been previously described (2). Briefly, four subclones were used for the construction of the recombinant full-length cDNA clones. 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 (Fig. 1). Recombinant full-length cDNAs of either CB4-P or CB4-V were generated from these subclones as *Xba*I-*Sac*I fragments. The chimeric cDNA was made by the exchange of a *Hin*cII-*Eco*RI fragment of CB4-P with that of CB4-V. This resulted in a single-nucleotide substitution at position 791 (T→A) causing a replacement of Ser-16 of VP4 with Arg-16. The recombinant virus, vCB424, was obtained by transfection of LLC-MK2(D) cells with in vitro-derived RNA transcripts as previously described (2). Virus was harvested when cells exhibited 80 to 100% cytopathic effect and were subsequently plaque purified. Both the chimeric cDNA and the viral, genomic RNA were characterized by limited sequencing of the 5′ UTR and the coding regions of VP4, VP2, and VP1. The mutation at nucleotide position 791 in both the chimeric cDNA and the viral, genomic RNA was confirmed. Furthermore, the nucleotide at position 2833 which also influences virulence (2) was the same as that of the avirulent virus, CB4-P.

Infection of mice. Previous studies had shown that of the B10 H-2 congenic mice tested, B10.T6(R) mice were very susceptible to infection with CB4-V (18). Mice were injected intraperitoneally with 10^4 PFU of virus diluted in phosphate-buffered saline (PBS). Control mice were injected intraperitoneally with PBS. All infected mice were monitored daily. Animals found to be moribund were euthanized immediately by CO_2 overdose. Mortality rates were determined from the numbers of mice that became moribund. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center for Laboratories and Research. Nonfasting mice were bled from the tail vein on various days postinfection. Serum glucose concentrations were determined

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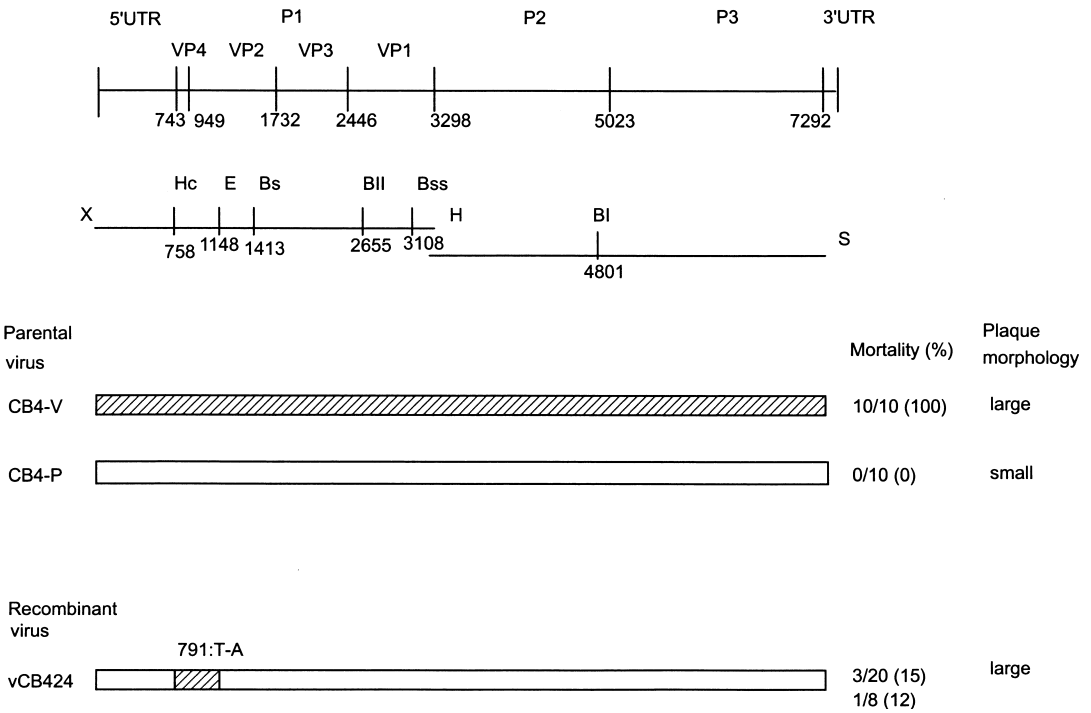

FIG. 1. Genotypes of the parental and recombinant chimeric coxsackieviruses. The top line depicts the structural organization of the coxsackievirus B4 (JVB strain) genome (8). A partial restriction map of the subclones used for the construction of chimeric cDNAs is shown. One pair of subclones contained the 5′ UTR 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′ UTR 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; Bl, *Bgl*I; S, *Sac*I.

by the glucose oxidase test (14). Serum amylase activity was assayed by using a modification of the *p*-nitrophenol-D-maltoheptaoside chromogenic method described by Barger and Craighead (1). Mice were sacrificed at various times postinfection, pancreatic tissues were fixed in phosphate-buffered formalin, and sections were stained with hematoxylin and eosin. Extensive characterization of the stock, parental viruses (CB4-P and CB4-V), and viruses derived from the cloned cDNAs (vCB4-P and vCB4-V) revealed no differences between the stock virus and the recombinant virus with respect to virulence and plaque phenotype (2, 15). In this study, the chimeric virus vCB424 was compared with the parental viruses CB4-P and CB4-V. The average plaque sizes of CB4-P and CB4-V were 0.4 cm (small) and 1.0 cm (large), respectively (15). The chimeric virus exhibited a large-plaque phenotype (Fig. 2), suggesting that an arginine substitution at position 16 of VP4 affected virus replication in vitro. Mice infected with vCB424 displayed a phenotype intermediate between those of CB4-V and CB4-P (Table 1). Infected mice developed hypoglycemia, similar to that observed in CB4-V-infected mice (Fig. 3), resulting in a mortality rate of 12 to 15%. However, serum amylase levels were comparable to those of CB4-P-infected mice (Fig. 4). Histological assessment of pancreatic tissues also supported the observation that vCB424 displayed an intermediate phenotype (Fig. 5). By 7 days postinfection, CB4-V-

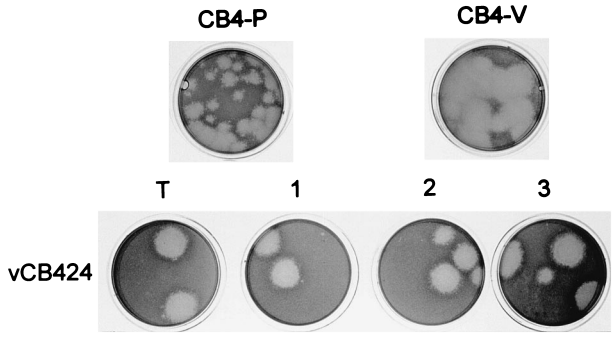

FIG. 2. Plaque morphology of parental and recombinant chimeric coxsackieviruses. The parental viruses, CB4-P and CB4-V, were from plaque-purified stocks. The vCB424 chimeric viruses from the original transfection (T) and from three plaque-purified, random isolates (plates 1 to 3) are also shown.

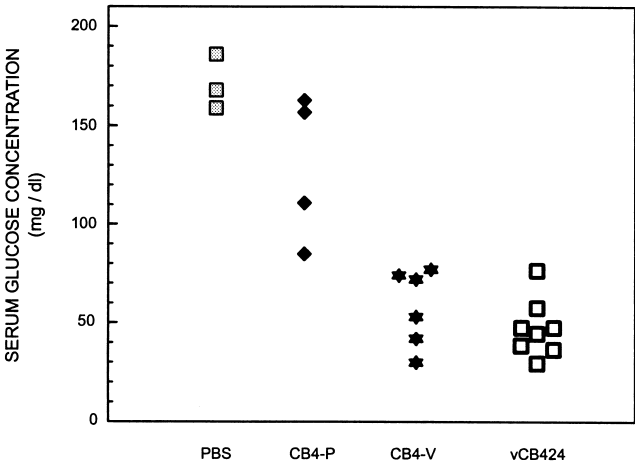

FIG. 3. Serum glucose concentrations of mice infected with parental and recombinant chimeric coxsackieviruses. Each value represents the serum glucose concentration in a single mouse 4 days postinfection.

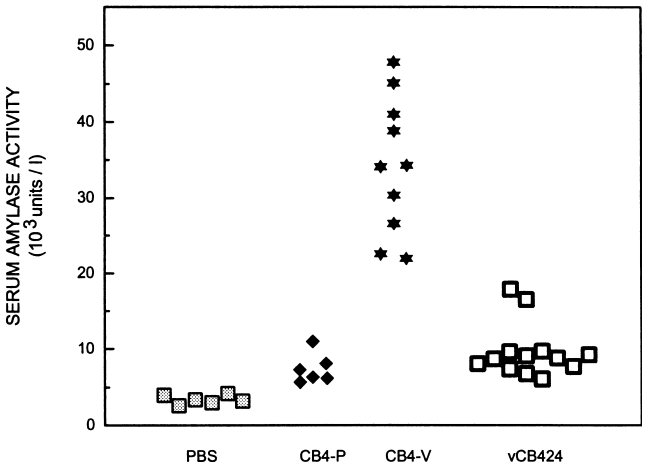

FIG. 4. Serum amylase activity of mice infected with parental and recombinant chimeric coxsackieviruses. Each value represents the serum amylase activity in a single mouse 2 days postinfection.

infected mice showed extensive, cytologic necrosis of the pancreatic acinar cells and had marked edema (Fig. 5C). Lymphocytes and plasma cells were observed in the interlobular tissues. In contrast, pancreatic tissues from CB4-P-infected mice contained well-preserved acini and islet cells. Sparse lymphocytic infiltration was observed in the periductal tissues (Fig. 5B). Mice infected with vCB424 showed some focal loss of groups of acini with the appearance of numerous small ductular structures of unknown origin. The inflammatory infiltrate consisted of lymphocytes and plasma cells (Fig. 5D).

TABLE 1. Phenotypes observed with CB4-P, CB4-V, and vCB424

Virus	Presence of:		% Mortality	Plaque morphology
	Hypoglycemia	Hyperamylasemia		
CB4-P	−	−	0	Small
CB4-V	+	+	100	Large
vCB424	+	−	12–15	Large

In our model system of coxsackievirus B4 infection, virulence is defined as the development of hypoglycemia, hyperamylasemia, and pancreatitis with morbidity in some strains of mice. Virulence is a multigenic phenotype mapping to at least two sites within the P1 region. Previous work identified Thr-129 of VP1 as a major determinant of virulence, because a recombinant virus, vCB420, containing a single-amino-acid substitution on a nonvirulent genetic background, recapitulated the phenotype of the virulent parental virus, CB4-V (2). In this report, we showed that a recombinant virus containing

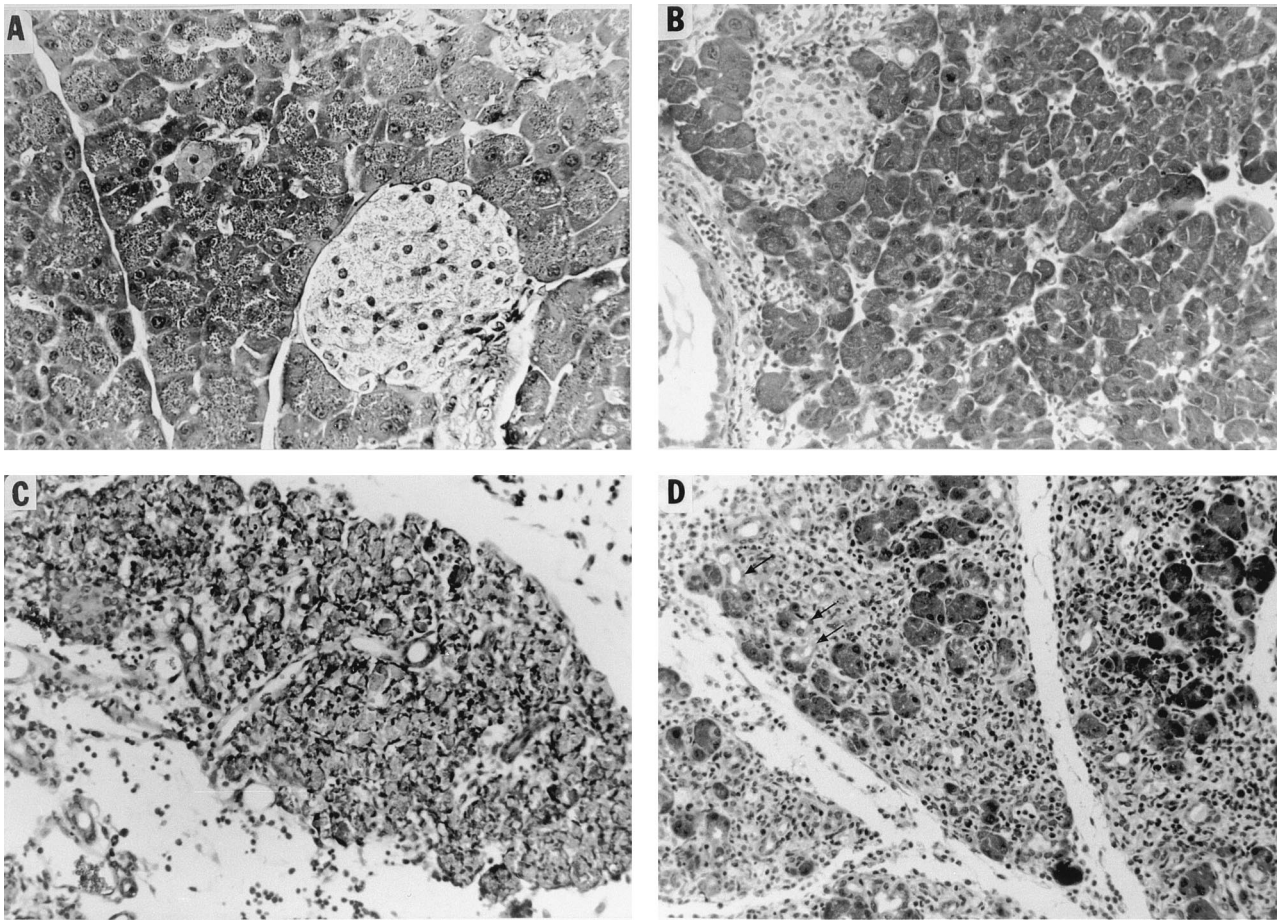

FIG. 5. Histopathology of pancreatic tissue from B10.T6(R) mice infected with parental and recombinant chimeric coxsackieviruses. Pancreatic tissues were harvested from mice at 7 days postinfection. (A) Mock-infected with PBS. (B) CB4-P-infected. (C) CB4-V-infected. (D) vCB424-infected. Arrows (D) show ductlike structures lined by flattened epithelium. Magnification: A, ×185; B to D, ×158.

Arg-16 of VP4 in an avirulent genetic background resulted in a phenotype that was intermediate between those of the two parental viruses. In vivo, Arg-16 of VP4 and Thr-129 of VP1 were associated with the development of hypoglycemia and hyperamylasemia, respectively, suggesting that these two sites may be affecting viral tropism. To date, we have not reconstructed the parental CB4-P virus from CB424 to test whether other sites in the genome (not assayed by sequence or restriction analyses) might have been altered, possibly affecting the virus phenotype. This experiment is planned. However, as noted earlier, sequence analyses of numerous chimeric CB4 strains have been consistent with the expected engineered results, suggesting that changes elsewhere do not likely play a role. Hyperamylasemia is possibly the result of infection of the pancreatic acinar cells. However, the underlying mechanism that results in the development of hypoglycemia is presently unknown. Serum insulin concentrations in mice infected with either parental virus were within the normal range (data not shown). Arg-16 of VP4 also affected virus replication in vitro, since the vCB424 recombinant had a large-plaque phenotype. Additional studies have shown that the 5′ UTR also influenced plaque size (15). The VP4-16 substitution occurs at the amino terminus of the protein. The VP4 capsid protein of picornaviruses is found on the inner surface of the virion and interacts with the flexible N-terminal extension of the other capsid proteins, forming an interwined network within the virion that contributes to virion stability (reviewed in reference 7). Previous studies with poliovirus have shown that mutations within the VP4 coding sequence can affect cell entry (13), host range, and pathogenicity (4). Moscufo et al. (13) demonstrated that although transfection of the cDNA of a nonviable VP4 mutant resulted in mature virus particles, the viruses were defective at a late stage of cell entry. Couderc et al. (4) showed that four amino acid substitutions in the capsid proteins, including residue 62 of VP4, extended the host range of the Mahoney strain to mice and conferred the neurovirulent phenotype. In vCB424, there is a gain of a positive charge at VP4-16, which may affect (i) the interaction of VP4 with the negatively charged viral genomic RNA, (ii) the interaction with the amino-terminal extensions of the other capsid proteins, or (iii) an early step in binding to the cell receptor. These interactions would be expected to affect virion assembly, stability, and/or tropism.

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