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SHORT COMMUNICATION

Deletion of Thymidine Kinase Gene Attenuates Channel Catfish Herpesvirus While Maintaining Infectivity

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A recombinant thymidine kinase (TK) gene deletion mutant of channel catfish herpesvirus (CCV) was constructed and compared to the parent virus for replicative ability in cell culture, for the ability to infect, replicate, and cause lethal disease in the channel catfish host, and for the ability to induce protective immunity in channel catfish fingerlings. There was no difference between parent CCV and the TK-negative mutant (CCVTK$^-$) in viral production in cell culture. However, in immersion challenge trials, 100-fold more CCVTK$^-$ than parent CCV was required to kill similar numbers of channel catfish fingerlings. The attenuation was confirmed as a TK-associated characteristic in marker rescue; the revertant regained the pathogenic characteristic of the parent virus. When catfish were immersion challenged with equal amounts of CCVTK$^-$ or CCV, the levels of virus isolated from the posterior kidney were similar through the peak production period which occurred 4 days postinfection. Subsequently, CCVTK$^-$ levels decreased dramatically when compared to CCV levels. Exposure to CCVTK$^-$ induced protective immunity against challenge with a lethal dose of wild-type CCV. The degree of protection provided by CCVTK$^-$ was related to the dose of CCVTK$^-$ given. This study demonstrates that the genetically distant CCV retains recombination properties and TK-associated attenuation properties of herpesviruses of higher vertebrates. Additionally, the unique characteristic of CCV containing two copies of the TK gene did not prevent the use of this gene as a selectable site of homologous recombination. © 1995 Academic Press, Inc.

Channel catfish virus (CCV) (*Ictalurid herpesvirus-1*) is a cytopathic herpesvirus that can cause an acute, hemorrhagic, and lethal disease in the channel catfish *Ictalurus punctatus* (*1*). Acute CCV epizootics in populations of fry and fingerling channel catfish can result in mortalities as high as 95% within 1 week (*2*). Yet, little is known about the mechanisms involved in the pathogenesis of this disease. Inferences from herpesvirus diseases of avian and mammalian species cannot be directly made because CCV represents a unique type of herpesvirus that has not been identified in higher vertebrates (*3*) and because the host is genetically distant from all terrestrial vertebrates. Thymidine kinase (TK) gene inactivation of other cytopathic large DNA viruses including α-herpesviruses reduces viral pathogenicity yet does not affect immunogenicity. This effect has been observed in marmoset herpesvirus (*4*), herpes simplex virus types 1 and 2 (*5–9*), equine herpesvirus type 1 (*10*), bovine herpesvirus type 1 (*11*), and suid herpesvirus type 1 (*12, 13*). The attenuation has been attributed to a reduced ability of the TK$^-$ virus to replicate in nondividing cells. Herpesvirus TK provides deoxynucleotide precursors for viral DNA synthesis in nonreplicating cells and hence enhances infectivity in these cells. Like the TK of other herpesviruses, CCV TK appears to have a broader substrate specificity than the host cell-encoded enzyme (*14*). However, CCV and its host are evolutionarily distant from previously studied herpesviruses and their respective hosts (*3*). Also, some TK deficient herpesviruses can retain a high level of virulence (*15*). Therefore, we deleted the TK gene of CCV to evaluate the effects of a TK$^-$ mutation on the pathogenicity of CCV and to determine if such a recombinant virus would be a viable candidate as a live virus vaccine.

A TK$^-$ mutant of the Auburn Clone A strain of CCV (American Type Culture Collection) was constructed by deleting the 663 bp immediately following the TK gene ATG-start codon. This was accomplished by first deleting the region in the plasmid pBSCV446, which is a pBluescript SK$^-$ (Stratagene, La Jolla, CA) construct containing the CCV genomic *SstI–XbaI* restriction fragment of plasmid pUCCV425 (*16*). Then, the plasmid construct was cotransfected with whole viral DNA into cells of the channel catfish ovary cell line (CCO) and recombinant CCV was selected. The deletion plasmid was generated by a combination of polymerase chain reaction (PCR) and traditional cloning techniques (Fig. 1). Vent polymerase (New England Biolabs, Inc. Beverly, MA) mediated PCR was accomplished in 50-μl reactions (1 × Vent buffer, 200 μM each deoxynucleotide triphosphate, 50 pM of

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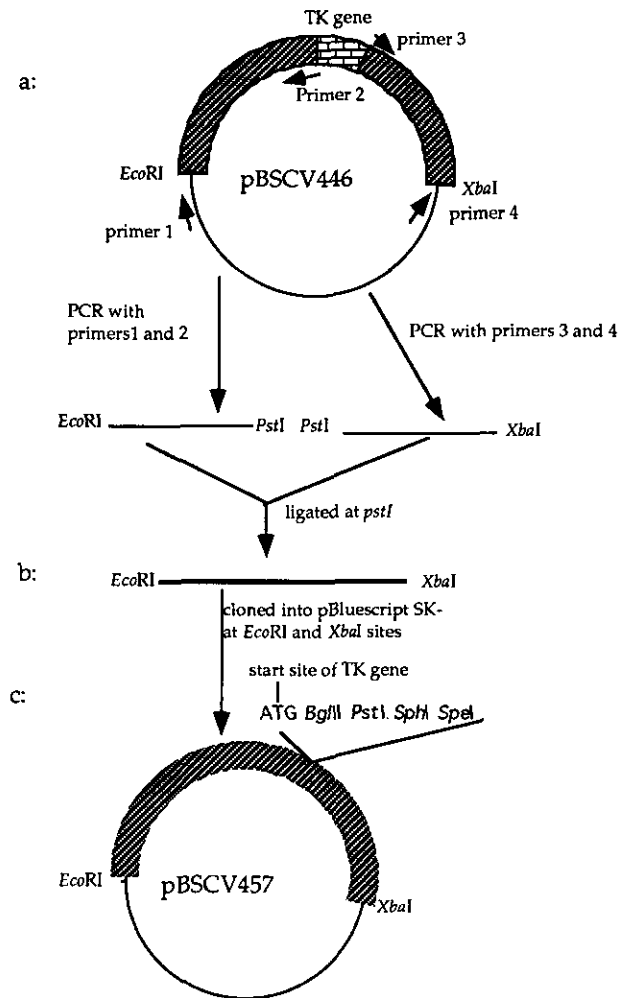

FIG. 1. Schematic of the construction of the transfer vector pBSCV457 in which 663 bp of the TK gene was deleted from the CCV fragment derived from pBSCV446. The sequences of primers 1, 2, 3, and 4 used in two separate PCRs (a) were 5'TGAGCGCGCGTAATACGACT3', 5'CTCCTGCAGATCTCATGTCTATAACAGCCCCGG3', 5'CTCCTGCAGCAGCATGCACTAGTACACCGAAAAAGTCAGTTATA3', and 5'CAGCTATGACCATGATTACG3', respectively. The 3' nucleotide of primers 1 and 4 correspond to position 616 on the positive sense strand and an 821 on the negative sense strand of pBluescript, respectively. Primer 2 contained *BglII* and *PstI* restriction sites at the 5' end and the 3' nucleotide corresponds to position 8798 the negative sense strand of the CCV genome (GenBank Accession No. M75136). Primer 3 contained *PstI*, *SphI*, and *SpeI* restriction sites at the 5' end and the 3' nucleotide corresponds to position 9465 of CCV. The products were *PstI*-digested and ligated (b) and then cloned into pBluescript SK$^-$ (c).

each primer, and 10 ng of template) in 30 cycles (1 min at 92°, 30 sec at 47°, and 3 min at 72°). Amplified products were purified by electrophoresis on 1% agarose gels, excised, and extracted using a DNA prep-A gene kit (Bio-Rad, Melville, NY). The construct was designated pBSCV457, amplified, and purified by CsCl gradient centrifugation (*17*).

Cationic liposome-mediated cotransfection of CCO cells with 10 μg of purified CCV DNA and 5 μg of pBSCV457 DNA was accomplished using the lipofectin reagent (BRL; Grand Island, MD) as previously described (*16*). Cell culture, virus production, and viral DNA purification were done according to previously described procedures (*16*). The cell culture medium used was Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 25 m*M* HEPES buffer, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The progeny of a TK negative recombinant (CCVTK$^-$) was isolated and cloned by three sequential plaque purifications of the transfected lysates in CCO cells with 0.1 m*M* arabinofuranosyl thymine (Ara-T) in the culture medium.

Virus from the single plaque was cultured in a 25-cm^2 flask of CCO cells and evaluated for purity and the designed deletion by PCR amplification of the TK region followed by direct sequencing of the products. Infected cells from 25-cm^2 tissue culture flasks were dislodged, pelleted by centrifugation 14,000 *g* for 1 min, and lysed and proteinase K digested (*18*). The DNA was isopropanol precipitated and resuspended in 80 μl distilled H_2O and 37.5 μl was used as template in a 50-μl *Taq* polymerase (Promega, Madison, WI) mediated PCR consisting of 30 cycles as described above. The positive strand primer was 5'GGAAAAATTGGGTTAAGAA3' (3' nucleotide corresponds to position 8739 in the CCV genome) located upstream of the CCV TK gene. The negative strand primer was 5'CGCCGTGATTGCTGTCGAA3' (3' nucleotide corresponds to position 9412 in the CCV genome) located downstream of the CCV TK gene.

Evaluation of the PCR products by electrophoresis on 4% polyacrylamide gel demonstrated a single band of 134 bp amplified from the CCVTK$^-$-infected cell extracts, whereas a 797-bp product was amplified from parent CCV. Sequencing of the CCVTK$^-$ PCR product by the *Taq* polymerase-mediated dideoxyribonucleotide chain termination method (dsDNA Cycler, BRL) using ^{32}P-5' end-labeled +8739 and −9492 primers showed that the 663 bp of the TK gene were deleted and the four new restriction enzyme recognition sites were inserted as predicted. [^{14}C]Thymidine-mediated plaque autoradiography using the TK$^-$ CCO cell line (CCOBr) (*16, 19*) demonstrated the lack of CCVTK$^-$-associated TK activity (Fig. 2). To determine if the TK deletion occurred in both terminal direct repeat regions of the CCV genome, the electrophoretic profile of *SalI*-digested CCVTK$^-$ DNA was compared to that of the parent on a 0.7% agarose gel. Then, CCV TK-specific Southern blot hybridization was performed using a random primed fluorescein-labeled PCR product generated from the region 8960 to 9229 of the CCV genome in a nonradiographic detection system (ECL, Amersham, Arlington Heights, IL), according to the manufacturer's instructions. The Southern transfer hybridization results demonstrated that the deletion occurred in both direct terminal repeat regions (Fig. 3).

The ability of the recombinant to replicate in cell culture was assessed using a one step growth curve on CCO cells. This demonstrated no detectable difference

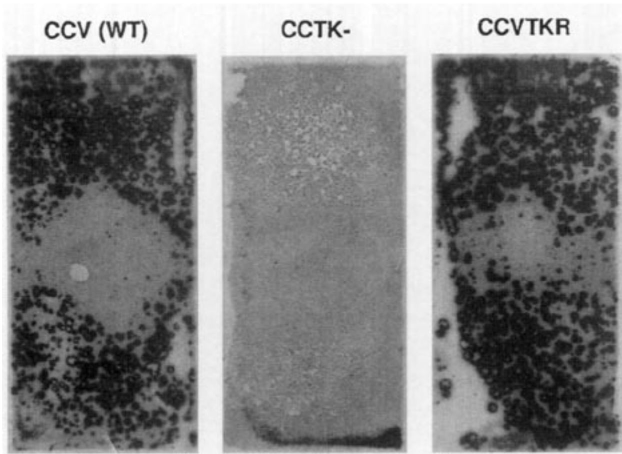

FIG. 2. Autoradiograph of CCV, CCVTK$^-$, and CCVTKR plaques produced in CCOBr cells in medium containing [^{14}C]thymidine.

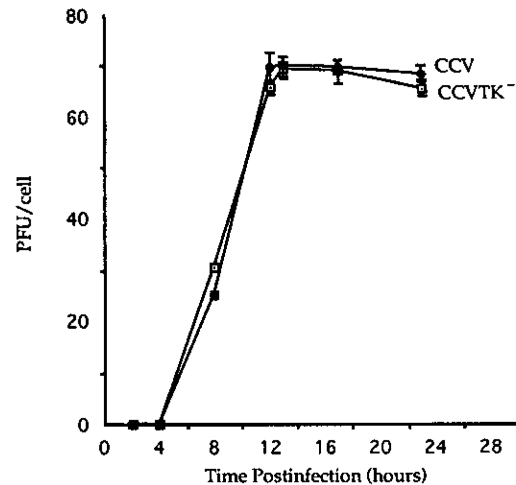

FIG. 4. One step growth curve of CCV and CCVTK$^-$ in CCO cells. Values are expressed in means ± standard deviation (SD). The respective virus was inoculated at 5 PFU/cell into wells of a 24-well plate containing cells at 80% confluency, allowed to adsorb for 1 hr at 29°. Then, the wells were rinsed three times with Hank's balanced salt solution, overlaid with 0.5 ml of cell culture medium, and incubated at 29°. Cells and medium were sampled every 4 hr, lysed by freeze–thawing, and virus was quantitated in the lysate using liquid overlay plaque assay on CCO monolayers (20).

between CCVTK$^-$ and the parent virus in either kinetics or progeny production ($P > 0.05$) (Fig. 4).

Immersion challenge-lethal dose trials on fingerling channel catfish were used to compare the virulence of CCV to CCVTK$^-$ to determine if the TK deletion was an attenuating mutation to the virus. Channel catfish (4–5 cm) were randomly distributed into 20 groups of 15 fish. Two groups were challenged with each of the following doses 3×10^7, 1×10^7, 3×10^6, 1×10^6, and 3×10^5 PFU of either CCVTK$^-$ or CCV in 400 ml of aerated water for 30 min at 30°. As a noninfected control, 15 fish were mock challenged using the DMEM viral diluent. Subsequently, the fish were transferred to 20-liter, aerated polypropylene tanks supplied with 30° dechlorinated municipal water. Fish were fed commercial aquarium flake food (Tetra Min) *ad libitum* once a day. Fish were observed twice daily and all dead fish were recorded, removed, and necropsied to confirm CCV disease as the cause of death. Both viruses were capable of causing lethal disease and there were no mortalities in the noninfected control groups. In two initial trials, the mortality ranges caused by both viruses did not span the 0 to 100% endpoints required for probit analysis statistical comparisons (data not shown). However, the number of fish killed by 3×10^5 PFU of the parent CCV was the same as that killed by 3×10^7 PFU of CCVTK$^-$. Therefore, a direct comparison of the two viruses was made using both doses, five replicate tanks per treatment. Data were transformed using the arcsine of the square root to account for nonnormal distribution of percentage values, evaluated by analysis of variance (ANOVA), and compared using the General Linear Models method (SAS software, SAS institute, Raleigh, NC). The results confirmed the earlier observation that 100-fold more CCVTK$^-$ was required to kill the same number of fish as the parent virus. The groups challenged with 3×10^7 PFU CCV

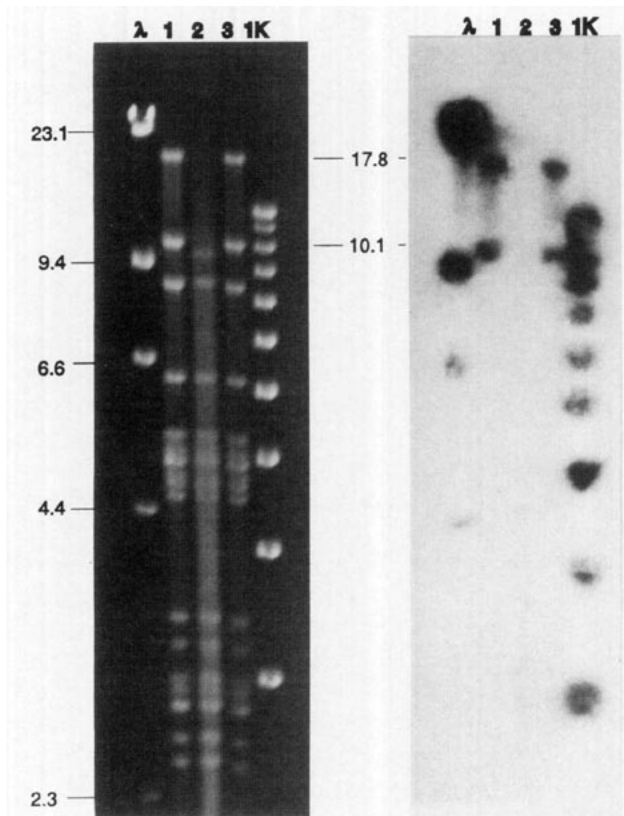

FIG. 3. *Sal*I restriction fragment electrophoretic profile of CCV (lane 1), CCVTK$^-$ (lane 2), and CCVTKR (lane 3) on a 0.7% agarose gel (left) and Southern blot hybridization of the same gel using a TK gene sequence specific probe (right). DNA size markers, *Hin*dIII-digested bacteriophage λ(λ), and a 1-kb ladder (1K) were detected in the Southern hybridization by adding marker-specific probes to the hybridization mixture.

experienced 100% cumulative mortality. This was significantly different ($P < 0.05$) from the mortality that occurred in the groups challenged with 3×10^6 PFU CCV and 3×10^7 PFU CCVTK$^-$, 44 ± 15 and 40 ± 7%, respectively. Likewise 9.3 ± 3.6% cumulative mortality in the groups challenged with 3×10^6 PFU CCVTK$^-$ was significantly different from the mortality experienced by the 3×10^6 PFU CCV and 3×10^7 PFU CCVTK$^-$ exposed groups. The mortality patterns were similar for both viruses. No deaths occurred in the noninfected control tanks during the experiment. Dead fish from CCV- and CCVTK$^-$-challenged groups demonstrated similar clinical signs, reddening of the fin bases, exophthalmia, ascites, and enlarged posterior kidneys. Histopathology on samples that were not autolytic demonstrated necrosis of hematopoetic cells in anterior and posterior kidneys and to a lesser extent renal tubules in the posterior kidney, and erythrophagocytosis was evident in the anterior kidney, posterior kidney, and spleen. No apparent differences were noted between the fish that died due to CCV or CCVTK$^-$ infections. Virus was cultured from the posterior kidney of all dead fish and produced CPE typical of CCV on CCO cells. Fish challenged with CCVTK$^-$ that were near terminal stages of the disease appeared to display less disorientated erratic swimming than those challenged with CCV; however, this difference was not quantified.

To determine if the attenuation of CCVTK$^-$ was associated with the TK$^-$ mutation and not caused by an inadvertent mutation that occurred during the generation or selection of the recombinant virus, a revertant TK positive CCV (CCVTKR) was generated by cotransfecting the CCVTK$^-$ genome with pBSCV446. The TK$^+$ progeny were selected and cloned using with 0.1 m*M* hypoxanthin, 0.4 m*M* aminopterin, and 16 m*M* thymidine (HAT) medium on infected CCOBr cells similar to procedures used on HSV-1 (*21*). The CCVTKR showed incorporation of [^{14}C]-thymidine in plaque autoradiography similar to CCV (Fig. 2) and demonstrated the requisition of the viral TK gene into both terminal repeat loci by Southern hybridization (Fig. 3). The pathogenicity of CCVTKR was compared to the parent CCV and CCVTK$^-$ in immersion challenge experiments as described above using fifteen 6- to 7-cm fingerlings in each of 35 polypropylene tanks. Five replica blocks of tanks containing seven randomly assigned experimental groups within each block were infected simultaneously. The experimental dosages were 1×10^7 PFU, 1×10^5 PFU of parent CCV, CCVTKR, or CCVTK$^-$, respectively or DMEM as a noninfected control group. The virulence of both the parent CCV and CCVTKR were the same as shown by both dosages. However, as was demonstrated earlier, 100-fold more CCVTK$^-$ was needed to kill the same number of fish as either CCV or CCVTKR (Fig. 5). No deaths occurred in the noninfected control tanks. This demonstrated that the TK deletion induced the attenuation property and that all detectable virulence lost in CCVTK$^-$ was regained by the revertant construct.

Attenuation may be an indication of a reduction in the ability of the virus to induce pathology in the host or a reduced ability to infect or replicate in the host. To determine if the attenuation observed in CCVTK$^-$ was due to reduced infectivity, we evaluated the kinetic properties of CCV and CCVTK$^-$ infections in immersion-exposed channel catfish fingerlings by quantifying virus levels in the primary target organ, the posterior kidney, over time. Five replicate groups of 20 fingerlings (5 cm mean length) were exposed to 3×10^5 PFU of either CCVTK$^-$ or wild-type CCV in 400 ml as described above. Three fish from each replicate were euthanitized by overdose immersion in tricane methane sulfonate at 0.5, 1, 2, 3, 4, 5, 7, and 9 days postinfection. Posterior kidney samples were removed, weighed, homogenized using a disposable plastic pestle (VWR Scientific, Sugarland, TX) in 200 μl cell culture medium, freeze–thawed three times to release cytoplasmic virus, and centrifuged at 8000 *g* for 10 min to remove cellular debris. The virus was quantitated in the supernatant by plaque assay on CCO cells. Both viruses infected the fish and replicated in the posterior kidney at the same rate through the period of peak infectious virus density which occurred on Day 4 postinfection (Fig. 6). However, the amount of CCVTK$^-$ in the posterior kidneys rapidly declined thereafter and was not detectable by Day 5 postinfection. In contrast, wild-type CCV could be detected through Day 9 postinfection. This indicated that the attenuation observed in CCVTK$^-$ was not due to a reduced ability to infect or replicate in the host overall but rather an altered ability of the TK$^-$ virus to induce lethal pathology. It is unlikely that the reduced pathogenicity was associated with the inability of CCVTK$^-$ to persist in the host because in the lethal dose trials, peak mortality occurred at 4 days postinfection at the time when peak virus concentrations occurred with

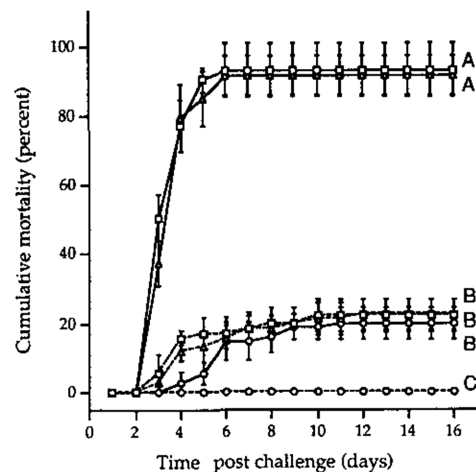

FIG. 5. Percentage cumulative mortality of fingerling catfish immersion challenged with CCVTKR(△), parent CCV(□), or CCVTK$^-$(O) at a dose of 1×10^7 PFU (solid line) or 1×10^5 PFU (dashed line) for 30 min. Values are expressed as means ± SD. Mortality is significantly different ($P < 0.05$) in groups labeled with different letters.

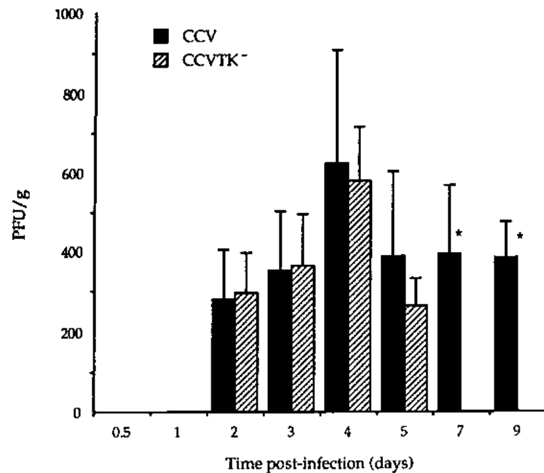

FIG. 6. Quantitation of infectious virus levels in the trunk kidney of channel catfish fingerlings after exposure to CCVTK⁻ or parent CCV. Values represent means ± SD (n = 9). An asterisk indicates that the values are significantly different at $P < 0.05$.

both virus types. Yet, the persistence of CCV may indirectly reflect an ability of the wild-type virus to replicate and cause damage in a more differentiated cell type, thus causing a more severe disease.

To evaluate the potential of using CCVTK⁻ as an attenuated-live CCV vaccine in channel catfish, experimental groups of 15 channel catfish fingerlings (5 cm mean length) were immersion exposed to 3×10^5, 1×10^5, 3×10^4, or 1×10^4 PFU of CCVTK⁻ or were immersed in DMEM as a negative control. Twenty-one days after CCVTK⁻ exposure the fish were challenged with wild-type CCV at 3×10^7 PFU/400 ml exposure. Dead fish were counted and necropsied as described above. Statistical comparison of mortality data were done as described in the virulence evaluations. CCVTK⁻ induced protective immunity to CCV in a dose dependent manner; i.e., the higher the CCVTK⁻ exposure, the lower the mortality of fish after challenge (Table 1). Even the lowest dose of CCVTK⁻ of 1×10^4 PFU/400 ml induced protective immunity that resulted in a 30% survival rate after challenge compared to 3% survival of the nonvaccinated control fish.

This research demonstrated that the TK gene of CCV could be used for positive and negative selection of recombinants similar to research with herpesviruses of higher vertebrates (*22, 23*). Although CCV is unique in that it contains two copies of the TK gene, one in both terminal direct repeat regions of the genome, the diploid nature of this gene presented no hindrance to recombinant virus construction. The PCR-mediated technique for precisely producing pBSCV457 was quick and relatively simple. To minimize the chances of unplanned mutations, we used the high fidelity Vent polymerase (*24*) in all PCR involved in the construction of the recombinant plasmid. The four unique restriction sites in pBSCV457 located immediately following the start codon of the TK gene will facilitate directional foreign gene insertion into the TK locus in future studies. The production of CCVTK⁻ reported herein demonstrated the utility and efficiency of this transfer plasmid in constructing recombinant CCV.

The demonstration that CCVTK⁻ is substantially less virulent than the parent CCV and the fact that nearly all of the pathogenic potential of the wild-type virus was regained in the revertant construct indicated that the loss of TK activity was the cause of attenuation. Nontheless, the loss of the production of an (as of yet undescribed) overlapping gene product could contribute to attenuation as has been described in HSV-1 constructs (*25*). However, it appears that CCV is similar to the other herpesviruses in the attenuating nature of TK⁻ mutations. The TK gene has been reported to play an important role in neuropathology of various α-herpesviruses (7, 11, 13). Although the direct cause of the reduced virulence is not known, TK may enhance the ability of CCV to replicate in differentiated nonreplicating cells as demonstrated in herpes simplex virus 1-infected neuron cells (*5*). Previous histopathological studies and virus kinetic studies do not support neuronal damage as an important mechanism of CCV pathogenesis (*26–29*). However, fish in terminal stages of CCV disease may exhibit signs of disorientation indicative of neurological damage (*1*). We observed that CCVTK⁻-infected fish at late stages of the disease only rarely displayed neurological clinical signs as compared to those infected with the wild-type CCV. The CCVTK⁻ and wild-type CCV reached similar viral progeny levels in the posterior kidney which is the primary replicative site of CCV (*26, 27*). Therefore, reduced virulence was not related to a reduced ability to infect or replicate during initial phases of the infection. Also, persistence in itself does not explain the pathogenic nature of the parent strain because peak mortalities generally occurred early in the progress of infection. A more likely explana-

TABLE 1

Protective Immunity in Channel Catfish to CCV Challenge 3 Weeks after Exposure to Various Levels of CCVTK⁻

Virus doses (PFU)[a]	Survivors (%)[b]	
3×10^5	100.0 ± 0.0[c]	A[d]
1×10^5	73.7 ± 5.0	B
3×10^4	60.0 ± 5.0	B
1×10^4	31.7 ± 3.3	C
Diluent	3.3 ± 3.8	D

[a] Channel catfish fingerlings (n = 4 tanks of 15 fish) were immersion vaccinated with the CCVTK⁻ at the designated dose for 30 min. Controls were immersed with the diluent (DMEM). At 21 days postinfection, they were challenged with 3×10^7 PFU parent CCV.

[b] Rates expressed as means ± SD.

[c] Dose (3×10^5) of CCVTK⁻ resulted in 4.9 ± 3.3% mortality. Percentage survival is calculated from the survivors of the CCVTK⁻ exposure.

[d] Percentage survival is significantly different ($P < 0.05$) in groups labeled with different letters.

tion is that the ability of the wild-type virus to replicate in differentiated cell types allowed it to be more pathogenic and more persistent. Our results are similar to the findings of Slater *et al.* (10) that demonstrated that a TK^- mutant of equine herpesvirus-1 replicated in the mice and foals at a rate similar to wild-type virus initially but replication did not persist. These parallel findings in genetically distant viruses and hosts indicate a possible common pathogenic function of TK in nonneurotropic herpesviruses. Detailed pathogenesis studies are underway which should help elucidate the mechanism involved in the attenuated nature of $CCVTK^-$.

The reduced virulence of the TK-deleted mutant and ability to protect against challenge with a lethal dose of the parent CCV demonstrates the potential use of this virus as a vaccine. However, because CCV is a problem in small channel catfish, the safety margin of $CCVTK^-$ must be evaluated in younger hosts before its true value as a vaccine agent can be assessed.

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