1990

Biochemical Characterization and Gene Mapping of the Channel Catfish Herpesvirus (CCV)-encoded Thymidine Kinase, a Selectable Site for Homologous Recombination.

Larry Allen Hanson
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Biochemical characterization and gene mapping of the channel catfish herpesvirus (CCV)-encoded thymidine kinase, a selectable site for homologous recombination

Hanson, Larry Allen, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1990
Biochemical Characterization and Gene Mapping of the Channel Catfish Herpesvirus (CCV)-Encoded Thymidine Kinase, a Selectable Site for Homologous Recombination

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences

by

Larry Allen Hanson
B.S., Auburn University, 1981
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May 18, 1990
Acknowledgements

The author expresses gratitude to Dr Konstantin G. Kousoulas for his advice in molecular virology and the use of his laboratory facilities, and to the author's wife, Lora, for typing this dissertation and providing support throughout this degree. The author thanks Dr Jack Nunberg (Cetus Corporation, Emeryville, CA) for performing polymerase chain reaction analyses using CCV-DNA. The Louisiana State University Alumni Federation Fellowship program made this innovative research possible by awarding the author a 4 year stipend independent of established research grants. Funding for equipment and supplies were largely provided by competitive intradepartmental grants. The support and freedom granted to the author, to pursue his research interests by Dr Ronald L. Thune is appreciated.
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Abstract

The channel catfish herpesvirus (CCV) encodes a thymidine kinase (Tk) which is biochemically distinguishable from it's host, the channel catfish ovary (CCO), and other herpesvirus Tk's. Tk deficient CCO cells (CCOBr) were developed by propagating CCO cells in increasing concentrations of 5-bromo-2'-deoxyuridine (B UdR). CCV infection produced high Tk activity in these Tk deficient cells. This activity was compared to CCO-Tk in ATP and CTP phosphate donor assays, nucleoside substrate-competition and dTTP feedback inhibition assays. The viral Tk was more competitively inhibited by deoxypurines than cellular Tk and showed less dTTP mediated feedback inhibition. CCO-Tk utilized lower concentrations of ATP more effectively than CCV-Tk. Neither CCV-Tk nor CCO-Tk utilized CTP as a phosphate donor. This is in contrast to other herpesvirus Tk's and indicates a divergence from mammalian herpesviruses. To map the CCV-Tk gene within the CCV genome, a Tk-negative mutant of CCV was isolated by passing the virus in the presence of B UdR and then selecting an isolate (CCVAR) that was resistant to 1 mM 1-β-D-
arabinofuranosylthymine (Ara-T). A CCV incomplete genomic library was constructed by cloning restriction endonuclease EcoRI digested viral DNA into plasmid pUC-19. A complete library was constructed into cosmid pHC-79 by cloning a partial EcoRI digest of viral DNA. Four cosmid clones were isolated that collectively encompassed 98% of the genome. The cosmid CCV-DNA clones and the pUC-19 subfragments were used in cationic-liposome mediated co-transfections with whole CCVAr-DNA in marker-rescue assays. CCVAr rescue, scored by $^{14}$CdT mediated plaque autoradiography on CC0Br cells, mapped the mutation within the direct repeat ends of the genome.
Introduction

Immunization has long been used as a disease control in human and veterinary medicine, but only recently has work been started on vaccines for aquatic animals. Presently the only vaccines registered for aquatic animals are for cold-water fish. This is because most cold water operations are intensive systems with readily accessible stocks. Work on the immunization of warm-water fish and other aquatic animals is needed to give aquaculturists another tool to combat disease problems and increase production.

Most warmwater aquaculture operations are extensive operations containing vast numbers of fish. Individual fish have a relatively low value and there is very little direct handling of the stocks. Therefore, any successful warm water vaccination program must use extensive vaccine delivery techniques such as immersion or oral application. Using these methods with killed antigen preparations can induce protective immune responses in fish, but require large volumes of antigen that are taken up in relatively low doses and persist in the target animal for a short period. The result is a relatively
weak and short lasting immune response. Live attenuated vaccines are effective immunogens because the attenuated pathogen enters the target animal via its natural invasion mode, resulting in efficient uptake. Subsequent replication of the agent results in antigen amplification and persistence, which induces a stronger, longer lasting response.

Live attenuated vaccines have not been used in aquaculture due to the potential of the agent to revert to a virulent form. Reversion occurs when the attenuating mutation is masked by a second mutation that makes the agent more like the wild type pathogen (Almond and Cann, 1984). Traditionally, attenuated viral vaccines have been developed by culturing the virus in cells from non-host species or under unnatural environmental conditions. This selects for mutations that make the pathogen less virulent. Most of these mutations are simple base changes which can revert. However, recent advances in genetic recombinant technology enable live vaccine agents to be constructed which can not revert. Pathogens can be permanently attenuated by deletion or insertion mutations in genes coding for virulence factors. These mutations cannot revert by simple base changes. In addition, the insertion and expression of foreign genes in these agents have led to a rapid expansion of live vaccine research. The techniques that were developed to produce recombinant, attenuated poxvirus and herpesvirus vaccine vectors for mammalian hosts, can be used to develop a safe
attenuated channel catfish virus vaccine. Furthermore, this engineered virus can be used as a vector to express other protective immunogens, providing a broad spectrum vaccine for the catfish industry.

Effective recombinant viral vaccine vectors require both a large genome which can accommodate foreign genes, and a selectable genetic site for the insertion of foreign genes. The viral thymidine kinase (Tk) gene has been effectively exploited as a selectable site of insertion for poxvirus and herpesvirus vectors (Shih et al., 1984, Mackett et al., 1984). Insertions or deletions of genetic material at the Tk gene locus inactivates the Tk gene and results in attenuation (Kit et al., 1985a, Kit et al., 1985b, Buller et al., 1985, Roizman et al., 1984).

Channel Catfish Virus (CCV) is a herpesvirus that causes disease (CCVD) in young channel catfish fingerlings. CCV can cause high mortality, decreased growth, and a predisposition of the catfish to bacterial diseases in heavily stocked fingerling ponds. Although large juvenile and adult fish are susceptible to CCV (Bowser et al., 1985; Hedrick et al., 1987), CCVD outbreaks are rare in these populations (Plumb, 1978; McGlamery and Gratzek, 1974). The CCV genome is large (130,000 base pairs), suggesting that it can accommodate foreign gene insertion. Isolating the CCV Tk gene is the first step in constructing a permanently attenuated, Tk negative CCV for initial use as a vaccine. Once developed,
the attenuated CCV could be used as a vector for the engineering of polyvalent live vaccines against other catfish diseases. Accordingly, the purpose of this project was to determine if CCV induces Tk activity in the infected cell, to distinguish the viral Tk from the cellular Tk, and to locate the Tk gene on the genome. In the process, methods for producing recombinant CCV was established which will allow the production of Tk negative recombinant CCV for vaccine research.
Literature Cited


The channel catfish virus

The channel catfish virus (ictalurid herpesvirus 1) is an α herpesvirus that can cause significant disease (CCVD) in channel catfish (Ictalurus punctatus) fry and young fingerlings (Fijan, 1968; Fijan et al., 1970; Wolf and Darlington, 1971). The acute form of CCVD can decimate a heavily stocked pond of small fingerlings or fry (up to 10cm) in 7 days. However, the host range of CCV is very limited. The channel catfish is the only known natural host of CCV. Fingerling blue catfish (I. furcatus) and channel x blue catfish hybrids can be experimentally infected by intraperitoneal injection but are resistant to oral and immersion exposures of CCV (Plumb and Chappell, 1978). Brown bullheads (I. nebulosis), yellow bullheads (I. natalis), white catfish (I. catus) (Plumb, 1978), and European sheatfish (Silurus glanis) (Plumb et al., 1985) are resistant to oral
and immersion exposures and intraperitoneal injections of CCV. Disease is characterized by a viremia, with highest replication occurring in the head and trunk kidneys and liver. Late in the infection titers increase in the intestine and brain (Plumb, 1971). Clinical signs include exophthalmia, ascites and neurological disorders that induce corkscrew swimming. Survivors are considered to be carriers for life as indicated by DNA-DNA hybridization studies (Wise and Boyle, 1985), and reactivation of virus from leukocytes of immunosuppressed adult fish (Bowser et al., 1985). Evidence strongly suggests vertical transmission of the disease from the carriers. Serum neutralization titers from stocks of adult channel catfish that produced CCV diseased offspring for two consecutive years were 10 to $10^5$ fold higher than control fish (Plumb, 1973b). Also, immunoflourescence analysis of primary cultures from spent ovarian tissue of suspected CCV carriers showed the expression of CCV antigens (Plumb et al., 1981), suggesting possible transmission of CCV in ovarian tissue. Experimental evidence indicates CCVD survivors have reduced growth rates. Fingerlings 2 g in size were intraperitoneally challenged with CCV and survivors had reduced growth rates (McGlamery and Gratzek, 1974). Larger unexposed juveniles and adults are susceptible to experimental infections and can develop an acute form of disease (Bowser et al., 1985; Hedrick et al., 1987) but natural epizootics in large fish are rare (McGlamery and Gratzek, 1974; Plumb,
Recent evidence suggests that CCV may infect many fish populations without any overt signs of disease (Wise et al., 1985). DNA-DNA hybridization data indicate many catfish populations carry the virus even though they have no history of CCVD. The expression of overt disease may be dependent on high stocking density, predisposing environmental stress (McConnell and Austen, 1978; Plumb, 1978) and strain susceptibility. Plumb et al. (1975) showed that CCVD mortality in different strains of channel catfish after oral CCV exposure ranged from 10 to 71% with hybrid strains showing the lowest mortality. Another factor that may affect disease expression is the environmental temperature at the time of exposure. Plumb (1973a) showed that at 19 C, CCV produced much lower mortalities in fingerlings than at 28 C.

Catfish develop a strong immune response to CCV when exposed to live virus. A patented, live, attenuated strain of CCV (Noga and Hartman, 1981) was produced by passage through walking catfish (Clarius batrachus) cells, Walczache et al. (1981) showed significant protection in fry. Awad (1988) found that eggs and 1 week old fry were immunized for CCVD after exposure to a soluble envelope preparation from CCV. However, the characteristics of the protective immunity have not been studied. Much research has been focused on neutralizing antibody activity in survivors of CCVD, with variable results. Fingerlings that have survived outbreaks of CCV may or may not produce detectable neutralizing
antibodies (Amend and McDowell, 1983). Some adult survivors retain detectable neutralizing antibodies years after the productive infection (Plumb, 1973). Some of the variability in production of neutralizing antibodies can be caused by differences in the ambient temperature, which affect the fish's immune response (Hedrick et al., 1987). Although neutralizing antibodies are probably a minor portion of the fish's protective immune response to CCV, high titers are an indicator of antigen expression from latent infections. Other important portions of the antiviral immune response that have not been addressed are cytotoxic T lymphocyte responses and non-neutralizing antibodies that play a role in antibody dependent cell cytotoxicity and complement mediated lysis.

The host specificity of CCV is maintained in cultured cells. CCV only replicates in cell lines derived from catfish, with the highest titers produced in two Ictalurid cell lines. Brown bullhead cells (BB) produce up to $10^{7.5}$ PFU/ml (Wolf and Darlington, 1971) and channel catfish ovary cells (CCO) produce up to $10^8$ PFU/ml (Bowser and Plumb, 1980), while walking catfish (Clarius batrachus) cells (K1K) produce up to $10^{5.5}$ PFU/ml (Walczak et al., 1981). The virus replicates at temperatures ranging from 10 to 33 C in cell culture, with highest titers at 30 C in CCO cells (Bowser and Plumb, 1981). CCV induces cytopathic effect (CPE) characterized by syncytia that contract to clumps with radiating spindles.
The replication cycle from adsorption to first infectious progeny takes 4 hours at 30 °C and as typical of α herpesviruses, temporal regulation of protein synthesis and a shutdown of host macromolecule synthesis occurs (Dixon and Farber, 1980). Denaturing polyacrylamide gel electrophoresis of infected cell extracts has been used to identify at least 32 unique viral induced proteins. These proteins were expressed in three temporal groups and kinetic analyses indicate that their synthesis is coordinately regulated (Dixon and Farber, 1980). The virion contains 18 of these polypeptides. Non-denaturing electrophoretic analysis of infected cell lysates (Huston, 1979), and susceptibility to viral thymidine kinase activated antiherpetic agents (Buck and Loh, 1985) indicate this herpesvirus codes for a thymidine kinase like other α herpesviruses.

The physical characteristics of the CCV genomic DNA have been well characterized. Buoyant density analyses indicate the overall base composition is 56% G+C (Goodheart and Pummer, 1975). Chousterman et al. (1979) used restriction enzyme mapping to show the genome is 86 x 10⁶ daltons composed of a 62 x 10⁶ dalton unique sequence flanked by 12 x 10⁶ dalton direct repeats. These results were supported by Dixon and Farber (1980) who analyzed the genome by electron microscopy, sucrose gradient centrifugation and reassociation kinetics. They also found the virus genome contained alkaline labile sites similar to other herpesviruses. A comparison of
intracellular and extracellular viral DNA restriction-enzyme-fragment electrophoretic patterns indicates that the intracellular replicative DNA does not contain the terminal restriction fragments. Instead, it contains a new fragment the size of a product produced if the ends of the virion DNA molecules were fused (Cebrian et al., 1983). The size of the new fragment requires the loss of one of the terminally repeated sequences. This "endless" form of replicative DNA is a typical form of herpes viral DNA and could be caused by a circularization of the viral DNA followed by a rolling circle mechanism of replication that produces concatamers that are cleaved and packaged into virions. Sequence analysis of portions of the 18 kb direct repeats show the following: The right end of the genome has a 3' single base overhang and the left end is blunt. There are sequences with homology to putative herpesviruses packaging sequences within 50 bp of each end. The repeat region contains an open reading frame for a 230 amino acid polypeptide with sequence conservation to herpesvirus protein kinases (Lacosa et al., 1989).

**Viral Vectors**

Much of the early work in molecular virology was performed on small DNA viruses, especially the Polyoma SV-40 group (Sambrook et al., 1973, Sebrung et al., 1971). This basic research focused on genome structure, gene regulation
and the function of gene products in infected cells, as well as the structure of the genome. Early viral vector research concentrated on the small DNA viruses (SV-40 and polyoma) and retroviruses (reviewed by Rigby, 1982). These viruses were used as eukaryotic expression vectors since no natural plasmids occur in higher eukaryotes. This research has been important in identifying useful promoter, enhancer and origin of replication sequences which led to the development of very efficient shuttle vectors. The shuttle vector carries DNA sequences that enable it to replicate in prokaryotic and eukaryotic cells. Thus, it can be manipulated in vitro, amplified in a prokaryotic host, then transferred to a eukaryotic host for expression of cloned eukaryotic genes. The vector can then be transferred back to a prokaryotic host for further manipulations. The basic components of shuttle vectors consist of a prokaryotic origin of replication and a selection system (generally derived from bacterial plasmids or bacteriophage), gene regulatory elements and an origin of replication (generally derived from viruses which infect eukaryotic cells). By taking advantage of the characteristics of each viral system, highly specialized plasmid or viral shuttle vectors have been established for transient high level expression, stable constitutive expression or stable inducible expression in a variety of cell types. Retroviral and adeno-associated viral shuttle vectors have been especially useful for inserting small sequences of cloned DNA into cell
genomes.

The current system of choice for producing large amounts of eukaryotic proteins is the insect baculovirus system (Emery and Bishop, 1987; Luckow and Summers, 1988). Baculoviruses are large DNA viruses that produce large intranuclear proteinaceous occlusion bodies for protecting the progeny virions from adverse environmental conditions. The occlusion body protein constitutes 1 mg/ml per 10^6 cells of infected cell cultures and is non-essential. By replacing the occlusion body (polyhedrin) gene of Autographa californica nuclear polyhedrosis virus (AcMNPV) with the desired foreign gene, the foreign gene is expressed in copious amounts. Research from mosquito cell lines indicate that the post-translational glycosylation in insect cells includes only the simple manose rich "backbone" of the complex glycosylation found in vertebrates (Buttrose and Hughes, 1981). Yet, the recombinant mammalian cell products expressed from baculovirus systems are functionally active.

Vaccine Vectors

In the early 1980's, researchers began to use recombinant techniques on large DNA viruses to study gene and protein functions (Knipe et al., 1978; Moss et al., 1981; Weir et al., 1982; Nakano et al., 1982; Sam and Dumbell, 1981). It became apparent that members of the poxvirus and herpesvirus families were uniquely suited to express large
amounts of genetic information. This property is extremely useful for live virus vaccine vectors, because the genes encoding protective antigens from several pathogens could be expressed in one vector inducing protective immunity to each pathogen. Initial research was focused on the vaccinia virus of the poxviridae and herpes simplex virus (HSV) of the herpesviridae. These viruses were the most characterized of their respective families allowing for rapid progress and refinement of techniques. Manipulation of genetic sequences of these viruses requires the in-vitro manipulation and cloning of a viral genomic fragment into E. coli vectors using molecular cloning techniques, followed by the transfection of this fragment into a live cell containing the replicating viral DNA. During the replication process, homologous recombination may occur between the viral DNA and the unaltered viral sequences flanking the altered DNA in the cloned fragment, allowing the insertion of the manipulated sequence into a complete viral genome. The most efficient recombination systems involve insertion into a selectable marker so recombinants can be quickly identified and characterized.

The earliest and most commonly used selectable marker for insertion is the viral thymidine kinase (Tk) gene (Mocarski et al., 1980; Post et al., 1982; Mackett et al., 1982; Smith et al., 1983). In this system the foreign gene is inserted into the Tk gene giving the recombinant virus a Tk
negative phenotype. The Tk negative recombinants can then be selected based on their resistance to Tk-activated, toxic thymidine analogs such as 5-bromo-2'-deoxyuridine (B UdR). Other recombination systems use non-essential glycoprotein genes in conjunction with black plaque selection (Saur et al., 1987) or inserted reporter genes, such as E. coli lac Z gene with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) staining (Spaete and Mocarski, 1987; Panicali et al., 1986) as selectable sites of insertion. These systems use visual selection to identify the glycoprotein or lac Z negative recombinants. An alternative method for selecting recombinant viruses is to include a dominant selectable gene as part of the insertion sequence, such as host range genes into a host range mutant virus (Perkus et al., 1989), neomycin resistance gene (Franke et al., 1985) in conjunction with Genticin 148 selection and E. coli lac Z gene with X-gal color selection (Chakrabarti et al., 1985).

Vaccinia virus has been the vector of choice for recombinant live virus vaccine research for several reasons: 1. it has been well established as an effective agent in the eradication of smallpox (WHO, 1980) 2. it generally causes only a localized epidermal infection 3. the virus is resistant to desiccation making it a good vaccine agent for third world countries where storage problems are common 4. it does not establish a latent infection 5. it is non-oncogenic 6. it has a large stable genome that can
accommodate as much as 25,000 bases of foreign sequences (Smith and Moss, 1983). It has a broad host range, making it a candidate for human and veterinary vaccines as well as providing non-primate animal models for vaccine research.

The ability of poxviruses to retain their infectivity in the environment for prolonged periods and the broad host range of vaccinia have hampered field use of recombinant agents due to safety concerns. The broad host range also increases the threat to man and thus limits the use of vaccinia based veterinary vaccines. In rare instances vaccinia can cause serious complications especially in very young and immunocompromised individuals (Redfield et al., 1987; Lane et al., 1969). Researchers have addressed these problems by developing vaccine vectors for veterinary use from more species specific poxviruses (Esposito et al., 1988; Boyle and Coupar, 1986) and by developing less virulent forms of vaccinia (Buller et al., 1985; Flexmer et al., 1987; Ramshaw et al., 1987).

Research into increasing the efficiency of vaccinia virus as a vaccine vector includes increasing the levels of foreign gene expression and increasing the immunogenicity of the products. Fuerst et al. (1987) obtained high level foreign gene expression using a bacteriophage T7-vaccinia hybrid. This virus codes for a T7 RNA polymerase and contains the foreign gene just below a T7 promoter. The result is an extremely high level of foreign gene mRNA production and
concurrent high level protein expression. Langford et al. (1986) and Vijaya et al. (1988) have increased the immunogenicity of secreted proteins by attaching a transmembrane anchor sequence to the gene.

Because of human risk and the ability to establish latency, HSV vaccine vectors have met with more skepticism than vaccinia vectors. The focus on HSV based vaccine vectors has been to establish a safe live vaccine to HSV-1 and HSV-2 infections as well as immunize humans against other important pathogens. Much of this research has been concerned with making HSV vectors safe for humans (Roizman et al., 1984; Meigner et al., 1988). By inactivating non-essential viral genes and subsequently assessing the effects on virulence in rodents and lower primates, novel, less virulent vectors which have been created (Longnecker et al., 1988). The recombination techniques developed by Knipe et al., (1978) allowed the pioneering of Tk based deletion and insertion systems (Mocarski et al., 1980; Post et al., 1981; Post et al., 1982). Early investigations showed that spliced as well as unspliced eukaryotic genes could be efficiently expressed from HSV vectors (Post et al., 1982). This lead to the production of recombinant vectors expressing protective antigens from other pathogens for vaccine purposes (Shih et al., 1984).

The knowledge gained from initial recombinant research has been used in the development of HSV based episomal genetic
elements for gene therapy (Palella et al., 1989; Geller and Breakefield, 1988). HSV based gene therapy is especially attractive for treatments of genetic nervous disorders such as Lesch-Nyhan syndrome which is caused by a rare mutation in which the neurons lack dehydrofolate reductase expression. The HSV vectors are large enough to accept eukaryotic genes containing all of the control elements and splice sequences. Also, HSV readily infects and establishes latency in neurons. Therefore, the overall goal is to construct replication deficient HSV vectors that can infect and establish latency as a stable episomal element expressing foreign genes in a cell-controlled, regulated fashion.

The techniques developed for HSV research have also been applied to other herpesviruses, leading to the development of several unique vector systems. The Epstein-Barr virus (EBV) has been genetically dissected and important control elements have been used to create efficient eukaryotic plasmids with wide host ranges (Yates et al., 1985; Jalanko et al., 1988; Young et al., 1988; Hammerschmidt and Sugden, 1988). Because the EBV derived plasmids are stable in cell culture and can express inserted cDNA, they have been used as efficient shuttle vectors for screening eukaryotic cDNA libraries (Margolskee et al., 1988). In this system, positive cells are selected by fluorescent activated cell sorting and cultured to increase the number of positive cells. The plasmids are then extracted from the cells and transferred into E. coli
where they are amplified for further analyses.

Recent cytomegalovirus (CMV) research indicates that this virus has great potential as an expression vector. As much as 1% of the total cellular protein has been expressed from foreign genes under the control of B 2.7 promoter in the CMV genome (Spaete and Mocarski, 1987). Because the CMV does not contain a Tk gene, an inserted E. coli lac Z gene was used as a selectable marker. The CMV used in this research was the Towne strain which is attenuated and appears to be incapable of establishing latency in human trials. Therefore, the CMV recombinants are candidates for human vaccine vectors.

Another non-pathogenic human strain of herpesvirus with potential as a vaccine vector is the Oka strain of the varicella-zoster virus (VZV). This live virus vaccine strain has been shown to be safe even in immunocompromised patients. Therefore, Lowe et al. (1987) developed a Tk based recombination system for this virus with an initial goal of establishing a combination VZV-EBV vaccine.

A non-oncogenic deletion construct (Desrosier et al., 1985a) of the T lymphotrophic γ herpesvirus, herpesvirus saimiri, has been used to express foreign genes in primates (Desrosiers et al., 1985b). Because this construct is non-pathogenic and readily establishes latency in T lymphocytes, researchers are using this vector system for gene therapy and immunological studies (Clark, 1987).

The pseudorabies virus (PRV) has been the most studied
herpesvirus candidate for a veterinary vaccine vector. Two selectable sites for homologous recombination have been identified in PRV: the Tk locus and the glycoprotein X(gX) locus. Thymidine kinase negative, bioengineered PRV are highly attenuated and effective vaccines for pigs (Kit et al., 1985a). The use of the gX insertion in addition to the Tk gene site allows the production of a polyvalent vector and because the vaccine strain would be gX negative, rapid humoral screening could be used to determine if stocks of pigs have been exposed to the wild type virus (Thomsen et al., 1987a; Marchioli et al. 1987; Thomsen et al., 1987b). Furthermore, rearranging the location of an essential gene into the Tk locus should prevent any genomic recombination with a wild type virus that could make the vector Tk positive (Post and Thomsen, 1988). One unique tool developed by Sauer et al., (1987) is a bacteriophage P-1 Cre-lox mediated, site specific insertion system for accurately inserting foreign DNA sequences into viral DNA in vitro. Another alternative to in vivo insertion of foreign DNA is the manipulation of cloned fragments in vitro, then the production of live virus by transfecting overlapping cosmid cloned genomic fragments (van Zijl et al., 1988).

Other herpesvirus vaccine vector candidates being developed for veterinary use are bovine herpesvirus type 1 (BHV-1) (Kit and Kit, 1986), bovine herpesvirus type-2 (BHV-2) (Sheppard and May, 1989), and feline herpesvirus (FHV)
(Nunberg et al., 1989). All of these vector systems are based on using the Tk gene as a selectable site of recombination and as a deletion site for attenuation.

**Thymidine Kinase**

By coding for a large portion of the metabolic enzymes needed for replication, herpesviruses are less dependent on host cell functions and can productively infect non-replicating, differentiated cells such as neurons. Many of the non-structural genes that herpesviruses encode are enzymes that are important in nucleotide metabolism and DNA replication. Herpesviruses have been found to encode a DNA polymerase (Weissbach et al., 1973; Boezi et al., 1974), exonuclease (Preston and Cordingly, 1982), uracil DNA glycosylase (Carradonna et al., 1987), deoxyuridine triphosphatase (Preston and Fisher, 1984), dihydrofolate reductase (Trimble et al., 1988), ribonucleotide reductase (Ponce de Leon, 1977; Dutia, 1983), thymidine kinase (Dubbs and Kitt, 1964) and thymidylate synthase (Honess et al., 1986; Bodemer et al., 1986). Thymidine kinase is the most studied of these enzymes because herpesvirus Tk plays a central role in activating several herpesvirus specific antiviral agents such as 9-(2-hydroxyethoxymethyl)-guanine, (acyclovir) (Elion et al., 1977), 1-β-D arabinofuranosylthymine, (Ara-T) (Aswell et al., 1977) and 5-bromodeoxycytosine (Doberson and Greer, 1978).
Tk is a pivotal enzyme in the salvage pathway for the synthesis of deoxythymidine triphosphate (dTTP), a necessary precursor for DNA synthesis. Tk phosphorylates deoxythymidine (dT) to deoxythymidine monophosphate (dTMP) which is further phosphorylated to dTTP by thymidylate kinase. Eukaryotic cells contain two Tk isozymes, a cytoplasmic Tk and a mitochondrial Tk (Kit et al., 1973; Lee and Cheng, 1976). Cells contain a thymidine transport mechanism on the cell membrane (Plageman et al., 1988) to transport the hydrophilic thymidine molecule into the cell. Herpesvirus infected cells have enhanced uptake of nucleosides via viral induced diffusion mechanism, possibly due to increased "leakiness" of the cell membrane (Palu et al., 1990).

Of seventeen herpesviruses analyzed, all but cytomegalovirus and herpesvirus aotus code encode a Tk (Table 1). Generally, herpesvirus encoded thymidine kinases are less substrate specific and less regulated than the cellular counterparts (Table 1). The cytoplasmic Tk of eukaryotic cells are strongly inhibited by dTTP, do not phosphorylate dC or Ara-T and cannot use CTP as a phosphate donor. Herpesvirus Tk's are not strongly inhibited by dTTP, they phosphorylate Ara-T and can use CTP as a phosphate donor. Some herpesvirus Tk's can phosphorylate dC, others cannot. The mitochondrial Tk's are more similar to herpesvirus Tk in substrate specificity. They are less inhibited by dTTP, phosphorylate dC and Ara-T and can utilize CTP as a phosphate donor.
However, mitochondrial Tk's are strongly inhibited by dCTP.

The low substrate specificity of herpesvirus Tk in conjunction with the increased uptake of nucleosides imparts the herpesvirus specificity of the nucleoside analog antiviral agents such as acyclovir, 5-halogenated dC and Ara-T. Other large DNA viruses including Poxviridae (Esposito et al., 1988; Boyle and Coupar, 1986; Barbanti-Brodano et al., 1968; Kit et al, 1975) and Iridoviridae (Scholz et al., 1988; Aubertine and Longchamps, 1974) also code for viral thymidine kinases. Poxvirus Tk is more similar to cytoplasmic Tk in substrate specificity (Kit et al., 1975).

In the early 1980's there was a surge of interest in viral thymidine kinase isozymes for use as selectable sites of homologous recombination. This enzyme is not essential for viral replication and Tk negative mutants are resistant to Tk activated, antiviral agents. Therefore, the use of the Tk gene as a site of foreign gene insertion allows rapid selection of recombinants due to their Tk-negative, antiviral-resistant phenotype. In addition, Tk negative mutants were found to be less pathogenic than the wild type parent virus. This gave impetus to the development of Tk deletion and Tk based recombination systems for viral vaccine vectors. To use the Tk gene for recombinant experiments the gene must first be located and cloned. Thirteen herpesvirus Tk genes have been cloned and sequenced. When amino acid sequences of herpesvirus Tk's are compared, only three small
regions of conservation can be located. Only one of these regions show homology to the cytoplasmic and poxvirus Tk sequences (Figure 1). The amino acid sequences of the Tk's have been valuable references in herpesvirus evolutionary comparisons. These comparisons have led to the re-evaluation of herpesvirus groupings. For example, the turkey herpesvirus and Marek's disease virus, once thought to be γ herpesviruses due to their lymphocyte specificity, are now considered more related to α herpesviruses (Honess et al., 1989).
Table 1. Characteristics of Thymidine kinase isozymes.

<table>
<thead>
<tr>
<th>virus</th>
<th>size (daltons)</th>
<th>inhibition</th>
<th>substrate</th>
<th>P donor</th>
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<tbody>
<tr>
<td></td>
<td>monomer</td>
<td>active</td>
<td>dTTP</td>
<td>P acceptor</td>
</tr>
<tr>
<td>HSV-1,</td>
<td>41,000</td>
<td>low</td>
<td>low</td>
<td>+</td>
</tr>
<tr>
<td>HSV-2,</td>
<td>41,000</td>
<td>low</td>
<td>low</td>
<td>+</td>
</tr>
<tr>
<td>VZV,</td>
<td>35,000</td>
<td>low</td>
<td>low</td>
<td>+</td>
</tr>
<tr>
<td>EHV-1,</td>
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<td>low</td>
<td>low</td>
<td>+</td>
</tr>
<tr>
<td>EHV-3,</td>
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<td>low</td>
<td>+</td>
</tr>
<tr>
<td>BHV-1,</td>
<td>85,000</td>
<td>low</td>
<td>low</td>
<td>+</td>
</tr>
<tr>
<td>HVT,</td>
<td>40,000</td>
<td>low</td>
<td>low</td>
<td>-</td>
</tr>
<tr>
<td>IILT viene</td>
<td>80,000</td>
<td>low</td>
<td>low</td>
<td>-</td>
</tr>
<tr>
<td>Mar HV,</td>
<td>80,000</td>
<td>low</td>
<td>low</td>
<td>-</td>
</tr>
<tr>
<td>HVS,</td>
<td>90,000</td>
<td>high</td>
<td>low</td>
<td>-</td>
</tr>
<tr>
<td>PRV,</td>
<td>90,000</td>
<td>high</td>
<td>low</td>
<td>-</td>
</tr>
<tr>
<td>MDV,</td>
<td>90,000</td>
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<td>low</td>
<td>-</td>
</tr>
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<td>FHV,</td>
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</tr>
<tr>
<td>BHVB,</td>
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<td>high</td>
<td>low</td>
<td>-</td>
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<tr>
<td>CMV,</td>
<td>no thymidine kinase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HV Aotus,</td>
<td>no thymidine kinase</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Human C,</td>
<td>90,000</td>
<td>high</td>
<td>low</td>
<td>-</td>
</tr>
<tr>
<td>Mouse C,</td>
<td>mod</td>
<td>high</td>
<td>high</td>
<td>+</td>
</tr>
</tbody>
</table>

1Jamieson and Subak-Sharpe, 1974. 2Doberson et al., 1976. 3Shiraki et al., 1985.
4Turenne-Tessier et al., 1986. 5Allen et al., 1979. 6McGowan et al., 1980.
7Weinmaster et al., 1982. 8Kit et al., 1975. 9Otsuka and Kit, 1984. 10Hones et al., 1982.
### Herpesviridae

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Amino Acid Sequence</th>
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</thead>
<tbody>
<tr>
<td>HV Consensus</td>
<td>dG--G--GKtt<strong><strong><strong><strong><strong>EPM-YWr</strong></strong></strong></strong></strong>DRHp</td>
</tr>
<tr>
<td>HSV-1</td>
<td>DGphGmGKTT<strong><strong><strong><strong><strong>EPMtYWq</strong></strong></strong></strong></strong>DRHP</td>
</tr>
<tr>
<td>HSV-2</td>
<td>DGphGvGKTT<strong><strong><strong><strong><strong>EPMtYWq</strong></strong></strong></strong></strong>DRHP</td>
</tr>
<tr>
<td>Mar HV3</td>
<td>DGphGvGKsT<strong><strong><strong><strong><strong>EPMaYWt</strong></strong></strong></strong></strong>DRHa</td>
</tr>
<tr>
<td>BHV-24</td>
<td>DGphG1GKTT<strong><strong><strong><strong><strong>EPMsYWt</strong></strong></strong></strong></strong>DRHP</td>
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<tr>
<td>VZV</td>
<td>DGayG1GKTT<strong><strong><strong><strong><strong>EPMsYWt</strong></strong></strong></strong></strong>DRHP</td>
</tr>
<tr>
<td>PRV7</td>
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<tr>
<td>EHV-19</td>
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</tr>
<tr>
<td>MDV10</td>
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<tr>
<td>HVT10</td>
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</tr>
<tr>
<td>EBV11</td>
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</tr>
<tr>
<td>HSV12</td>
<td>eGsiGvGKTT<strong><strong><strong><strong><strong>EPMaYWt</strong></strong></strong></strong></strong>DRHP</td>
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### Poxviridae

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>vaccinia</td>
<td>iGpmfsGKsT</td>
</tr>
<tr>
<td>FPV14</td>
<td>tGpmfsGKsT</td>
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<tr>
<td>SFV15</td>
<td>iGpmfaGKsT</td>
</tr>
<tr>
<td>variola16</td>
<td>iGpmfsGKsT</td>
</tr>
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</table>

### Vertebrate cytoplasmic

<table>
<thead>
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<th>Vertebrate</th>
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<tbody>
<tr>
<td>human17</td>
<td>lGpmfsGKsT</td>
</tr>
<tr>
<td>chicken18</td>
<td>fGpmfsGKsT</td>
</tr>
<tr>
<td>hamster19</td>
<td>lGpmfsGKsT</td>
</tr>
</tbody>
</table>

Figure 1. Amino acid sequence homologies among thymidine kinases.

Literature Cited


Chapter Two

Identification and Characterization of a Channel Catfish Herpesvirus (CCV) Encoded Thymidine Kinase

Abstract

This investigation demonstrated that channel catfish virus (CCV) encodes a thymidine kinase (Tk) which is biochemically distinguishable from the Tk of the host cell line, channel catfish ovary (CCO), as well as other herpesvirus Tk's. A Tk deficient CCO cell line (CCOBr) was developed by propagation in increasing concentrations of 5-bromo-2'-deoxyuridine. CCV infection of the Tk deficient cells produced high Tk activity. CCV was found to be sensitive to the Tk activated antiherpetic agent, 1-β-D arabinofuranosylthymine (Ara-T). A mutant of CCV (CCVAr), isolated by serial passage in BUdR followed by Ara-T, was found to be resistant to Ara-T and Tk negative. Extracts of CCO and Tk negative cells infected with CCV were assayed for differences in competitive inhibition, thymidine triphosphate (dTTP) mediated feedback inhibition and phosphate donor specificity. CCV-Tk was more competitively inhibited by deoxypurines than CCO-Tk and showed less dTTP mediated feedback inhibition. CCV-Tk was unique among herpesvirus Tk's in its inability to utilize CTP as a phosphate donor.
Introduction

Thymidine kinase (Tk) phosphorylates thymidine (dT) into thymidine monophosphate (dTMP). This reaction is part of the salvage pathway for producing thymidine triphosphate (dTTP), a necessary precursor for DNA synthesis. The Tk gene is a normal component of cells and many large DNA viruses, yet it is not essential under normal culture conditions because the cell can rely on de-novo dTTP synthesis, where dTTP is produced by methylation of deoxyuridine monophosphate. As a result the Tk gene can be used as a doubly selectable marker for genetic recombination experiments. If supplied dT, Tk positive cells and viruses can grow in the presence of aminopterin, an inhibitor of de-novo dTTP synthesis. On the other hand, Tk negative mutants can be selected by their resistance to toxic thymidine analogs, such as 5-bromo-2'-deoxyuridine (BUDR), which are activated by Tk mediated phosphorylation. In addition, herpesvirus isozymes are often less specific than cellular Tk, resulting in the selective antitherpetic action of nucleoside based antiviral agents such as 1-β-D arabinofuranosythymine (Ara-T) and 9-(2-hydroxyethoxymethyl) guanine (acyclovir). These agents are first phosphorylated by the viral Tk and then cellular enzymes phosphorylate the monophosphate compounds to triphosphate compounds that interfere with viral DNA
Tk based selection has greatly facilitated the molecular dissection of mammalian herpesviruses. Herpesvirus Tk genes have been used as reporter genes to analyze promoter elements (Pater and Pater, 1984). Also, Tk genes have been used in homologous recombination experiments as selectable sites for the insertion of foreign genes (Mocarski et al., 1980) and as selectable insertion sequences to interrupt other viral genes (Post and Roizman, 1981).

Previous work by others (Buck and Loh, 1985; Huston, 1979) indicated that CCV may encode its own Tk. The purpose of this investigation was to confirm the presence of a CCV encoded Tk, clearly distinguish it from cellular Tk and to develop Tk deficient mutants of CCV and the host cell for future marker-rescue based Tk gene mapping.

Materials and Methods

CCO cells were routinely grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 25 mM HEPES buffer and 50 μg/ml gentamycin or 100 IU/ml penicillin and 100 μg/ml streptomycin. The mutation and selection of Tk deficient CCO cells was done by passage in medium containing increasing levels of BUdR: 5 mg/l for 23 passes, 50 mg/l for 21 passes, 150 mg/l BUdR for 7 passes, 250 mg/l for 2 passes and 500 mg/l for 8 passes.

The CCV used in this experiment was the ATCC type strain
(Auburn clone A). The sensitivity of CCV to Ara-T was determined by comparing the median tissue culture infective dose (TCID 50) of a viral suspension in ten fold serial dilutions of 1 mM Ara-T to non-exposed controls. An Ara-T resistant CCV mutant was selected by passage of the virus in the presence of 20 mg/l BUDR, followed by 50 mg/l BUDR and 0.1 mM Ara-T and finally by dilution cloning three times in 0.1 mM Ara-T.

Cell extracts for Tk analysis were prepared from confluent cell sheets in 150 cm² tissue culture flasks by rinsing the cells with Hank's balanced salt solution and scraping the cells into 2 ml of ice cold lysis buffer containing 10 mM tris (pH 7.5), 10 mM KCl, 1.5 M MgCl₂, 0.1 mM phenyl-methlysulfonyl flouride (PMSF). Unless otherwise specified, viral Tk extracts were prepared in a similar manner from confluent Tk negative cells infected with 0.1 plaque forming unit (PFU)/cell, and incubated 18 hours at 30 C. The cells were lysed by freeze-thawing two times, followed by sonication on ice on a Branson Sonifier (Cell Disrupter 200) using a microtip with a 50% interval setting at power level 6 for 30 seconds. The lysate was then mixed with 200 µl of 50% glycerol, 1.5M KCl, 12.5mM MgCl₂, 3mM 2-mercaptoethanol (2ME) and cleared by centrifugation at 100,000g for one hour at 4 C. The cleared lysates were stored for a maximum of three weeks at -70 C for use in Tk assays.

The Tk activity was determined by quantifying the amount
of tritium labeled thymidine ($^3$HdT) phosphorylated by the extracts. Basically, equal volumes of cell extract and reaction solution (10.6 mM MgCl$_2$, 8 mM KCl, 120 mM Tris pH 8.0, 0.5 mCi/ml $^3$HdT, 20 mM ATP, 10 mM NaF, 0.16 mM 2ME) were combined, incubated at 27°C for 3 hours and 20 µl of the mixture was spotted on a 1 cm$^2$ DEAE paper square. The negatively charged $^3$HdTMP bound to the cationic substrate and the unphosphorylated $^3$HdT was washed off the paper by rinsing it three times in 4 mM ammonium formate pH 3.6, three times in distilled water, once in 95% ethanol and once with 100% ethanol. The paper squares were dried at 60°C for two hours, and the bound $^3$HdTMP quantified by liquid scintillation. In all assays 1 mg/ml bovine serum albumin (BSA) was used as a negative control. Variations of the above protocol included the addition of 0.5, 0.05 or 0.005 mM final concentration of BUdR, Ara-T, dT, deoxyadenosine (dA), deoxycytosine (dC), or deoxyguanosine (dG) to the reaction mixture for competitive inhibition assays, the addition of 0.5, 0.05 or 0.005 mM final concentration of deoxythymidine triphosphate (dTTP) to the reaction mixture for feedback inhibition assays, and the replacement of the 10 mM ATP with 0.5, 1.5, 4.5 or 13.5 mM final concentration of ATP or cytosine triphosphate (CTP) for phosphate donor assays.
Results

Development of Tk deficient CCO cells.

Normal CCO cells die when cultured in the presence of 25 mg/l BUdR within seven passes. However, after 23 passes in 5 mg/l BUdR the cells could be continuously cultured in 50 mg/l BUdR. After 21 passes in 50 mg/l BUdR, the cells retained a relatively high Tk activity (8270 cpm) compared to normal CCO extracts (14,850 cpm). Cells that were successively cultured in 150 mg/l BUdR, 250 mg/l BUdR and 500 mg/l BUdR were resistant to 500 mg/l BUdR (CCOBr) and had significantly reduced Tk activity when compared to normal CCO cell extracts (Figure 1).

High Tk activity was found in the Tk negative cells infected with CCV (Figure 1). Tk activity was highest 16 hours after infecting CCOBr cells with one plaque forming unit per cell (figure 2), at which time the cell sheet was characterized by total involvement of the cells in syncytia (4+ cytopathic effect).

Sensitivity of CCV to Ara-T and development of a resistant mutant.

The infectivity of CCV was reduced 1000 fold in $10^{-6}$ M Ara-T and was completely eliminated in $10^{-4}$ M Ara-T (Figure 3). The sensitivity of CCV to the Tk activated, antiherpetic agent
Figure 1. Tk activities of CCO cells, CCOBr cells, CCV infected CCOBr cells and CCVAr infected CCOBr cells (mean±SD). The reactions were run using three extracts from each Tk type prepared from 25 cm² flasks in a total volume of 300 μl.
Figure 2. The elevation of Tk activity in CCOBr cells as CCV infection progresses. CCOBr cells were infected with 1 PFU/cell and sampled at 0, 2, 4, 8, 10, 12.5, 16 and 18 hours post infection. All samples were diluted to 700 μg/ml total protein, and incubated at 25 C for 12 hours.
Figure 3. Effect of Ara-T on infectivity of CCV. A stock CCV culture was dilution plated on CCO cells and incubated in medium containing ten fold dilutions of 1 mM Ara-T. The tissue culture infective doses (TCID 50) were calculated using the methods of Reed and Meunch (1938).
Ara-T, combined with the induction of Tk activity in Tk deficient cells, indicates that CCV contains a Tk gene. Subsequent passage of the virus in the presence of BUdR and Ara-T resulted in selection of an Ara-T resistant mutant of CCV (CCVAR). This mutant virus replicated efficiently in Tk negative cells, yet did not induce detectable Tk activity (Figure 1). Therefore, CCVAR was considered a thymidine kinase deficient CCV mutant.

**Characterization of cellular and viral isozymes**

The cellular and viral induced Tk isozymes were compared by assessing the nucleoside competitive inhibition profiles, ATP and CTP phosphate donor specificity, and dTTP mediated feedback inhibition. Unlabelled dT, used as a reference for competitive inhibition comparisons, produced significantly different inhibition profiles for each isozyme (Figure 4), indicating different enzyme kinetics. Therefore, to simplify alternate substrate competitive inhibition analyses, the relative percent inhibition by each substrate was determined by dividing the inhibition induced by the inhibition induced by a similar level of dT (Figure 5). CCO-Tk and CCV-Tk showed similar competitive inhibition profiles for dC. Ara-T and BUdR induced inhibition of each isozyme was statistically different, but quantitatively similar. The viral Tk was much less specific for the deoxypurines dA and dG than the cellular Tk.
Figure 4. Competitive inhibition of $^3$HdT phosphorylation by unlabelled dT. Values are expressed in percentage of maximum activity. Statistics were performed using the Student's T test on arcsin of the square root transformed data to account for non-normal distribution of percentage values.
Figure 5. Relative competitive inhibition of Tk mediated $^3$HdT phosphorylation by thymidine analogs and deoxynucleosides in CCO-Tk extracts (O) and CCV-Tk extracts (V). Values are expressed in percentage of the inhibition induced by a similar concentration of dT. Statistics were performed on SAS using the Student's T test on arcsin of the square root transformed data to account for non-normal distribution of percentage values.
Feedback inhibition was more pronounced in the CCO Tk than CCV Tk at all levels of dTTP concentration and was statistically significant at 0.5 mM dTTP (Figure 6). Both isozymes were inefficient at using CTP as a phosphate donor. Also, phosphate donor assays showed that the cellular enzyme required lower levels of ATP to reach peak Tk activity than the viral isozyme (Figure 7).

Discussion

The Tk deficient CCO cell line established in this research was pivotal in determining the presence and characteristics of the CCV expressed Tk. The CCOBr cell line had approximately 10% of the Tk activity of the normal CCO cells. A low residual Tk activity due to mitochondrial Tk activity is common among BUdR resistant cell lines (Kit et al., 1973a; Kit et al., 1973b). The apparent initial BUdR resistance of the CCO cells that maintained high Tk activity while being continuously cultured in 50ug/ml BUdR may be explained by a mutation in the thymidine transport system similar to a mutation described in ³HdT resistant Chinese hamster cells (Breslow and Goldsby, 1969). Increasing the
Figure 6. A comparison of dTTP feedback inhibition of CCO-Tk and CCV-Tk. Values are expressed as a percentage of maximum activity. Statistics were performed using the Student's T test on arcsin of the square root transformed data to account for non-normal distribution of percentage values.
Figure 7. CTP and ATP phosphate donor specificity of CCO-Tk and CCV-Tk. Values are expressed in percentage of maximum activity. Statistics were performed using the Student's T test on arcsin of the square root transformed data to account for non-normal distribution of percentage values.
BUdR levels to 500μg/ml probably induced an additional mutation making the cell Tk deficient.

The induction of high Tk activity in CC0Br cells by CCV and the production of a mutant virus which does not have a Tk inducing activity indicates CCV encodes a thymidine kinase. Only 2 of 17 herpesviruses analyzed to date, the human cytomegalovirus and herpesvirus aegyptius, do not contain a Tk gene. Generally herpesvirus Tk's have less restricted substrate specificity and dTTP feedback inhibition than the cell's cytoplasmic Tk. The phosphate donor specificity, competitive inhibition and dTTP mediated feedback inhibition assays used in this investigation clearly distinguished the cellular Tk from the CCV Tk. Competitive inhibition assays were used as indicators of substrate specificity because direct measurement of alternate substrate phosphorylation would require significant enzyme purification to discount the effects of other enzymes. Competitive inhibition profiles indicate which substrates bind to the active site and block Tk activity, but do not indicate whether the alternative substrate is phosphorylated. These investigations showed that the cellular Tk used low levels of ATP more efficiently, exhibited different enzyme kinetics as indicated in the dT inhibition profile, was not competitively inhibited by deoxypurines and was more inhibited by dTTP than the viral Tk. The ATP and dT profiles indicate cellular Tk has a lower processivity and a higher substrate binding activity than the
viral enzyme, making it most efficient in conditions of low substrate concentrations. The cellular Tk competitive inhibition profiles for Ara-T and dC were high compared to the profiles for bovine, equine, rabbit, mouse and human cytoplasmic Tk (Turenne-Tessier et al., 1986; Kit and Qavi, 1983; McGowan et al., 1980; Allen et al., 1979), indicating that catfish cytoplasmic Tk may be less specific than mammalian Tk and may be a more primitive enzyme. The most distinctive feature of the CCV-Tk was its strong competitive inhibition by deoxypurines, especially dG. The moderate specificity of the cellular Tk to Ara-T and dC in conjunction with the selective viral Tk inhibition by deoxypurines indicate that deoxypurine analogs such as acyclovir may be more selective antiviral agents for CCV than deoxypyrimidine analogs such as Ara-T or Ara-C. CCV-Tk was similar to other herpesvirus Tk's in nucleoside competitive-inhibition profiles and low dTTP inhibition but was unusual in it's low efficiency in using CTP as a phosphate donor. The Tk's of other herpesviruses such as herpes simplex 1 and 2 (Kit et al., 1975), Epstein-Barr virus (Turenne-Tesner et al., 1980), equine herpesvirus 1 (Weinmaster et al., 1982), herpesvirus of turkeys and infectious laryngotracheitis virus (Kit et al., 1975), characteristically use CTP effectively as a phosphate donor. In this respect CCV-Tk is more similar to poxvirus and cytoplasmic cellular Tk (Kit et al., 1975).

This investigation demonstrated that CCV encodes a unique
Tk which is clearly distinguishable from cellular Tk activity. It also indicated that the CCV-Tk has phosphate donor specificity that is unique among herpesvirus Tk's, which, in conjunction with the evolutionarily distant nature of the host, would make the CCV-Tk gene sequence a uniquely valuable reference to compare with higher vertebrate herpes Tk gene sequences for enzyme and herpesvirus evolution studies. In addition the sequence could be a key reference in studying the structure function relationships in the herpesvirus Tk group. Research is currently underway using the thymidine kinase deficient, CCVAR mutant in marker-rescue assays to locate the Tk gene on the CCV genome for subsequent sequence analysis.
Literature Cited


Chapter Three

The Channel Catfish Herpesvirus (CCV) Thymidine Kinase Gene is Located in The Terminal Direct Repeat Regions of the Genome

Abstract

The gene encoding the previously identified unique channel catfish herpesvirus (CCV) thymidine kinase (Tk) was preliminarily located on the CCV genome. CCV genomic DNA libraries were constructed into plasmid pUC 19 and cosmid pHC 79. Analysis of CaCl₂, DEAE and cationic liposome mediated transfection techniques using β-galactoside expressing plasmid pON 105 on the channel catfish ovary cell line (CCO), a Tk deficient mutant of CCO (CCOBr), and the brown bullhead cell line (BB) revealed cationic liposome transfection of CCO cells to be most effective combination. More importantly, cationic liposome mediated transfection of whole CCV-DNA onto CCO or CCOBr cells was the only method that effectively produced infectious viral progeny. This is the first account showing purified CCV-DNA to be infectious. Subsequently, cationic liposome mediated co-transfection of cloned wild type CCV DNA with the Tk deficient mutant of CCV (CCVAr) in marker rescue assays, mapped the mutation within the 18 Kb direct repeat
ends of the genome. In addition, the polymerase chain reaction, (PCR), was used (Jack Numberg, Cetus Corporation) to amplify regions flanked by sequences with homology to degenerate primers corresponding to conserved amino acid sequences among herpesvirus Tk's. Three PCR generated fragments were isolated, cloned into pUC 19 and sequenced. The corresponding amino acid sequence of the presumptive coding strand of one sequence (405) showed limited homology to the mammalian cytoplasmic and poxvirus Tk. A weak specific hybridization signal was located on the Eco RI L fragment, which is located on the direct repeat ends of the CCV genome, when DNA-DNA hybridization analysis was performed using the purified $^{32}$P nick-translation labelled 405 fragment. The combined marker rescue and PCR data map the Tk gene within the direct repeat region of the CCV genome. This location is unique among herpesviruses, indicating significant divergence from previously identified herpesvirus gene arrangements.

Introduction

Because thymidine kinase (Tk), is a non-essential, readily detectable, selectable trait in herpesviruses, the herpesvirus Tk genes have been the center of much recombinant molecular virology research. Herpesvirus Tk genes have been used as selectable sites of homologous recombination to insert foreign genetic material (Mocarski et al., 1980), as
selectable insertion sequences to interrupt other genes (Post and Roizman, 1981), as reporter genes to analyze promoter elements (Pater and Pater, 1984) and as reference sequences for evolutionary comparisons (Honess et al., 1989). Also, Tk deletion mutants have been used as permanently attenuated live vaccines.

Recent mutagenesis and enzyme substrate specificity data indicate that channel catfish virus (CCV) (Ictalurid herpesvirus 1) contains a Tk gene (Chapter Three) that is unique among herpesviruses in its inability to utilize CTP as a phosphate donor. The evolutionarily distant host and unique enzyme characteristics of this Tk compared to previously analyzed herpesviruses Tk's make this gene a valuable reference for evolutionary and structure function comparisons. In addition, Tk negative mutations in herpesviruses generally attenuate the pathogen making them suitable as live vaccine agents. The purpose of this investigation was to map the viral Tk gene on the CCV genome for future evolutionary comparisons and vaccine development.

Materials and Methods

Channel catfish ovary cells (CCO) were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 25 mM HEPES buffer and 100 IU/ml penicillin and 100 μg/ml streptomycin (growth medium). The Tk negative mutant of CCO (CCOBr) (Chapter 3) was
maintained in growth medium supplemented with 500 µg/ml of 5-bromo-2′-deoxyuridine (BUdR). The CCV strains used in this experiment were the ATCC type strain (Auburn clone A) and a Tk negative mutant of this strain, CCVAR (Chapter 3).

Stock virus was produced by infecting CCO cells with 0.01 plaque forming units per cell, disrupting the cell sheet after 18 hours at 30 C, freeze thawing the suspension two times and storing aliquots at -70 C. Stock CCVAR was produced under similar conditions except for the presence of 0.1 mM Ara-T in the growth medium. Concentrated virus for DNA isolation were produced from roller bottle cultures of CCO cells. Infected cells were dislodged from the bottle and centrifuged at 500g for 5 minutes at 4 C. The supernatant containing the extracellular virus was saved and the pellets were resuspended in TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5) and lysed by one cycle of freeze thawing, followed by dounce homogenization. The cellular debris was again centrifuged at 3000g for 15 minutes at 4 C and the supernatent containing the intracytoplasmic virus was pooled with the extracellular virus. The pooled virus was pelleted from the medium at 18,000 g for one hour at 4 C.

Concentrated virus from 8 roller bottles was purified on 20 to 60% sucrose gradients at 26,000 rpm in a Beckman SW27 rotor for 1 hour at 4 C using tritiated thymidine labelled virus as a tracer. For library construction, CCV-DNA was purified from fractions containing high counts. The fractions
were digested with 5 μg/ml DNase free RNase, 5μg/ml proteinase K in 0.5% SDS at 45 C for 4 hours, followed by gentle extraction two times with two volumes of 50 mM Tris, pH 8.0, 1 mM EDTA saturated, buffered phenol and three times with water saturated ether. Residual ether was driven off the solution at 70 C for 15 minutes. This solution was dialyzed in three changes of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) at 4 C. Purity of concentrated CCV-DNA was checked by agarose gel electrophoresis of Eco RI digested fragments, and only fractions producing clean distinct Eco RI restriction fragments of CCV were used for subsequent library construction.

Infectious CCVAr-DNA was purified by sodium iodide gradient centrifugation. The total concentrated virus from 4 roller bottles were suspended in 15 ml of TNE and digested with RNase and proteinase K in SDS as described above. This suspension was added to 24 mls of NaI saturated TE buffer containing 0.3 mg ethidium bromide and centrifuged at 48,000 rpm for 40 hours at 20 C in a Beckman VTi 50 rotor. The distinct viral DNA band was visible under long wave UV light, and was gently harvested using a syringe with a 14 gauge needle, gently extracted with isobutanol to remove the ethidium bromide, and dialyzed in TE buffer.

An incomplete genomic library of CCV was made by digesting purified viral DNA to completion with Eco RI and 'shotgun' cloning these fragments into the Eco RI site of
plasmid pUC-19. The recombinant plasmids were transformed into DH5α *E. coli* using the calcium chloride method of Hanahan (1983). Transformants were isolated on LM agar medium containing 50 μg/ml ampicillin and coated with 2 μg of X-gal. White colonies, indicative of transformants containing pUC-19 with an insertion in the Lac Z polylinker site, were subcultured. The respective plasmids were purified using a rapid plasmid isolation procedure (Holmes and Quigley, 1981) and then cut with Eco RI. These DNA fragments were analyzed by agarose gel electrophoresis. The size of inserted viral DNA fragments were compared to the published Eco RI restriction map (Chousterman et al., 1979) to determine its location on the genome.

A more complete genomic library of CCV was produced by cloning a partial Eco RI digest of viral DNA into the cosmid pHC-79. First, 12 μg of CCV DNA was digested with 2.4 units of EcoRI for 60 minutes in 30 μl at 37°C and 3.6 μg of this DNA was mixed with 0.35 μg of calf intestine alkaline phosphatase-dephosphorylated Eco RI digested pHC-79 DNA. The DNA mixture was ethanol precipitated, reconstituted to 10 μl and ligated. This methodology was similar to Maniatis et al. (1982). The ligation mixture was subsequently packaged into λ capsids (Stratagene, Gega Pack Gold, La Jolla, CA) and transfected into DH5α *E. coli*. Transformants were selected on ampicillin plates and the inserts were analyzed by Eco RI digestion followed by agarose gel electrophoresis. The
corresponding location of the cloned fragments on the CCV genome were determined by comparing the Eco RI electrophoretic profile to the published Eco RI restriction map of CCV (Chousterman et al., 1979).

A comparison of CaCl$_2$, DEAE and cationic liposome mediated transfection methods on CCO, CC0BR and brown bullhead (Ictalurus nebulosis) (BB) cell lines at 60 to 80% confluency in 6 well plates was performed using the plasmid pON 105 which contains the E. coli lac Z gene under the control of the herpesvirus simplex virus (HSV) immediate early promoter from the IE 4 gene (Ho and Mocarski, 1988). At 72 hours post-transfection cells were fixed in 0.5% glutaraldehyde in PBS for 15 minutes, rinsed three times with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM HPO$_4$.7H$_2$O, 1.4 mM KH$_2$PO$_4$, pH 7.4) then stained in a solution of 2.4 mM X-gal, 12.5 mM K$_3$Fe CN$_6$, and 12.5 mM K$_4$FeCN$_6$.3H$_2$O in PBS overnight. $\beta$-galactosidase (B-gal) positive transfected cells stained blue and were counted in 7 fields of view at low power on an inverted microscope (12 mm$^2$/view). The mean counts of positive staining cells per view were divided by the mean number of cells in the viewing area to determine the transfection efficiency.

The CaCl$_2$ mediated transfection procedures were modifications of Ausubel et al. (1987). First, 7 $\mu$g of DNA and 7 $\mu$l of 2.5 M CaCl$_2$ were mixed and diluted to 60 $\mu$l with distilled water. Sixty $\mu$l of 2X BBS was slowly added under constant agitation. This mixture was held at room temperature
for 20 minutes, diluted with 3 ml of growth medium, and dispersed over the cells. Cells were incubated 4 to 5 hours at 30 C, shocked for 3 minutes with 15% glycerol or 25% DMSO in TBS, rinsed with TBS and overlayed with growth medium. Modifications of the above procedures included a DEAE pretreatment, in which the cells were rinsed with TBS, overlayed with 1.5 ml of 500 μg/ml DEAE dextran (MW 500,000) for 3 minutes and rinsed with TBS before the CaCl₂ precipitate overlay, or a 100 μM chloroquine treatment during the incubation with the CaCl₂ precipitate.

DEAE dextran transfection procedures followed the methods of Ausubel et al. (1987). The cells in each well of a 6 well plate were rinsed three times with serum free medium (SFM) and overlayed with a mixture of 200 μg DEAE dextran and 2.5 μg DNA in 1 ml of SFM. After four hours, the cells were either glycerol or DMSO shocked as previously described. A modification of the above procedure was the addition of 100 μM Chloroquine to the treatment mixture.

Cationic liposome mediated transfections were performed using the Lipofectin reagent (Bethesda Research Laboratories, Bethesda, MD) according to the methods described by the manufacturer or by method C of Felgner and Holm (1989). Marker rescue assays were performed using cationic liposome mediated co-transfection of CCVAR-DNA and cloned CCV-DNA by a modification of transfection method C of Felgner and Holm (1989). Basically, 50 to 70 % confluent CCO cells in a 25 cm²
tissue culture flask were rinsed 3 times with 1.5 ml of serum free growth medium (SFM) and overlayed with 1.5 ml SFM containing 50 μg Lipofectin for 30 minutes. Subsequently, 1.5 ml of SFM containing 5 μg CCVAR DNA and 7 μg cloned CCV-DNA was added and incubated at 30 C for 20 hours, after which 3 ml of growth medium containing 20% FCS was added. Cytoplastic effect (CPE) was usually evident 4 days later, and virus was harvested when CPE covered the entire cell sheet.

Tk marker rescue was identified by plating the virus on CCOBr cell monolayers on single well glass slides using the Tk plaque autoradiography method of Tenser et al. (1983). The percentage of recombinants were scored by placing the slide on the autoradiograph and comparing the number of plaques with a black background to the number of plaques on a light background under a dissecting microscope.

Purified CCV-DNA was used in PCR by Dr. Jack Nunberg (Cetus Corporation) to amplify regions flanked by sequences with homology to degenerate primers corresponding to conserved amino acid sequences among herpesvirus Tk's using the primers and methods previously described (Nunberg et al., 1989). Herpes simplex virus type 1 (HSV-1) and feline herpesvirus 1 (FHV-1) DNA were used as positive controls. The PCR products were phenol-ether extracted, ethanol precipitated and resuspended in 30 μl of 5 mM Tris (pH 8.0), 0.1 mM EDTA. Ten μl of each product was separated by vertical 5% polyacrylamide gel electrophoresis (PAGE) and stained in 0.5 μl/ml ethidium
bromide. Bands of approximately 330 bp (co-migrating with HSV-1 and FHV-1 Tk specific bands) from CCV PCR products were excised and placed in 0.5 ml of 0.5 M ammonium acetate for elution as described by Maniatis et al. (1982). The eluted PCR fragments were Eco RI and Hind III digested and cloned into Eco RI, Hind III cut pUC 18 (purified from the polylinker fragment by low melt agarose electrophoresis and phenol ether extraction). Plasmids were isolated from β-galactosidase negative transformed colonies using the rapid purification method described above, digested with Eco RI and Hind III, and analyzed by 5% PAGE. Large scale plasmid extracts were made of three clones (405, 409 and 398) containing Eco RI/Hind III restriction fragments of the proper size and purified by CsCl2 gradient centrifugation (Maniatis et al., 1982).

The inserts of purified plasmids were double stranded, dideoxyribonucleotide sequenced using a T7 DNA polymerase sequencing kit (Sequenase 2.0, United States Biochemical Corporation, Cleveland, OH) with M13 positive and negative strand primers and α35SATP (1000 Ci/mM) as a label according to the manufacturers methods. The reaction mixtures were electrophoresed on 35 X 60 cm X 0.4 mm, 6% polyacrylamide-urea gels (Gel-Mix 6, Bethesda Research Laboratories, Bethesda, MD) at 200 W, 55 C. The gels were dried onto paper supports and autoradiographed at room temperature for two days using XAR-5 film (Kodak, Rochester, NY).

DNA-DNA hybridization analyses of Eco RI cut, 0.7%
agarose gel electrophoresed CCV and cosmids 395, 394, 389, 388 DNA were performed using $^{32}$P labelled purified inserts of plasmids 405 and 409 as probes. The agarose gels were electrophoresed at 1 v/cm overnight, then vacuum blotted (Trans-vac, Hoeffer Scientific, San Francisco, CA) onto Gene Screen Plus (DuPont Inc., Boston, MA) nylon membranes using the methods described by Sambrook et al. (1989). The PCR derived inserts were excised from plasmids 409 and 405 by Eco RI and Hind III digestion, purified by 5% PAGE and eluted in sodium acetate buffer as described earlier. Approximately 7 ng of each insert were nick translation labelled with $\alpha^{32}$PCTP using the kit NEK-004 (Dupont, Boston, MA) and purified using a sephadex G25 spin column. The blots were prehybridized in 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl and 0.2 µg/ml denatured salmon sperm DNA at 42 C for 2 hours followed by hybridization at 45 C overnight using one half of the heat denatured probe in 10 ml of prehybridized solution. The blots were then washed two times in 2X SSC (3 M NaCl, 0.03 M sodium citrate, pH 7) at 65 C for 15 minutes, rinsed in SSC, wrapped in plastic film and autoradiographed with Kodak XAR-5 film at 70 C using intensifying screens.

Results

The genomic library developed in pUC19 was incomplete because several large fragments, specifically A, C and F, were resistant to cloning after several attempts. A more complete
library was constructed using the cosmid pHc79 system because of its selectivity for larger DNA inserts. Sixteen ampicillin resistant transformant colonies were analyzed and all contained cosmids with 30 to 40 Kb viral DNA inserts. Four unique clones, covering 98% of the genome, were selected for the initial marker rescue assays (Figure 1).

The most efficient and reproducible method of transfecting CCV permissive cell lines was the cationic liposome mediated technique of Feigner and Holme (1989). This method produced 0.1% transfectants in CCO and CCOBr cell lines, which was a 10 to 200 fold higher frequency than the various CaCl₂ and DEAE mediated transfection techniques. BB cell transfection experiments consistently produced transfection efficiencies at least 10 fold lower than the respective CCO and CCOBr transfection experiments. Early attempts to produce infectious progeny by CaCl₂ mediated transfection of CCV-DNA were unsuccessful. However, the first cationic liposome mediated transfection of CCOBr cells with 10 μg of CCV-DNA produce infectious CCV progeny. In subsequent marker rescue assays using CCVAR-DNA, infectious progeny were produced 80% of the time.

Of the four initial cosmids used in marker rescue assays, both end cosmids 395 and 389 rescued the CCVAR mutation very efficiently. Cosmid 395 induced 67% rescue and 389 induced 82% rescue, while cosmids 388 and 394 produced no detectable
Figure 1. A. Agarose gel electrophoretic profile of Eco RI digested cosmids containing the CCV DNA inserts used in marker rescue experiments. The letters on the left designate the Eco RI fragments as defined by Chousterman et al., (1979). The right lane contains Hind III digested bacteriophage λ DNA as size markers, the corresponding sizes in Kb are designated adjacent to this lane. B. Location of the CCV DNA inserts of the cosmids in A on the Eco RI restriction map (Chousterman et al., 1979). C. H3T autoradiograph analysis of viral plaques from rescue experiments.
rescue (<0.1%). Cosmids 395 and 389 contain DNA from opposite ends of the genome and thus contain common sequences from the terminal direct repeat region. Subsequently, cosmids overlapping the CCV unique portion of cosmid 395, 383 and 386 (Figure 1), were used in marker rescue and were also found to be negative.

Three PCR generated fragments that were produced using degenerate Tk primers were cloned into pUC 18 and sequenced. The sequence of one fragment (409) corresponded to the FHV-1 Tk gene and was probably a contaminant generated by the PCR reaction or isolated by cross contamination during PAGE purification. The corresponding amino acid sequences of the presumptive coding strand of fragment 389 showed no homology to published Tk sequences and contained several stop codons. The deduced amino acid sequence of the presumptive coding strand of fragment 405 (Figure 2) showed limited homology to vertebrate cytoplasmic and poxvirus Tk sequences (Figure 3). Hybridization analyses of Eco RI digested, agarose gel electrophoresed CCV and cosmid DNA using PCR generated fragments 409 and 405, showed some specific binding of fragment 405 to the Eco RI K, L fragments of CCV and the Eco RI fragment of cosmid 389 (Figure 4). Fragment 409 showed no homology to viral sequences.
Discussion

This investigation showed that CCV-DNA is infectious, established a method for marker rescue analysis of CCV and mapped the Tk negative mutation in CCVAr to the terminal direct repeat regions of the CCV genome. If this represents the Tk gene, the Tk of CCV is diploid. This diploid nature would explain the very high percentages of rescue found with cosmids 389 and 395 because only 1 of the 2 genes in the genome would need to be rescued to impart a Tk positive phenotype. A Tk gene located in the repeat regions is unique among the herpesviruses thus far investigated. Herpes simplex 1 and 2 (Wagner et al., 1981; Swain and Galloway, 1983), psuedorabies virus (Kit, 1985), bovine herpesvirus 1 and 2 (Mittal and Field, 1989; Sheppard and May, 1989), Herpesvirus saimiri (Honess et al., 1989), marmoset herpesvirus (Otsuka and Kit, 1984), varicella zoster virus (Littler et al., 1986), herpesvirus of turkeys, Marek's disease virus (Scott et al., 1989), and Epstein-Barr virus (Littler et al., 1986) all contain their respective Tk genes in the unique long regions of the genome. Generally the Tks are contained in a gene 'cassette' which also contains the genes coding for the major capsid protein and the homolog of HSV glycoprotein H (Albrecht and Fleckenstein, 1990; Davison and Taylor, 1987). In other α herpesviruses the short repeat regions code for regulatory proteins. These results, along with the unique
Figure 2. The nucleotide sequence and deduced amino acid sequence of the presumptive coding strand of the PCR generated fragment 405.
405 GKSTGGKEPRQLATKAARKSAPYTGKKPHRYKPGTVALERIRRYQKSELIC

**** * ****

VAC\textsuperscript{1} GKSTEL.............................................IRRVRQIAQYKCVT
VAR\textsuperscript{2} GKSTEL.............................................IRRVRQIAQYKCVT
SFV\textsuperscript{3} GKSTEL.............................................IRLVRRQIAKHKCLV
MFV\textsuperscript{4} GKSTEL.............................................IRRVRQIAQYKCVT
FPV\textsuperscript{5} GKTSEL.............................................VRRIKRFMLSNKCI
HUM\textsuperscript{6} GKSTEL.............................................MRRVRFQIAQYKCLV
MUS\textsuperscript{7} GKSTEL.............................................MRRVRFQIAQYKCLV
CHK\textsuperscript{8} GKSTEL.............................................MRRVRFQIAQYRC

KLHFQHVLVRKIAQDLKDLCFQXALGALQEASEAYLVGLFEDTNLCSIHAKGVIGI

******

IKYSNDNRY....GTGLNTHDKNN....FEALEATKLDVL.E....SITDFSIGI
IKYSNDNRY....GTGLWTHDKNN....FEALEATKLDVL.EA....ITDFSIGI
VKYEKDIRY....GNGVCTHDNMS....ITAVCTPSLDKIDSVA....ENAEGIGI
IKYSNDNRY....GTGLWTHDKNN....FAALEVTKLDVL.EA....ITDFSIGI
IKHCDNRYNEEDINKYYTHDLFL....MEATASSNLVLVTPLL....NGVQVIGI
IKYAKDTRY....SSSFCTHDRNT....MEALPACLRDVQGAL....GVAIGI
IKYAKDTRY....SSSFSTHDRNT....MDALPACDLTVQELL....GVAIGI
VKYAKDTRY....CTTGVSTHDRNT....MEARPAQDVQGAL....GSAIGI

Figure 3. A comparison of the deduced amino acid sequence of fragment 405 with the amino acid sequences poxvirus and vertebrate cytoplasmic Tk's. * indicates regions of 405 with homology to Tk sequences.

\textsuperscript{1} vaccinia virus, Hruby et al., 1983 \textsuperscript{2} variola virus, Esposito and Knight, 1984 \textsuperscript{3} Shope fibroma virus, Upton and McFadden, 1986 \textsuperscript{4} monkey pox virus, Esposito and Knight, 1984 \textsuperscript{5} fowl pox virus, Boyle et al., 1987 \textsuperscript{6} human, Bradshaw and Deininger, 1984 \textsuperscript{7} mouse, Lewis, 1986 \textsuperscript{8} chicken, Kwoh and Engels, 1984.
Figure 4. DNA-DNA hybridization of Eco RI digested 0.7% agarose gel electrophoresed CCV and cosmid DNA using $^{32}$P labelled PCR fragment 405 as a probe.
substrate specificity of CCV-Tk (Chapter Three), indicate that the channel catfish herpesvirus may show considerable divergence from the mammalian herpesviruses. The results of the degenerate based PCR experiments provide some support for the marker-rescue results. The one sequenced PCR fragment that showed some homology to cytoplasmic and poxvirus Tk's hybridized to the Eco RI K and L fragments of CCV and to the Eco RI L fragment of cosmid 389. These fragments are within the direct repeat regions. The PCR data is non definitive because the reactions were performed under non-stringent conditions and the reactions are sensitive to minute quantities of contaminants. If the sequenced PCR product does represent the Tk gene, the CCV-Tk is more related to vertebrate cytoplasmic and poxvirus Tk's than herpesvirus Tk's. Research is currently in progress to sequence this region of the genome to confirm the presence of the Tk gene. A comparison of CCV-Tk and other viral Tk sequences may give insight into the evolution of this enzyme and the herpesviruses.


deletion of specific genes in large genomes: a gene 22
of herpes simplex virus 1 is not essential for growth.

virus as a vehicle for the isolation and characterization
of unknown mammalian promoters and enhancers. J. Mol. and

Cloning, a laboratory Manual 2nd edition" Cold Spring

Scott, S.D., N.L.J. Ross and M.M. Binns. 1989. Nucleotide and
predicted amino acid sequences of the Merek's disease
virus and turkey herpesvirus thymidine kinase genes and
comparison with thymidine kinase genes of other

Sheppard, M. and J. T. May. 1989. Location and
colorization of the bovine herpesvirus type 2

Swain, M. and D. Galloway. 1983. Nucleotide sequence of the
herpes simplex virus type 2 thymidine kinase gene. J.

Thymidine plaque autoradiography of thymidine kinase-
positive and thymidine kinase-negative herpesviruses. J.
Clinical Micro. 17:122-127.

Upton, C. and G. McFadden. 1986. Identification and nucleotide
sequence of the thymidine kinase gene of Shope fibroma

of the thymidine kinase gene of the herpes simplex virus
Chapter Four
The Use of Ciprofloxacin to Cure
Mycoplasma Contaminated Channel Catfish Ovary Cells

Introduction

Mycoplasmas (MP) are common, yet often inapparent contaminants of cell culture. Their effects on cultured cells can be subtle or extreme, ranging from altered nutritional requirements and changes in physical appearance to metabolic changes and altered virus production characteristics (reviewed by Barile, 1979). Elimination of mycoplasma contamination is essential in any experiments involving detailed analysis of cellular enzymes.

During an investigation into cellular and channel catfish virus induced thymidine kinase isozymes, MP contamination was discovered in both the channel catfish ovary cell line (CCO) and a thymidine kinase deficient mutant of the same cells (CCOBr). The contaminant evaded detection during initial screenings using standard MP culture methods but was detected using a commercially available genetic probe to MP ribosomal RNA. Schmitt et al. (1988) efficiently eliminated MP from mammalian cell lines using the 4-flouroquinalone,
Ciprofloxacin (CIP). The ability of CIP to cure contaminated CCO cell lines was investigated.

**Materials and Methods**

All cell lines were routinely passed in Dulbecco's modified minimal essential medium + 10% fetal calf serum + HEPES buffer + 50 μg/ml gentamicin or 100 IU/ml penicillin and 100 μg/ml streptomycin. CCOBr cells were cultured in the presence of 500 μg/ml 5-bromo-2'-deoxyuridine. Ciprofloxacin tablets were dissociated in distilled water at a concentration of 10 mg/ml. This suspension was dissolved in antibiotic free growth medium at the appropriate dilution and filter sterilized. To find the highest dose of CIP tolerated by CCO cells, MP contaminated CCO cells were cultured in the presence of 10 μg/ml, 50 μg/ml and 100 μg/ml CIP for 10 days at 30 C. Mycoplasma cultures were prepared by centrifuging antibiotic free culture medium from 7 day old cell cultures at 5000g for 30 min, resuspending the pellet in 100 μl of medium and inoculating it on modified Hayflick's agar medium (Stalheim, 1973). To identify any bacterial contaminants in the cell cultures, a portion of the centrifuged cell culture medium was cultured on 5% blood agar medium at 30 C. Ribosomal RNA based hybridization analyses were performed according to the manufacturer's recommendations (Mycoplasma T.C., Gene Probe) except that 10 ml of tissue culture medium were centrifuged for analysis and hybridization times were increased to 16
hours to improve sensitivity.

Results

Doses of 50 and 100 μg/ml of CIP produced sublethal toxic changes characterized by reduced growth and a flattened appearance of the cells. Cells in 10 μg/ml CIP did not produce the distinct morphological changes observed in the higher doses. Because the 100 μg/ml dose cells survived, this level was used to evaluate the MP elimination. The 100 μg/ml CIP treated cells were cultured for two weeks in antibiotic free medium and again analyzed for MP. Table 1 shows that CIP treated cells were negative for MP while untreated cultures and cultures grown in medium containing 50 μg/ml gentamicin remained MP positive. Bacterial screening plates revealed tiny <0.5 mm hemolytic colonies on the blood agar after two weeks, even though the MP plates grown at 37 C remained negative. When replated on MP medium and incubated at 30 C instead of the standard 37 C, tiny fried-egg shaped colonies characteristic of MP appeared after two weeks. Subsequently, CCOBr cells cultured in the presence of 100 μg/ml CIP for 10 days at 30 C followed by two weeks in antibiotic free media were also free of MP contamination. Treated CCO and CCOBr cells subcultured in normal growth medium and intermittently analyzed for MP contamination remained MP free after 2 or 4.5 months of continuous culture (Table 1) and were considered cured.
Table 1. Percent Hybridization of Mycoplasma Assays. All results above 0.6% are positive. Positive and negative controls are supplied by the manufacturer.

<table>
<thead>
<tr>
<th>sample</th>
<th>1 month</th>
<th>2 months</th>
<th>4. 5</th>
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</thead>
<tbody>
<tr>
<td>months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCO</td>
<td>51.60</td>
<td>37.00</td>
<td>ND¹</td>
</tr>
<tr>
<td>CCO Gentamycin</td>
<td>53.80</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CCO Ciprofloxacin</td>
<td>0.15</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>CCOBr</td>
<td>45.20</td>
<td>33.40</td>
<td>ND</td>
</tr>
<tr>
<td>CCOBr Ciprofloxacin</td>
<td>ND</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>negative control</td>
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<td>0.49</td>
</tr>
<tr>
<td>positive control</td>
<td>64.00</td>
<td>73.60</td>
<td></td>
</tr>
</tbody>
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¹ND- not determined.
During the 100 μg/ml CIP treatments all cells became flattened and grew slowly. Two to three passes in normal medium were required for the cells to regain the normal growth characteristics.

Discussion

CCO cells are the cell line of choice for studying channel catfish virus (CCV) (Bowser and Plumb, 1980). It produces higher titers of infectious virus, replicates faster and is less sensitive to variables in tissue culture medium than other CCV permissive cell lines. Personal communications with other labs indicate that mycoplasma contamination is prevalent in this cell line, contraindicating the use of these cells in enzyme studies. Our results indicate that Ciprofloxacin at 100 μg/ml of culture medium can be used to effectively eliminate MP contamination from fish cell lines. Schmitt et al. (1988) were successful in eliminating 7 different species of MP from experimentally contaminated mammalian cell lines using 10 μg/ml CIP for 10 days at 37 C. They found that levels higher than 10 μg/ml affected protein synthesis in specialized cell lines such as hybridomas and lymphokine producing tumor cells. In this study the minimal inhibiting concentration of CIP on the contaminating MP at 30 C was not known and the maintainance of a specialized cell type
was not necessary. Therefore, contaminated cells were cultured in the presence of the highest CIP levels tolerable. A gradient of doses of CIP is recommended in treating other fish cell lines that may be more sensitive or have specialized functions.

The temperature sensitive nature of the MP contaminating these piscine derived cell lines indicates a possible piscine origin rather than the human, bovine or porcine species that commonly contaminate cell cultures. This temperature sensitivity also illustrates the need for incubating MP diagnostic cultures at the normal incubation temperature for the host cell. The genetic probe kit proved to be a simple, sensitive and fast method for screening poikilothermic vertebrate cell lines for MP and could be used routinely in laboratories with access to a liquid scintillation counter.
Literature Cited


Summary

Live vaccine agents should improve the efficiency of vaccination for extensive aquaculture programs. Recombinant live vaccines can be constructed which are safer, more efficient and provide a broader spectrum of protection than naturally attenuated pathogens. This research established the foundation for developing a channel catfish virus (CCV) based vaccine vector system. First, CCV was shown to encode thymidine kinase (Tk). This gene has been shown to be a highly efficient selectable and attenuating marker for selectable recombination in other herpesviruses. Thymidine kinase negative mutants of CCV (CCVAr) and the channel catfish ovary (CCO) cell line (CCOBr) were developed that were pivotal in identifying and biochemically characterizing the viral Tk. Next, the CCV genome was cloned into cosmid and plasmid prokaryotic vectors, allowing the viral Tk gene to be mapped by identifying the cloned fragments which recombined with the Tk negative virus, making it Tk positive. This established the methods for producing recombinant CCV, provided a valuable library for future CCV research and tentatively located the Tk gene.
Vita

Larry Allen Hanson was born to Kay Darlene Harding and Allen Fred Hanson on February 28, 1959 in Portland, OR. He graduated from West Anchorage Sr. High School in Anchorage, AK in June, 1976. He obtained his Bachelor of Science degree on biology from Auburn University, Auburn, AL, in June, 1981. On September 12, 1981, he married Lora Jane Petrie. He worked as a Fish Disease Specialist at the Alabama Fish Farming Center in Greensboro, AL from January, 1983 to September, 1985. He obtained his Master of Science degree in Fish Pathology from Auburn University in December, 1983. He was awarded an Alumni Federation Fellowship and began his Doctoral studies at the School of Veterinary Medicine at Louisiana State University in September, 1985.
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Major Field: Veterinary Medical Sciences

Title of Dissertation: Biochemical Characterization and Gene-Mapping of the Channel Catfish Herpesvirus (CCV) - Encoded Thymidine Kinase, a Selectable Site for Homologous Recombination.

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

April 27, 1990