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論文題目　　Molecular Biological Studies on Equine Herpesvirus type 4 infection

（ウマヘルペスウイルス 4 型感染の分子生物学的研究）

Summary

Equine herpesvirus 4 (EHV-4) is a major cause of respiratory disease in horses throughout the world. It causes significant economic losses to the equine industry because of respiratory problems and lost time for training and performing. Infections of horses with EHV-4 are clinically difficult to be distinguished from those caused by equine herpesvirus 1 (EHV-1), the close relative of EHV-4. Although both viruses cause respiratory disease, only EHV-1 has been established as a major cause of epidemic and sporadic abortion, prenatal mortality and neurological disorders that can range in severity but often including complete paralysis.

The differences in pathogenicity between EHV-1 and 4 seem to be reflected in the cell culture host range for both viruses. EHV-1 can propagate well in many cell lines including, equine, bovine, rabbit, hamster, mouse, monkey, pig and cat derived cell lines while EHV-4 appears to be restricted mainly to equine-derived cells. Primary fetal horse kidney (FHK) cells have been reported to be the most useful type of cells for replication and isolation of EHV-4. However, preparation of primary FHK cells is laborious and finding a permanent source of freshly prepared FHK cells is difficult. Moreover, in vivo studies of EHV-4 pathogenesis have been restricted owing to the lack of suitable small-animal models for this virus.

As a result, generation of recombinant viruses with subsequent understanding of the viral gene functions has been hampered.

The study of thymidine kinase (TK)-deficient mutants of other herpesviruses has received considerable attention for many years. Deletion of this viral encoded enzyme in alphaherpesviruses, such as herpes simplex virus (HSV) types 1 and 2, bovine herpesvirus 1 (BHV-1), and pseudorabies virus (PrV), resulted in a reduction in virulence and the rate of re-activation of latent viruses. Previous reports of EHV-1, the close relative of EHV-4, TK-deficient mutants suggested that they are markedly less pathogenic than the wild-type virus, despite being able to replicate in the host.

Herpesvirus-encoded TKs also have an important role in the mode of action of several acyclic nucleoside analogues [e.g. acyclovir (ACV) and ganciclovir (GCV)] which are potent anti-herpetic drugs. The ultimately active metabolites of most nucleoside analogues are their triphosphates. Studies on the mode of action of ACV have shown that it is phosphorylated in infected cells by the virus-induced TK to ACV monophosphate (ACV-MP). After monophosphorylation, host cellular kinases convert ACV-MP to the di- and triphosphate (ACV-TP). ACV-TP is the active form that inhibits herpesvirus DNA polymerase. Although antiviral chemotherapy is a standard practice in the management of herpesvirus infection in humans, the veterinary use of antiviral drugs is relatively uncommon. Perhaps the most frequently reported use of antiviral drugs in veterinary medicine is for the treatment of feline herpesvirus 1 infections. However, there are some reports declared the efficacy of antiviral drugs against EHV-1, but not EHV-4, in vitro.

Many alphaherpesviruses, such as HSV-1, varicella-zoster virus (VZV), PrV, BHV-1, and EHV-1 attach to target cells by interaction of the virion with the cell-surface heparan sulfate. In HSV-1, PrV, BHV-1 and EHV-1, the viral glycoprotein C (gC) is primarily responsible for this interaction. Evidence for this includes the following: (i) these viruses do not bind to cells surfaces devoid of heparan sulfate, regardless of whether this deficiency is due to enzymatic treatment with heparinase or heparitinase or mutant cells that fail to synthesize heparan sulfate. (ii) Heparin, which is structurally and chemically very similar to heparan sulfate, and heparin binding proteins (e.g. platelet factor 4 and neomycin) inhibit the binding of virions to cells. (iii) Deletion of the gC coding genes of HSV-1, PrV, BHV-1 or EHV-1 results in virions that are impaired in their ability to bind to cells. In contrast to these viruses, HSV-2 gC does not play the key role in viral binding, however, HSV-2 gC is a heparin-binding glycoprotein. Interestingly,

HSV-1 gB is also a heparin-binding glycoprotein and does participate in the binding process to cell surface heparan sulfate, at least when gC is absent from the virion envelope.

Recently, many laboratories reported the cloning of large herpesvirus genomes as bacterial artificial chromosomes (BACs). This technique allows the maintenance and mutagenesis of herpesvirus genomes in *Escherichia coli* (*E. coli*). The previous requirements for selection and rounds of plaque purification in eukaryotic cells to generate recombinants are no longer required. Targeted and random mutagenesis can be introduced easily into the viral genomes while being maintained in *E. coli*. The reconstitution of progeny virions is achieved by transfection of the BAC plasmid into permissive mammalian cells, thereby allowing the generation of mutant viruses more quickly.

Several features make EHV-4 attractive as a backbone for use as a recombinant vaccine and/or as a model for further fundamental studies. Efficient generation of mutants of EHV-4 would significantly contribute to the rapid and accurate characterization of the viral genes. However, generation of recombinant viruses with subsequent understanding of the viral gene functions has been hindered by the absence of suitable cell lines and small-animal models of the infection.

In the current study, the author described the cloning of the genome of EHV-4 as a stable and infectious BAC without any deletions of the viral genes. The feasibility to introduce targeted mutations into the BAC cloned EHV-4 genome will be addressed.

In chapter 1, the genome of EHV-4 strain TH20p was cloned as a stable and infectious BAC without any deletions of the viral genes. Mini F plasmid sequences flanked by *loxP* sites were inserted into the intergenic region between genes 58 and 59. Following electroporation into *E. coli*, the EHV-4 BAC was stably maintained over multiple generations and able to produce infectious viruses when transfected into permissive cells. The *loxP* flanked BAC cassette was excised from the genome of reconstituted virions by growing them in FHK cells that was previously infected with a recombinant adenovirus, AxCANCre, expressing Cre recombinase. Importantly, the resulting recombinant EHV-4 replicated comparably to the wild-type virus *in vitro*.

In chapter 2, the feasibility to introduce targeted mutations into the BAC cloned EHV-4 genome was demonstrated by deleting the gene encoding thymidine kinase. Our findings reported here revealed no significant difference between wild-type EHV-4 and TK-negative strain in their replication cycle in cell

culture. GCV displays a potent activity against both EHV-4 and 1 infection in vitro. On the other hand, EHV-4 appeared to be relatively insensitive to ACV; however, it acquired sensitivity in the TK-transformed cells, in which the transfected EHV-1 TK was able to phosphorylate the drug. Therefore, the inefficient phosphorylation of ACV by EHV-4 TK was responsible for the observed in vitro insensitivity of this virus to the antiviral drug action of ACV.

In chapter 3, the role of gC in EHV-4 infection was analyzed. Our results showed that gC-negative mutant did not exhibit markedly lowered virus titers and the plaque sizes were reduced by around 12 % when compared to parental viruses. Although gC is nonessential for EHV-4 replication in cell culture, yet it plays a role in the adsorption of the virus to cell surface component that includes a heparinlike moiety. Furthermore, we found that gC protects the virus from complement-mediated neutralization.

As described above, we described the cloning of EHV-4 genome as infectious BAC for the first time. The EHV-4 BAC clone produced here will be an invaluable resource for the production of recombinant viruses. This system will be a very useful tool for analyzing the function of different genes of this important equine viral pathogen and may allow the production of recombinant viral vaccines to control EHV-4 and EHV-1. Furthermore, the construction of EHV-4 BAC is an important step towards understanding the aspects of difference in pathogenicity between EHV-1 and EHV-4. We also declared that despite the high sequence and structural similarity, more than 90 %, between EHV-4 TK and its EHV-1 homologue, ACV was more efficiently phosphorylated by EHV-1 TK than by EHV-4 TK. This explains why ACV is an attractive candidate as antiviral drug against EHV-1, but not EHV-4, infection in horses. However, GCV displayed a potent activity against both EHV-4 and 1 infection in vitro. But there is no direct clinical application due to its high price cost. Finally, we described experimentally the role of EHV-4 gC in the adsorption of the virus to cell-surface heparan sulfate. However, we also demonstrated that gB, another heparin-binding glycoprotein, could also mediate the adsorption of EHV-4 to the cells. Moreover, our findings reported here revealed that gC may play roles in the protection of EHV-4 from the host immune system.