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STRUCTURE AND ORGANIZATION OF THE GENOME OF FELINE HERPESVIRUS-1

BY

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ABSTRACT

STRUCTURE AND ORGANIZATION OF THE GENOME OF FELINE HERPESVIRUS-1

by

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The structure and organization of the genome of feline herpesvirus-1 (FHV-1) were studied using a variety of biochemical techniques. The genome of FHV-1 was found to be similar in structure and general organization to the genomes of other alpha herpesviruses. The FHV-1 genome is approximately 134 kilobase pairs (kb) in size and is composed of two covalently joined segments. The longer segment is 103 kb in size and composed of unique DNA. The shorter segment is 31 kb in size and is composed of an 8 kb unique region which is flanked by 11 kb inverted repeat regions. The short region can invert relative to the long giving the genome two possible isomeric forms. A set of recombinant bacteriophage clones containing most of the FHV-1 genome was isolated and used to construct Sal I, Hind III and Eco R1 restriction endonuclease cleavage maps of the viral genome. These clones and restriction maps were used in subsequent studies to map the immediate early and late genes of FHV-1.

Radiolabeled FHV-1 immediate early RNA hybridized to restriction fragments of FHV-1 DNA which are located primarily within the inverted repeat regions in the short segment of the viral genome. Two RNA species with sizes of 5 and 1.8 kilobases were transcribed from the FHV-1 immediate early gene region. In addition, two immediate early polypeptides

with molecular weights of 155 and 120 kilodaltons (kd) were identified by in vitro and in vivo translation of FHV-1 immediate early RNA.

FHV-1 late RNA was hybrid selected by the individual cloned restriction fragments and the in vitro translation products of the eluted RNA were analyzed by SDS-PAGE. The results indicated that FHV-1 late genes are distributed throughout the genome but are primarily found in the unique long region. The region of the genome contained within Sal I fragment E contained a cluster of 3-4 late genes two of which may code for precusors to viral glycoproteins.

Five herpesvirus strains which were isolated from dogs and were antigenically related to FHV-1 were analyzed by restriction endonuclease digestion, Southern blotting and immunoprecipitation. The results indicated that the canine isolates were antigenically and genetically very similar to FHV-1. Comparison of the restriction digest patterns of DNA from the C-27 prototype strain of FHV-1, FHV-1 field isolates, and FHV-1-like canine isolates indicated that the genome of FHV-1 is very stable. Most of the restriction fragments showing size heterogeneity were located at the viral termini and within the inverted repeat sequences.

to Susan

and

my family

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INTRODUCTION

Feline herpesvirus-1 is a significant cause of upper respiratory tract disease in cats. Because of the worldwide distribution and severity of FHV-1 infections, the biological properties of FHV-1 have been well studied and several modified live and attenuated virus vaccines have been developed. Few studies, however, have been conducted to investigate the biochemical structure and function of this virus. The goal of the research presented in this thesis was to characterize the basic structure and organization of the FHV-1 genome. The experimental approaches to achieve this have been established in the analysis of other herpesviruses.

There are several reasons that justify the initiation of detailed biochemical studies on this new herpesvirus system. The first reason is that FHV-1 is a significant pathogen of cats. Increased understanding of the biochemical structure of FHV-1 will allow for a more complete understanding of the pathogenesis of this virus. For example, once the basic structure of the viral genome has been established, studies can be conducted to detect FHV-1 DNA in tissues from latently infected cats and to determine the physical state of the latent DNA. Restriction digests of DNA from a number of field isolates and vaccine strains could be analyzed to identify the sites of potential virulence determinants. This type of information would be useful in the development of new attenuated virus vaccines and in the evaluation of new strains. Also, FHV-1 antigens can be expressed via recombinant DNA techniques and used for the construction of more effective and economical vaccines against FHV-1.

The second reason is that the information obtained in these studies will contribute to the rapidly expanding pool of information available concerning the molecular biology of herpesviruses. There is evidence that the genomes of various members of this important group of viruses are arranged in a similar manner. Some highly conserved genes are located at similar postions on the genomes of different herpesviruses. To expand these studies, gene mapping data is needed for a variety of herpesviruses rather than from a few well-characterized viruses.

Finally, the FHV-1 infection of the cat may provide a way to study herpesvirus infections in the natural host. Herpesviruses which cause disease in humans are usually studied in experimental animals which are not the natural hosts of these viruses. The results obtained in these animal models might not reflect the true nature of the virus-host interaction. It will be especially important to test the efficacy of new vaccines and antiviral agents in natural host system where all aspects of the infection can be measured.

Each of these points is discussed in greater detail in the Literature Review (Chapter 1). Chapters 2-5 contain the research data and are presented in manuscript form. Unfortunately, this format results in some repetitiveness since certain information is included in the Introductions and Methods sections of all of the manuscripts.

Chapter 2 describes the physical structure of the FHV-1 genome, the isolation of the recombinant bacteriophage clones, and the construction of restriction maps of the viral genome. This information forms the basis for the work completed in Chapters 3 and 4. Chapter 3 describes the identification of the immediate early genes and polypeptides of FHV-1 while Chapter 4 contains an analysis of FHV-1 late gene organization.

In Chapter 5, several herpesvirus isolates that were obtained from clinically ill dogs were examined and found to be nearly identical to FHV-1. The experiments in this chapter provided an interesting way to compare the restriction digest patterns of a number of FHV-1 isolates and investigate genetic heterogeneity in FHV-1. The locations of the restriction fragments showing size variation could be determined using the map generated in Chapter 2.

Chapter 1

Literature Review

Paul A. Rota

Part I. Feline Herpesvirus-1

Introduction

Feline herpesvirus-1 (FHV-1) was first isolated by Crandell and Maurer (1958) from kittens with acute upper respiratory tract disease. The disease was later designated feline viral rhinotracheitis and the viral agent referred to as feline rhinotracheitis virus (Crandell and Despeaux, 1959). Subsequent work by Ditchfield and Grinyer (1965) revealed that this virus was a herpesvirus. Based on its biological properties, feline rhinotracheitis virus was placed in the alpha herpesvirus subfamily and classified as feline herpesvirus-1 (Roizman, 1980). FHV-1 has been shown to be antigenically distinct from FHV-2, a herpes-like virus that causes urethral obstruction in cats (Fabricant, 1984). For simplicity, the term FHV-1 will be used throughout this review.

FHV-1 has a worldwide distribution and is responsible for a large proportion of upper respiratory tract disease in cats (Povey, 1976). The other virus which is a major cause of respiratory disease in cats is feline calicivirus (a picornavirus) (Studdert, 1978).

The most recent review of FHV-1 was written by Povey (1979). This article provides a very complete source of information concerning the morphology and physical properties of FHV-1 and gives an excellent overview of FHV-1 induced disease in cats. The most interesting research on FHV-1 since this review has focused on the characterization of the biochemical structure of FHV-1, identification of the tissue or tissues of FHV-1 latency, and evaluation of the immune response to FHV-1 in cats at defined times post vaccination and during reactivation. My goal in this

literature review is to discuss both the earlier work on FHV-1 and incorporate the more recent research about this virus.

Viral Morphology, Growth Characteristics and Host Range

The morphology of FHV-1 is similar to that of most herpesviruses (Ditchfield and Grinyer, 1965). The viral capsid is hexagonal and has an average diameter of 108nm. A membranous envelope surrounds the capsid giving the complete virion an average diameter of 178 nm. The envelope contains viral glycoprotein antigens that are important in virus-host cell interaction. As is the case for other herpesviruses, the infectivity of FHV-1 is greatly reduced by exposure to lipid solvents such as ether, chloroform (Johnson, 1966), and sodium deoxycholate (Bartholomew and Gillespie, 1968). The infectivity of FHV-1 is also destroyed by treatment with proteases such as trypsin (Johnson, 1966).

FHV-1 is very sensitive to heat and humidity (Povey, 1979). Cell culture fluids lose viability rapidly at ambient temperaturers. At 23°C, the virus losses 99% of is viability in 5 minutes if the relative humidity is greater than 30%. Virus stored at -50°C will still lose 90% of its viability in 5 months.

FHV-1 will replicate in primary, secondary or established cells lines of feline origin (Lee, et al., 1968). Cells of non-feline origin will not support the growth of FHV-1. For most *in vitro* studies, FHV-1 has been grown in Crandell-Rees feline kidney (CRFK) cells. At an input multiplicity of 1 pfu/cell, infective virus was first detected within CRFK cells at 6 to 8 hours post-infection and in the extracellular fluid at 9 hours post-infection. Direct cell to cell spread of infectious virus was detected at 6 to 7 hours post-infection (Wardley, et al., 1976). By 30

hours, the average yield was 200 virions per infected cell (Tegtmeyer and Enders, 1969) and most of this progeny virus remained cell associated. The growth characteristics of FHV-1 were different in tracheal ring organ cultures (Milek, 1976). In this system, the viral growth rate was slower and the final viral titer was lower than in cell culture monolayers. This indicates that growth studies using monolayer cultures might not accurately reflect the in vivo growth situation. It would be interesting to compare the in vitro growth characteristics of field strains to attenuated vaccine strains. Unfortunately, at this time, no studies of this type have been attempted.

The cytopathic effect induced by FHV-1 is similar to that of other herpesviruses. Changes in infected cells include syncytia formation and/or a rounding of cells followed by degeneration. The nature of these virus induced changes depends on the input multiplicity of virus. FHV-1 can spread from cell to cell by the intracellular route (Wardley, 1976). Infected cells may detach from the monolayer leaving holes in the monolayer which can reach 1 cm in size (Crandell et al., 1960). Plaque assays have been developed for FHV-1. Differences in plaque morphology in CRFK cells have been observed and can be used to differentiate between vaccine and field strains of virus (Gaskell, et al., 1985).

The only species susceptible to FHV-1 infection is the cat. Experimental infections of many other mammals have failed to produce disease or evidence of virus replication (Povey, 1979). Because of its limited in vivo and in vitro host range FHV-1 is often compared with varicella-zoster virus (VZV), the causitive agent of chicken pox in humans. In contrast, other alpha herpesviruses, such as pseudorabies virus

and herpes simplex virus will infect a wide variety of cells in culture and experimental animals (Roizman, 1980).

Serological studies using polyclonal antisera on a number of FHV-1 isolates from different parts of the world revealed that the virus has only one serotype. All of the test isolates were serologically identical to the C-27 prototype strain of FHV-1 (Metianu and Viart, 1974). Strains of modified virulence do exist and several attenuated virus vaccines have been developed (Slater and York, 1976) . These vaccine strains have no observable antigenic differences from field isolates based on the serological methods used. Serological studies have also shown that there is no cross reactivity between FHV-1 and FHV-2 (Fabricant, 1984), herpes simplex, pseudorabies (Crandell and Weddington, 1967), and infectious bovine rhinotracheitis virus (Johnson and Thomas, 1966). However, Evermann et al. (1982) has reported that antiserum against FHV-1 was able to neutralize the infectivity of canine herpesvirus. The biochemical basis for this cross reactivity has not been defined.

Biochemical Properties

While the biological properties of FHV-1 have been studied in great detail, little is known about the biochemical structure of the virus. Recently, two groups have reported on the analysis of FHV-1 virion structural components. Maes et al. (1984) identified at least 17 virion associated polypeptides on the C-27 strain of FHV-1. Three of these polypeptides were glycosylated and had approximate molecular weights of 105, 68 and 60 kilodaltons (kd). These glycoproteins were present on the membranes of infected cells and the 105 kd glycoprotein was detected in the supernatant medium. All three glycoproteins were recognized by a goat

anti-FHV-1 serum which had a high virus neuralization titer. In another study, Fargeaud et al. (1984) used gradient gel analysis to detect 23 polypeptides on virions of the IFFA RTF-CO strain. FITC-labeled Con A was used to identify 6 glycosylated peptides on FHV-1 virions with sizes of 125, 116, 112, 83, 70, and 62 kd. The sizes of the glycoproteins detected on virions by Fargeaud et al generally correspond to the sizes detected by Maes et al. with the exception of the 83 kd molecule. It is possible that this molecule is a breakdown product of one of the larger glycoproteins or represents an antigen unique to the strain studied. It is unclear, as to whether the largest glycoprotein band or bands identified in both studies (125-105 kd) contains only one or a mixture of several different glycoproteins. Often, variations in glycosylation cause individual glycoproteins to migrate as more than one band in SDS-PAGE analysis. Further studies using monospecific or monoclonal antibodies directed against individual viral antigens will be needed to construct a more complete antigenic profile of FHV-1. Generally, the number and size distribution of FHV-1 virion proteins and glycoproteins is consistent with the antigenic profiles of other herpesviruses (Spear, 1976).

The DNA of FHV-1 has been found to have a density of 1.705 g/cm^3 corresponding to a G+C content of 46% (Roizman, 1980). Only one report has been published on the molecular biology of FHV-1 (Hermann et al., 1984). In this study, restriction enzyme digestion patterns of DNA from a number of FHV-1 clinical isolates and vaccine strains from Europe, the United Kingdom and the United States were compared. The results showed that there was little difference in the restriction digestion patterns of the DNA from various isolates. The restriction patterns of DNA from virus isolated from tissues other than the upper respiratory tract were not different

from the digestion patterns observed for DNA from respiratory isolates. Also, no major differences were found between the restriction patterns of DNA from field isolates and DNA from a temperature sensitive vaccine strain. The restriction patterns of DNA from a trigeminal ganglion isolate showed no differences when compared to DNA from the original input virus suggesting that the virus is not altered during latency. The results of this study indicated that FHV-1 has a very stable genome and this is another characteristic which it shares with VZV (Martin et al., 1982). It appears that it may be difficult to differentiate between various strains of FHV-1 based on restriction endonuclease analysis as is possible with other herpesvirsus such as bovine herpesvirus (Engels et al., 1981) and pseudorabies virus (Paul et al., 1982). However, more studies on the molecular epidemiology of FHV-1 need to be conducted using a variety of restriction enzymes on a large number of isolates.

From the results of restriction enzyme analysis of Hermann et al. (1984), the size of the FHV-1 genome was estimated to be approximately 80 megadaltons. It is obvious that further basic research is needed to characterize the physical structure of the FHV-1 genome.

The Disease

FHV-1 occurs on a worldwide basis and 50-70% of cats in Europe, North America and Asia have virus-neutralizing antibodies to FHV-1 (Povey and Johnson, 1971, Ellis, 1981). The clinical syndrome produced in cats infected with FHV-1 was recently reviewed by Gaskell and Goddard (1984). Upper respiratory tract disease in cats is generally uniform and the clinical signs include depression, sneezing, fever, and copious ocular and nasal discharge. Conjunctivitis may also develop. The severity of the

clinical signs and the length of the incubation period (usually 2-6 days) depend on the infecting dose (Gaskell and Povey, 1979). The disease is usually resolved within 10-20 days but may become chronic in some cats. The mortality rates are high among kittens and debilitated cats which may develop a generalized infection (Gaskell and Goddard, 1984). Less common clinical signs include skin ulcerations and nervous system disorders (Flecknell et al., 1979).

Cats are highly susceptible to FHV-1 infection and the virus is naturally spread by direct contact via the oral, intranasal and conjunctival route. The intranasal route is most often used to induce experimental infections. Congential infection of kittens following vaginal instillation of virus has also been demonstrated (Bittle and Peckham, 1971). Abortions associated with FHV-1, however, are most likely secondary effects of the severe debilitation caused by the upper respiratory disease since virus was not isolated from aborted fetuses (Hoover and Griesemer, 1971).

During the acute phase of the disease, virus can be readily isolated from the nasopharynx, tonsils and upper trachea at titers of up to 10^6 $TCID_{50}$/ml. Only rarely has FHV-1 been found in the serum (Gaskell and Povey, 1979).

FHV-1 infection has more severe osteolytic effects in kittens which often suffer necrosis of the long bones in addition to the necrosis of the turbinate bones which frequently occurs as a result of infections in adult cats (Povey, 1979).

Despite its susceptibility to the environment, FHV-1 remains the most significant viral upper respiratory tract pathogen of cats. Indirect transmission through contact with infected material probably only occurs

in areas where with high cat density such as boarding catteries (Gaskell and Goddard, 1984). The majority of viral spread occurs as a result of close contact bewteen animals. Like for many other herpesviruses, the successfull persistence of FHV-1 in the cat population is partially dependent upon the ability of the virus to establish latent infections. Susceptible cats are infected horizontally either by virus shed from cats with acute disease or by asymptomatic carrier cats which are shedding virus from a reactivated latent infection. Cats with latent FHV-1 infection shed virus intermittently (Gaskell and Povey, 1977) and this shedding is not always accompanied by a recrudescence of clinical signs (Gaskell and Goddard, 1984). During such a reactivation phase, virus can be isolated from the oro-phanyngeal secretions though at lower titers (10^5 $TCID_{50}$/ml) than during acute disease (Gaskell and Goddard, 1984).

FHV-1 Latency

The presence of latent FHV-1 infections has been demonstrated only in a very preliminary way. Latent FHV-1 virus has been experimentally reactivated in vivo following administration of corticosteroids. A recent study (Ellis, 1981) revealed that virus could be reactivated from 25.8% of healthly cats by this method. Reactivation can also be triggered by stress and experimenatlly initiated rehousing stress was sufficient to induce virus shedding in 18% of carrier cats (Gaskell and Povey, 1982). In this same study, corticosteroid treatment was able to induce reactivation in 64% of these carrier cats. Approximately 40% of latently infected queens shed virus during the post partum period. Though none of the kittens examined in this study developed clinical signs, 50% developed latent

infections. Since there was no evidence for in utero infection, it appears that the kittens were infected by virus shed by the queen during the immediate post partum period and that passive antibody was able to prevent the disease in the kittens but not the establishment of a latent infection.

Current research efforts have focused on attempting to identify the anatomical site of FHV-1 latency. Since herpesviruses frequently establish latent infections in neural tissue (Hill, 1985), these tissues were the first to be examined for FHV-1 latency. Gaskell and Povey (1979a) were able to isolate FHV-1 from homogenized trigeminal ganglia or olfactory bulbs (Gaskell and Goddard, 1984) of shedding carrier cats. However, Ellis (1982) examined a number of tissues, including trigeminal ganglia, from carrier cats using homogenization, explant and co-cultivation techniques and was unable to recover FHV-1 from any of these tissues even though the cats were shedding at the time of sampling. More recently, Gaskell et al. (1985) have been able to recover FHV-1 from the trigeminal ganglia of 3 of 17 cats latently infected with field virus using a tissue fragment culture technique. Interestingly, these nervous tissue isolates had plaque morphologies similar to a vaccine strain of FHV-1 and not the input virus. Though similar results have been reported for bovine herpesvirus (Nettleton et al., 1984), Gaskell's study was complicated by the fact that the cats from which the virus was isolated were being treated with BVdU ((E)-5-(2-bromovinyl)-2'-deoxyuridine).

The inability to consistently detect latent FHV-1 by explantation or co-cultivation techniques may reflect a deficiency of these methods to recover FHV-1 rather than an absence of virus in the explanted tissue. Only a small proportion of the cells in a tissue sample may contain virus

and the amount of virus shed from these tissues during reactivation may be very small. This low virus yield combined with the added problem of the extreme labilty of FHV-1 may result in levels of virus that are below the detection limits of a cell culture system. More sensitive techniques such as in situ hybridization with nucleic acid probes need to be done on a variety of tissues from carrier cats before the site of FHV-1 latency can be unequivocally determined.

FHV-1 Vaccines

Because of the prevalence and severity of FHV-1 infections in cats, a large effort has been directed towards the development of vaccines. One of the most useful attenuated vaccines was developed by Slater and York (1976) by serial low temperature passage (25°C) of FHV-1 in CRFK cells. Cats intranasally exposed to vaccine virus at the 171th passage level did not develop clinical signs and were clinically protected from challenge with a virulent strain of the virus. This vaccine also gave protection when administered by the intramuscular route. Later work (Orr et al., 1978) revealed that cats given the same type of vaccine by the intramuscular route did not develop clinical signs but replicated challenge virus and a proportion of them became carriers. In contrast, when the vaccine was administered via the intranasal route (Orr et al., 1980), there was little replication of a virulent challenge virus given 5 days post-vaccination. In addition, virus was not recovered from the intranasally vaccinated, challenged cats after corticosteroid treatment. Though only a small number of cats were tested for latent infection, the results of this study indicated that vaccine given by the intranasal route was able to prevent the disease and, more importantly, may have prevented

the cats from becoming carriers. This work also suggested that local immunity which was elicited by antigen stimulation of mucosal surfaces may have an important influence in determining the outcome of a herpesvirus infection and the frequency of latent infection in the natural host.

Immunity

Researchers in the United Kingdom have attempted to examine the nature of the immune response elicited by FHV-1 in cats. Challenge studies had shown that full protection was not achieved with an intranasal vaccine until 6 days post-vaccination (Cocker et al., 1984a). Later work was aimed at characterization of this response (Cocker et al., 1984b). Even though vaccinated cats were resistant to FHV-1 challenge by 6 days post-vaccination, analysis of the serum and nasal secretions of these cats revealed only low levels of neutralizing antibodies and interferon. This resistance was specific for FHV-1 since FHV-1-vaccinated cats challenged with feline calicivirus were infected and developed clinical signs. Lymphocytes from both blood and tonsil showed no proliferative response to FHV-1 antigens. It was concluded that "a local cytotoxic cell" response in the tonsil, an important site in primary virus replication (Gaskell and Povey, 1979a), was able to prevent viral dissemination and was responsible for the observed protection against FHV-1. Tonsils have been shown by other workers to be important sites of local immunity against infectious agents (Drucker et al., 1979).

In a study of the mechanisms involved in immunity to herpesviruses, Wardley et al. (1976) demonstrated that during the acute phase of the disease the spread of FHV-1 was limited by antibody-complement mediated

lysis and antibody directed cellular cytotoxicity (ADCC) mediated by both lymphocytes and macrophages. These responses were detectable by 6 hours post-infection when intracellular spread of the virus begins. Direct cytotoxicity was also noted but the responses were variable and did not occur early enough in the infection to limit viral spread. These workers postulated that defects in the immune responses needed to control viral dissemination within the host may lead to the establishment of latent infections and to more severe recrudescent disease.

A similar study by Goddard and Gaskell (1984) attempted to evaluate immune function in cats during reactivation of latent FHV-1 infections. A natural stimulus, rehousing stress, was used to induce reactivation. No significant suppression of specific or non-specific immunity was associated with FHV-1 reactivation and subsequent shedding. In the shedder cats, reactivation served to boost specific immunity. The shedder cats could be differentiated from the non-shedders in that the shedders had a lower baseline cytotoxic antibody levels, a greater stress response to rehousing and had experienced more severe primary disease episode than non-shedders. These results indicate that specific resistance mechanisms may not be important in controlling herpesvirus reactivation.

It has been noted in the study of other herpesvirus infections that the levels of effector functions may be more important than memory functions. For example, in HSV systems, levels of lymphokines and interferon changed markedly during virus reactivation (Sheridan et al., 1982). Such parameters still need to be examined in FHV-1 infections.

FHV-1 as a Natural Host Model

It is important to define all of the immune responses that affect the outcome of a herpesvirus infection (Nahmias and Coleman, 1984). Frequently there are diffuculties in establishing the validity of observation made in experimental animals. Therefore, there is great interest in developing suitable natural host models for herpesvirus infections. These systems could be used to fully evaluate all phases of the viral infection process including the establishment, maintenance and reactivation of latent infections. Also, the ability of candidate vaccines and vaccine delivery procedures to prevent clinical disease and latent infections could be tested in these systems. In the near future, it will be especially important to test the effectiveness of immunity induced by herpesvirus vaccines containing isolated viral antigens produced via recombinant DNA techniques in natural host systems. For example, a recombinant glycoprotein vaccine against pseudorabies produced protective immunity in mice but not in pigs (Robbins et al., 1984).

At this time, the major drawback to using the FHV-1 infection of the cat as a natural host model is a lack of information about the system. This can only be remedied by continuing research efforts. It will be important to have a detailed understanding of the biochemical and antigenic structure of FHV-1. Fortunately the methodology for completing these studies has been well established by other herpesvirus workers.

One of the major advantages to using FHV-1 as a natural host model is that reactivation of latent infections can be induced by natural, non-pharmacological means. This would allow for an accurate evaluation of immune function in cats during the reactivation process and would be completed without the complications of general immunosuppression caused by

corticosteroid treatment. Hopefully, this system will allow the molecular biological events responsible for the induction of virus reactivation in a natural host to be defined as well. Finally, FHV-1 causes widespread disease in cats. Therefore, any novel vaccine strategies can be tested in a large, naturally susceptible population. An added incentive is that these novel vaccines will have obvious commercial potential.

A few similarities can be found beteween FHV-1 infections of cats and HSV infections of humans. Both viruses cause severe disease in newborns exposed when the virus is shed from a reactivated infection in the mother. Both viruses also have been shown to cause conjunctivitis and severe encephalitis in newborns (Orr, 1983). FHV-1 may therefore be a good model for research on neonatal herpesvirus infections.

The first evidence for interest in FHV-1 as a natural host model for human herpesvirus infections is provided by a very recent paper in which FHV-1 was used to study inner ear infections (Falser, 1984). This is interesting since inner ear infections in humans are sometimes caused by reactivation of latent herpesvirus infections (Davis et al. , 1981).

Part II. The Herpesviruses: An Overview

Introduction

The purpose of the following section is to provide a general introduction to selected characteristics of herpesviruses. The topics covered are those which will be examined for FHV-1 in this thesis. Treatment of these topics can not be exhaustive because of the

considerable amount of research that has been published about herpesviruses. Rather, important principles will be illustrated using examples from well characterized herpesvirus systems such.

The herpesviruses include a vast family of DNA viruses which infect a large number of eucaryotic species. Herpesviruses have been observed to infect fungi, fish, oysters, amphibians, birds and many mammals including most domestic animals, monkeys and humans. The common features of the herpesviruses are: identical virion construction, a large double-stranded DNA genome, and a regulated reproduction cycle (Feldman, 1980). Another important property of this virus group is their ability to persist in the natural host for long periods of time after primary infection. These latent asymptomatic infections can periodically reactivate allowing virus to spread horizontally through direct contact with the individual in which reactivation occurred.

Herpesviruses have been divided into three subfamilies based on their biological properties (Honess and Watson, 1977). FHV-1 along with pseudorabies virus (PRV), herpes simplex virus (HSV) and varicella-zoster virus (VZV) belong to the alpha herpesviruses. Viruses in this subfamily cause self-limiting skin or upper respiratory tract infections. The site of virus persistence is usually the central nervous system. Beta herpesviruses are more commonly referred to as the cytomegaloviruses. They cause little overt disease except in neonates or immunocompromised adults. The gamma herpesviruses are composed of lymphotropic viruses such as Epstein-Barr virus and Marek's disease virus. Members of this group cause lymphoproliferative diseases and can persist in lymphocytes. They may cause fatal leukemias and lymphomas.

Morphological Characteristics

Early electron microscopic studies (Wildy, 1960) revealed that herpesvirions contained linear double-stranded DNA packaged within a hexagonal capsid composed of 162 capsomeres. This nucleocapsid is surrounded by tegument proteins which are contained within a lipid envelope (Wildy, 1971). Later, electrophoretic analysis showed that HSV had a total of 25-30 peptides present in the virion. As much as 40% of virion protein was found to be part of the viral envelope (Kaplan and Ben-Porat, 1970). Many of the envelope proteins are glycoproteins (Choppin and Scheid, 1980, Sormiento et al., 1979), and have important functions in viral infectivity. These molecules are involved in the attachment to and penetration of host cell membranes (Spear, 1979). In addition, because glycoproteins are expressed on the surface of the infected cell, they are important targets of the host's cellular and humoral immune response to viral infection. For this reason, there is a great deal of interest in developing glycoprotein vaccines against herpesviruses by various biochemical and molecular biological techniques. The most advanced work in this regard has centered on the use of glycoprotein D (gD) as a vaccine for herpes simplex infections in humans (Watson et al., 1982).

Herpesvirus capsid proteins can be purified from the virion envelope for biochemical analysis. Herpesviruses have one major capsid protein with a molecular weight between 110 and 150 kilodaltons. In HSV and PRV the capsid comprises 28% of total virion protein (Courtney et al., 1971).

Contained within the capsid are core proteins which are phosphorylated and associate with the viral DNA. These proteins are unstable and diffucult to purify once the capsid has been removed. HSV has been found to contain two core proteins (Olshevshi and Becker, 1970).

The capsid of HSV is surrounded by a series of eight globular proteins referred to as tegument proteins (Roizman, 1980). The function of these molecules is not well defined.

Viral DNA

Herpesvirus DNA is associated with the core proteins and located within the viral capsid. The physical properties of many herpesvirus DNA's have been determined using modern molecular biological techniques. The entire genomes of many herpesviruses have been cloned in plasmid or bacteriophage vectors. The availability of cloned viral DNA has allowed detailed restriction endonuclease cleavage maps and extensive DNA sequence data to be obtained for a number of important herpesviruses (Sullivan et al., 1984, Davison and Scott, 1983, Mayfield et al., 1983, Spector et al., 1982, Henry et al., 1981, Goldin et al., 1981, Hayward et al., 1975).

The G+C content of herpesvirus DNAs, as determined by isopycnic density gradient centrifugation, has been shown to vary considerably. Values range from a low of 33% for canine herpesvirus to a high of 77% for monkey B-virus DNA (Roizman, 1980). The molecular weights of the genomes of many herpesviruses have been determined by equilibrium sedimentation, electron microscopy, and restriction enzyme analysis. These values range from approximately 120 kilobase pairs (kb) for channel catfish virus DNA (Honess and Watson, 1977) to 230 kb for cytomegalovirus DNA (DeMarchi et al., 1977).

Varying amounts of genetic relatedness have been found between the genomes of various herpesviruses. HSV 1 and 2 share 50% homology (Feldman, 1980). Marek's disease virus has been found to have extensive homology with the vaccine virus, herpesvirus of turkeys, when lower stringency

hybridization conditions are used (Gibbs, et al, 1984). Limited regions of homology have been noted between the DNA's of other herpesviruses. Davison and Wilkie (1983) used molecular hybridization to identify regions of homology between the genomes of HSV-1, HSV-2, PRV, VZV, and equine herpesvirus (EHV). Their results indicated that herpesvirus genes are highly conserved and that their organization on the viral genome is similar. This homology has been useful in gene mapping studies. For example, a glycoprotein gene on the pesudorabies virus genome was recently identified by hybridization with a HSV DNA probe containing the HSV glycoprotein B gene (Robbins et al., 1985).

Herpesvirus genomes were placed into three classes depending upon the arrangement of unique and repeat DNA sequences on the molecule (Ben-Porat and Kaplan, 1982). These arrangements are shown in Figure 1. HSV has a class 3 genome which contains two sets of inverted repeats. One set of repeats brackets a long unique sequence and the other brackets a short unique region. In this arrangent, both the long and short unique regions can invert relative to each other giving rise to a genome with with four possible isomeric forms. PRV, VZV, and equine herpesvirus-1 have class 2 genomes. In this configuration, only the unique short region is bracketed by inverted repeats. The short region can invert relative to the long producing two possible genomic isomers. Class 1 genomes are found in Epstein-Barr virus and channel catfish virus. These molecules contain long and short unique DNA regions interspersed with repeat regions. These repeats are not in the inverted orientation and genomes with this structure have only one isomer. The location and arrangement of the repeat sequences within a herpesvirus genome can be determined by electron microscopy (Ruyechan et al., 1983) of denatured and reannealed DNA

Figure 1. Organization of the genomes of herpesviruses. Us and Ul designate the unique short and unique long sequences, respectively. Repeat sequences are designated by rectangles; for repeats with inverted complementarity rectangles are cross-hatched. Arrows indicate possible orientations of repeat sequences. (adapted from: Ben-Porat, 1982)

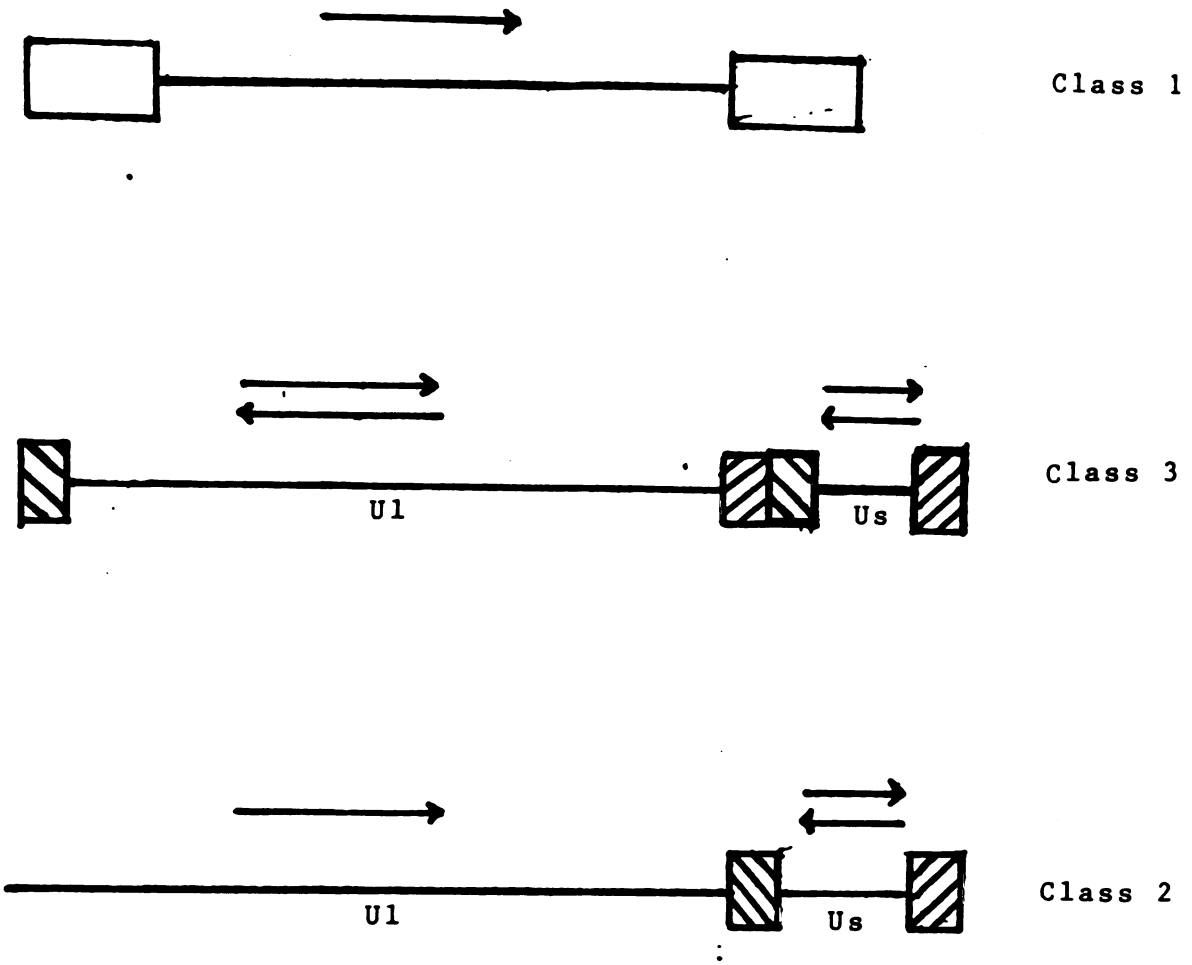


Figure 1.

molecules and by restriction endonuclease digestion and blot hybridization studies (Sullivan et al., 1984, Henry et al., 1981).

Recent studies have indicated that some herpesviruses might have genome structures belonging to more than one class. A vaccine strain of PRV has been shown to have inverted repeats bracketing the long unique region as well as the short (Lominiczi et al., 1984a). This strain has a four isomer genome. Kinchington et al., (1985) found that a small population of VZV genomes have inverted long unique regions. It will be interesting to determine if such novel genomic structures exist in other herpesviruses.

Although the issue is still being debated, most studies with HSV and PRV have shown that the different isomers of the genome are functionally equivalent (Roizman, 1979, Ben-Porat and Kaplan, 1985). Virions containing the different isomers were isolated from infected cells in equimolar amounts. Each isomer of HSV and PRV has been shown to be infectious since infection of cells with a single isomer yields progeny virus with equimolar amounts of the possible isomers.

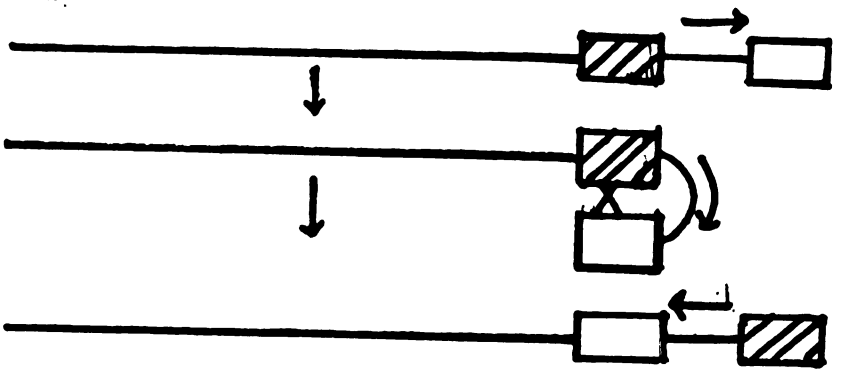
Two models for isomerization of PRV based on crossing over between different viral DNA molecules have been proposed by Ihara et al., (1982) and Ben-Porat (1982). These models are diagrammed in Figure 2.

Genetic Variability

The molecular epidemiology of herpesvirus infections has been studied using restriction endonuclease digestion analysis. This method is useful to accurately trace the source and monitor the spread of infections as well as provide a measure of genetic variability in herpesviruses. Varying amounts of restriction fragment heterogeneity have been found

Figure 2. Models for equalization of repeats in herpesviruses. Crosses indicate sites of recombination. Rectangles with and without cross-hatching indicate inverted repeats. (adapted from: Wagner, 1985)

Model I



Model II

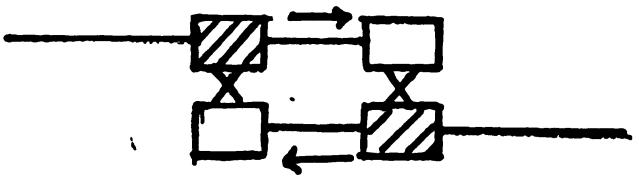


Figure 2.

between different isolates of a given herpesvirus. For example, VZV has a very stable genome and restriction digest analysis of DNA from a number of strains revealed very similar fragment patterns (Martin et al., 1982, Richards et al., 1979). The genomes of PRV (Paul et al., 1982) and HSV (Buchman et al., 1980) contain more variability between isolates. Most of the variability is limited to size differences in the restriction fragments which map within the inverted repeat regions of the genome (Ben-Porat and Kaplan, 1985, Roizman, 1979) rather than a gain or loss of partcular restriction sites. The genome of herpes saimiri has been shown to be even more variable than the viruses mentioned above (Desrosiers et al., 1982).

Restriction endonuclease digestion analysis of herpesvirus DNA has been used with varying amounts of success to evaulate the pathogenicity of various isolates and to map virulence determinants. A deletion in the unique short region of the genome of avirulent vaccine strains of PRV can be identified by restriction digest analysis (Lomniczi et al., 1984a). This deletion maps to a site which codes for a major glycoprotein antigen (Mettenleiter et al., 1985). However, further studies (Lomniczi et al., 1984b) revealed that attenuation in PRV is a multigenic event and that these vaccine strains had genetic alterations in other regions of the genome that were not evident by restriction digestion analysis and could only be detected using complementation analysis.

However, in some cases herpesvirus strains of varying pathogenicity can be identified using restriction digest analysis. For example, consistent differences between infectious bovine rhinotracheitis (severe infections including abortions) and infectious pustular vulvovaginitis (harmless infections) strains of bovine herpesvirus-1 have been detected

by restriction enzyme analysis (Engels, 1981) even though these strains appear anitgenically identical. In a similar study of this type, fetal and respiratory isolates of equine herpesvirus were differeniated based on restriction digest patterns (Studdert et al., 1981).

Herpesvirus Gene Mapping

A wide variety of techniques has been used to determine the locations of herpesvirus genes. Several of these methods will be discussed briefly in this section.

HSV 1 and HSV 2 can be differentiated by restriction endonuclease digestion analysis and by the SDS-PAGE migration patterns of viral glycoproteins. Ruyechan et al. (1979) were able to use this information to map the locations of several glycoproteins by analyzing the DNA and glycoproteins of intertypic recombinants between HSV 1 and 2.

Gene mapping studies in HSV were facilitated by the availibility of cloned restriction fragments of the viral genome. These fragments were used to specifically select infected cell RNA which hybridized to defined regions of viral DNA. Lee et al. (1982) were able to map HSV gD and gE genes within the S region of the genome by translating selected RNA *in vitro* and analyzing the translation products in immunoprecipitation assays with monoclonal antibodies directed against the glycoproteins. Anderson et al. (1980) purified individual mRNA species by a combination of hybrid-selection and gel electrophoresis and, by translating these RNAs *in vitro*, were able to precisely map HSV immediate early genes.

Other methods of gene mapping use the biological activity of the gene product of interest as an assay. The alkaline exonuclease of HSV was mapped by microinjecting hybrid-selected infected cell RNA or cloned viral

DNA into Xenopus oocytes (Preston and Cordingley, 1982). The injected oocytes were then assayed for exonuclease activity. The HSV thymidine kinase gene was mapped by both microinjection of oocytes (McKnight and Gavis, 1980), as described above, and by transfection assays (Wigler et al, 1977). In the transfection assays, cloned restriction fragments were tested for their ability to restore tk activity to tk- cells or used in marker rescue experiments with tk- mutant viruses.

Recently, herpesvirus researchers have coupled the availability of a large battery of monoclonal and monospecific antibodies with the use of a number of procaryotic expression vectors containing either random viral DNA fragments or viral cDNA to map viral genes. To map the gC gene of VZV to the Us region of the viral genome, Ellis et al. (1985) expressed randomly generated DNA fragments from a VZV plasmid clone library in an open reading frame (ORF) vector. Clones expressing the glycoprotein were identified using a monoclonal antibody and gene identity was confirmed by demonstrating that DNA from the positive clones could select RNA which coded for gC. In similar studies, a bacteriophage expression vector, !lgtll (Young and Davis, 1983), was used to map genes of CMV (Mocarski et al. 1985) and PRV (Petrovskis et al. 1985). An interestng aspect of the latter study was that only antibody prepared against denatured antigens was able to detect the expressed peptides.

Gene mapping strategies often employ a combination of these techniques (Rea et al., 1985). For example, the gene for a PRV glycoprotein that is found in the cell culture medium was mapped to a specific restriction fragment by translating hybrid-selected RNA in the presence and absence of a dog pancreas microsome extract. Peptides requiring futher processing, such as glycosylation, were identified by

comparing the translation products from the two reactions. The restriction fragment was then expressed in an ORF vector and antibody to the expressed peptide was prepared in mice. This antibody was used in immunoprecipitation studies to confirm the gene identification.

Herpesvirus Gene Expression and Regulation

Transcription in herpesviruses has recently been reviewed by Wagner (1985). In general, herpesvirus mRNA's appear to be unspliced and many mRNA species are transcribed from overlapping regions of the genome.

Honess and Roizman (1975) placed herpes simplex genes and gene products into three classes based on the temporal order of their expression. This "cascade" pattern of regulation is similar in other herpesviruses. Immediately upon viral entry into the host cell, immediate early genes are expressed. This expression occurs in the absence of de novo viral protein synthesis. The immediate early gene products function as positive regulators of early gene expression. The early gene products inhibit the expression of immediate early genes while stimulating the expression of late genes. In turn, expression of early genes is inhibited by the late genes products. To complete the cycle a late gene product which is a virion component has been shown to stimulate the expression of immediate early genes (O'Hare and Hayward, 1984).

The cascade is more complex than originally proposed, however, as some late genes are expressed at early times of infection but become more abundant at late times. These genes are referred to as "leaky late" genes (Spear and Roizman, 1980).

Immediate Early Genes

Immediate early genes can be more easily identified since they are the only viral genes transcribed in the presence of cycloheximide. Immediate early RNA is made up of only a few species. These all map in a limited area of the viral genome. The immediate early genes of HSV and PRV are clustered within the inverted repeat sequences (Wagner, 1985). In HSV, 5 immediate early peptides with molecular weights of 175, 110, 68, 64, and 12 kd have been identified (Wagner, 1985) while in PRV only a single peptide with a molecular weight of 180 kd was found (Ihara et al., 1983).

Immediate early peptides are often phosphorylated and though their function is still unclear, these peptides appear to function in the shut off of host cell protein synthesis through a mechanism that is not understood. HSV immediate early proteins have DNA binding activity (Freeman and Powell, 1982) and have been shown to effect the expression of heat shock peptides within the infected cell (Notarianni and Preston, 1982). Immediate early proteins may also function in the regulation of latency. Expression of HSV immediate early genes has been found in latently infected tissues from experimental animals (Green et al., 1981) but not in human tissues (Galloway et al., 1982). Recently, a model was proposed by O'Hare and Hayward (1985) in which HSV immediate early genes function to regulate their own expression as well as early gene expression.

Early and Late Genes

The early genes map throughout the genome but only a limited number of mRNA species are recognized. Early gene products function primarily in viral DNA replication. In HSV early genes have been found to code for a

thymidine kinase, alkaline exonuclease, DNA binding proteins, DNA polymerase, ribonucleotide reductase and deoxypyrimidine triphosphatase (Wagner, 1985).

"Leaky late" gene expression is detectable in infected cells in the absence of viral DNA replication while true late gene expression is sensitive to inhibitors of viral DNA replication. Both "leaky late" and true late genes map throughout the viral genome. Many of the late genes code for structural components of the virion such as the major capsid antigen and viral envelope glycoproteins (Wagner, 1985).

Figure 3 is a HSV transcript map (Wagner, 1985). This figure is presented to show the distribution of the three classes of genes on the HSV genome. There is increasing evidence that herpesviruses with similar genomic structure may have conserved the locations of important viral genes (Davis and Wilkie, 1983, Culliane and Davison, 1985). To further explore colinear genome organization in herpesviruses, the location of viral genes needs to be determined in a number of different herpesviruses.

Figure 3. Location of HSV mRNA species abundant at different stages of replication. Figure shows size of RNA transcripts and, if known, size of polypeptide coded for by the RNA transcript. (reproduced with permission from: Wagner, 1985)

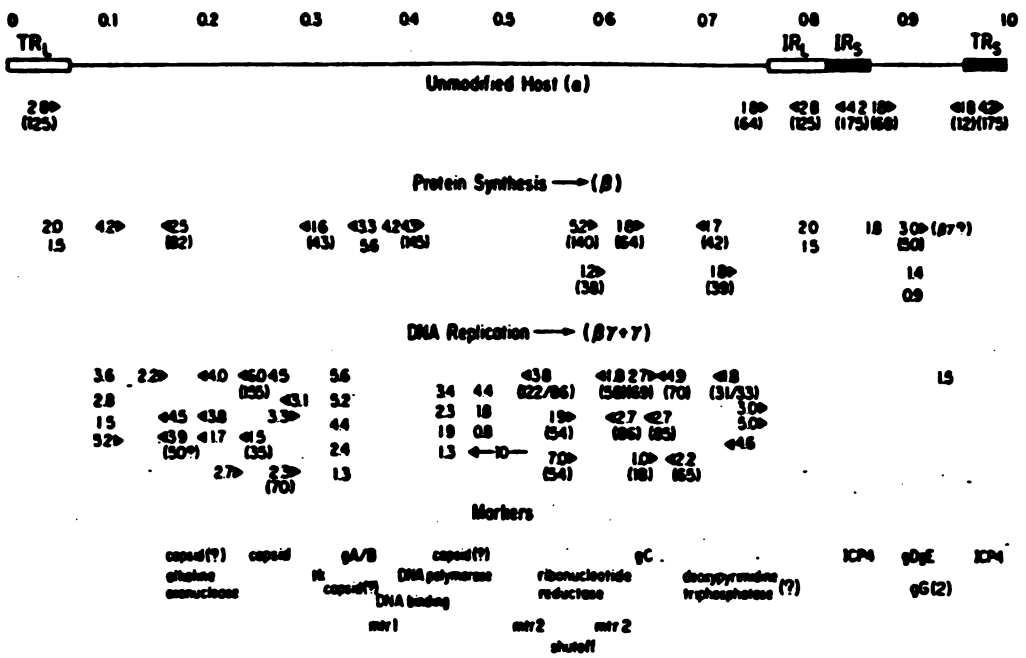

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
TR_L
IR_L
IR_S
TR_S
Unmodified Host (a)
Protein Synthesis ⟶ (β)
DNA Replication ⟶ (β7+γ)
Markers
capsid(?)
capsid
gA/B
capsid(?)
gC
ICP6
gD,E
ICP4
alkaline exonuclease
tk
capsid(?)
DNA polymerase
DNA binding
ribonucleotide reductase
deoxypyrimidine triphosphatase (?)
gG(2)
mtr1
mtr2
mtr2
shutoff

Figure 3.

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Research Data

Chapter 2

Physical Characterization of the Genome of Feline Herpesvirus-1

Paul A. Rota and Roger K. Maes

Abstract

The physical structure of the genome of feline herpesvirus-1, a major upper respiratory tract pathogen of cats, was studied. Restriction endonuclease analysis with Bam HI, Eco Rl, Hind III, and Sal I indicated that the size of the genome was approximately 134 kilobase pairs (kb). Restriction fragments containing the viral termini were identified by lambda 5′ exonuclease digestion of intact viral DNA prior to restriction endonuclease digestion. Blot hybridizations using the Sal I terminal fragments as probes indicated that one end of the viral genome contained both terminal and internal repeat sequences. Electron microscopic analysis of denatured and reannealed FHV-1 DNA revealed a single-stranded loop and double-stranded stem at only one end of the molecule and confirmed the presence of inverted repeat sequences. The size of the single-stranded loop region was determined to be approximatly 8 kb. To facilitate further studies, nine bacteriophage clones were isolated which contained 85% of the viral genome as Sal I inserts. These clones were used in blot hybridization experiments and as substrates for restriction digest analysis. Data from these studies permitted construction of Sal I and partial Hind III and Eco Rl restriction maps of the viral genome. FHV-1 DNA is composed of a long (L) and a short (S) segment. The long segment (Ul) is 103 kb in size and is composed of unique DNA. The adjacent S segment is approximately 31 kb in size and contains a central portion of unique DNA (Us) which is approximately 8 kb in size. The Us region is bounded by inverted repeat sequences which are 11 kb in size. The entire S region of the genome can invert relative to the L resulting in a genome with two isomeric forms.

Introduction

Feline herpesvirus-1 (FHV-1) is a major viral pathogen of cats. In adult cats, the virus causes upper respiratory tract disease. In kittens, the infection can generalize resulting in mortality rates of up to 50%. FHV-1 can also cause genital tract infections and has been associated with abortions (Povey, 1979, Pratt, 1983). Like other herpesviruses, FHV-1 can cause latent infections, and the site of latency appears to be the trigeminal ganglia (Gaskell et al., 1985). The prevalence and seriousness of FHV-1 have lead to development of both modified-live and inactivated virus vaccines. While these vaccines can prevent disease, they are unable to prevent exposed cats from becoming infected (Povey and Wilson, 1978). Therefore, vaccinated cats still develop latent FHV-1 infections, and periodic reactivation of these latent infections allows the virus to spread to both vaccinated and non-vaccinated cats (Gaskell and Povey, 1978).

FHV-1 has been classified as a member of the subfamily alphaherpesvirinae. Its host range *in vivo* is limited to Felidae and *in vitro* to cells of feline origin. The virus has a short reproductive cycle. The infection spreads rapidly in cell culture leading to mass destruction of cells (Roizman, 1980). Most isolates of FHV-1 are of uniform pathogenicity, although strains of modified virulence have been developed for vaccine use (Slater and York, 1976). Serological studies indicate that various clinical isolates have similar antigens (Johnson and Thomas, 1966). More recently, restriction enzyme analysis of viral DNA from a number of clinical isolates has shown that the genome of FHV-1 is

remarkably stable in comparison to that of other herpesviruses (Herrmann et al., 1984).

The biological properties of FHV-1 have been well studied but little is known about the biochemical structure of this virus. Two recent studies have characterized the major virion-associated structural molecules and identified three major antigenic glycoproteins on the virion and the surface of infected cells (Fargeaud et al., 1984, Maes et al., 1984).

We are interested in studying the molecular biology of FHV-1 as a first step in developing FHV-1 as a natural host model for herpesvirus infections. This report describes an initial characterization of the physical structure of the FHV-1 genome. Various biochemical methods were used to determine the size and isomeric arrangement of the genome. In addition, a number of recombinant bacteriophage clones containing most of the genome as inserts have been isolated. These clones were used in blot hybridization and double digestion experiments to generate the first restriction map of the viral genome.

Methods and Materials

Virus and Cells. The C-27 prototype strain of FHV-1 was obtained from the American Type Culture Collection, Rockville, MD (#VR 636). The virus was propagated in Crandell-Rees Feline Kidney (CRFK) cells which were maintained in Eagle's Minimum Essential Medium (EMEM) (K.C. Biologics, Lenexa, KS.) with 10% fetal bovine serum (Hyclone defined, Sterile Systems, Logan, UT).

Isolation of Viral DNA. Ten tissue culture roller bottles (490 cm, Corning Glass Works, Corning, NY) containing confluent monolayers of CRFK cells were infected with virus at a multiplicity of infection of .01. When the cytopathic effect was advanced, infected cells were shaken from the culture vessel surface and pelleted by low speed centrifugation (500 rpm, 10 min). Washed cells were resuspended in 10 ml of TE (10mM Tris-HCl pH 7.5, 1 mM EDTA) and left on ice for 15 minutes. NP-40 (Sigma, St. Louis, MO.) was added to a final concentration of .5%, and the cells were incubated for another 15 minutes on ice. Cell nuclei were pelleted by centrifugation at 2,000 x g for 10 minutes, and the supernatant fluid containing the viral nucleocapsids was adjusted to 1% SDS and 25mM EDTA. Pre-digested (80°C., 15 min.) pronase was added to a final concentration of 1 mg/ml and the virus preparation was incubated overnight at 37°C. Saturated sodium iodide (1.5 volumes) containing 1 ug/ml ethidium bromide was then added and the mixture was centrifuged for 48 hours at 44,000 rpm at 20°C. in an SW 50.1 Rotor (Beckman Instruments, Palo Alto, CA.). Viral DNA bands were visualized by u.v. illumination and harvested by side-puncturing the tube with a 20 ga. needle. DNA-containing samples

were extracted three times with isoamyl alcohol (5 volumes) before being dialyzed extensively against TE.

Restriction Enzyme Analysis. Viral DNA was incubated with 10 U/ug of either Bam HI, Eco R1, Sal I or Hind III (Bethesda Research Laboratories, Gaithersburg, MD.) using the reaction conditions recommended by the manufacturer. When the digestion was complete, the fragments were separated by agarose gel electrophoresis (20 volts, 20 hours in Tris-acetate buffer: .04M Tris-acetate, .002M EDTA) and the fragments were visualized by ethidium bromide staining and u.v. illumination. In some experiments, the fragments were end-labeled prior to electrophoresis by adding 1 uCi of the appropriate α^{32}P-labeled trinucleotide (Bam HI: dGTP, Eco R1 and Hind III: dATP, Sal I: dTTP, Amersham, Arlington Heights, Ill., sp. act. 800 Ci/mmole) to the reaction along with 1 unit of the Klenow fragment of DNA polymerase (BRL). Samples were incubated at room temperature for 45 minutes, heated to 68°C to inactivate the polymerase, and precipitated twice with ethanol to remove unincorporated label before being subjected to electrophoresis. When electrophoresis was complete, the gel was dried on a gel dryer for 45 minutes without heat and exposed to Kodak X-AR film. To determine fragment molarity, films exposed for various lengths of time were scanned with a densitometer equipped with an integrator (Ortec 4310, Oak Ridge, TN).

Double restriction digests with either Sal I and Eco R1 or Sal I and Hind III were carried out simultaneously in Sal I buffer (BRL).

Southern Blotting. After electrophoresis, restriction fragments were transferred to nitrocellulose filters by the method of Southern (1975).

Filters were prehybridized for 4 hrs at 42°C. in 20% formamide, .6M NaCl, .06M sodium citrate, .01M EDTA, .1% SDS, 5X Denhardt's solution (.1% ficoll, .1% polyvinylpryollidone, .1% bovine serum albumin), and 50 ug/ml denatured salmon sperm DNA. Hybridizations were performed at 42°C. in 50% formamide, 1X Denhardt's, 4X SSC (1X SSC: .15M NaCl. .015M sodium citrate, pH 7.0), .1M EDTA, .1% SDS, 50 ug/ml denatured salmon sperm DNA. After hybridization, filters were washed twice for 15 minutes in 2X SSC at room temperature followed by two 1 hr washes in .1X SSC at 68°C. The filters were allowed to dry and exposed to X-ray film.

^{32}P-labeled probes were prepared from either total viral DNA, total recombinant phage DNA or agarose gel purified restriction fragments by nick translation using standard methods (Maniatis et al., 1982). Restriction fragments for use as probes were purified from agarose gels by electrophoresis onto nitrocellulose filters (NA-45, Schleicher and Schuell, Keane, NH). Fragments were eluted from the filters by incubation at 68°C for 25 minutes in TE with 1M NaCl. Recovered fragments were extracted twice with water-saturated butanol and precipitated twice with ethanol. Usually, fragments were subjected to a second electrophoretic purification before being nick translated.

Terminal fragment identification. Identification of fragments containing the viral termini was done by two methods. First, 1 ug aliquots of viral DNA were incubated at 37°C. for 10, 20, 30, 40 and 60 minutes with lambda 5' exonuclease (BRL, 1.5U/ug) in 67 mM glycine pH 9.5, 2.5 mM MgCl. Reactions were stopped by heating the samples to 65°C. for 15 minutes followed by extraction with phenol:chloroform: isoamyl alcohol (25:24:1). After precipitation with ethanol, viral DNA was resuspended in the

appropriate restriction enzyme buffer and digested with the enzyme overnight. Fragments were end-labeled and separated by agarose gel electrophoresis. Untreated viral DNA was digested, end-labeled and run in an adjacent lane on the gel as a control.

In the second approach, synthetic Bam HI linkers (BRL) were end-labeled with gamma^{32}P ATP using polynucleotide kinase and ligated to intact viral DNA using the methods described by Spector et al.(1982). Unattached linkers were removed from the DNA by electrophoresis through .8% low melting point agarose (Seakem, FMC, Rockland, ME). The viral DNA band was visualized by staining the gel with ethidium bromide and the DNA was recovered by melting the excised band in TE at 65°C. followed by repeated phenol:chloroform extractions and ethanol precipitation. Labeled DNA was digested with restriction enzymes and the fragments were resolved by agarose gel electrophoresis. After electrophoresis, gels were stained with ethidium bromide, and photographic records were made. Gels were dried as described above and exposed to X-ray film. End fragments were identified by measuring the distance migrated by the labeled fragments and comparing the measurements to the photographic record.

EMBL-3 cloning. EMBL-3 DNA was prepared from a phage stock (gift of Paul Bates and Dr. Jerry Dodgson, MSU Dept. of Microbiology) by the methods described in Maniatis et al. (1982). Sal I cut FHV-1 DNA (2ug) was ligated to Sal I cut EMBL-3 DNA (1ug) at 16°C. overnight in 66mM Tris pH 7.2, 10 mM MgCl, 10 mM DTT, 1 mM ATP. Recombinant phage DNA was packaged *in vitro* according to the method described by Scalenghe et al (1978). Phage were titered on both Q358 and Q359 strains of *E. coli* to determine the number of recombinant phage. Libraries of recombinant phage were plate amplified

on Q359. Libraries were screened by picking individual, well isolated plaques growing on a lawn of Q359 and placing them into .5 ml of SM (.1M NaCl, .2% MgSO4, 50mM Tris-HCl pH 7.5, .01% gelatin). Phage from 1/2 to 1/3 of a resuspended plaque were amplified in 2 ml LB broth cultures and phage DNA was purified using the small scale procedure described by Leder, et al (1977).

For in situ plaque hybridizations, plaques were transferred to nitrocellulose filters (Millipore, HATF, Bedford, MA) and screened with nick-translated DNA probes as described by Benton and Davis (1977).

Electron Microscopy. Electron microscopic analysis of FHV-1 DNA was performed using the conditions previously described by Ruyechan et al (1982).

Results

Restriction enzyme analysis. FHV-1 DNA was purified as described above and digested to completion with either Bam HI, Eco R1, Hind III or Sal I. The end-labeled fragments were separated on agarose gels. After electrophoresis, gels were dried and exposed to X-ray film. Lambda phage DNA, digested with either Eco R1, Hind III or Sal I, was run in adjacent lanes on the gels to provide size markers. Since fragments were labeled according to their molar concentration in the digest and not their size, a large number of fragments could be resolved on a single gel. Figure 1 shows that Bam HI, Eco R1, Hind III or Sal I digestion of FHV-1 DNA produced a number of fragments with sizes ranging from approximately 30 kb to less than .5 kb. The size of each restriction fragment, based on the average measurements from three different gels, is given in Table 1. Sizes of smaller fragments were determined on high concentration gels (1.0% and 1.4%), while sizes of larger fragments were based on measurements from gels of lower concentrations (.5% and .7%).

The molarity of each fragment was determined by scanning autoradiograms with a spectrodensitometer equipped with an integrator. Films exposed for varying lengths of time were scanned to ensure linearity of the film. Figure 2 shows the results of a typical scan of each digest after electrophoresis on a .8% agarose gel. Molarity of the smaller fragments was determined by scanning gels of higher concentration (1.0% and 1.4%). The molarity of each fragment, based on the area under the peak, is shown in Table 1. Scanning results showed the presence of only one , two or three molar fragments.

Figure 1. Restriction endonuclease digestion patterns of FHV-1 DNA. FHV-1 DNA was digested with either Bam HI, Hind III, Eco R1 or Sal I and the fragments were end-labeled and separated by agarose gel electrophoresis. Hind III digest fragments of lambda DNA with sizes of 23.3, 9.4, 6.6, 4.3, 2.2 and 2.0 kilobase pairs are shown as size markers.

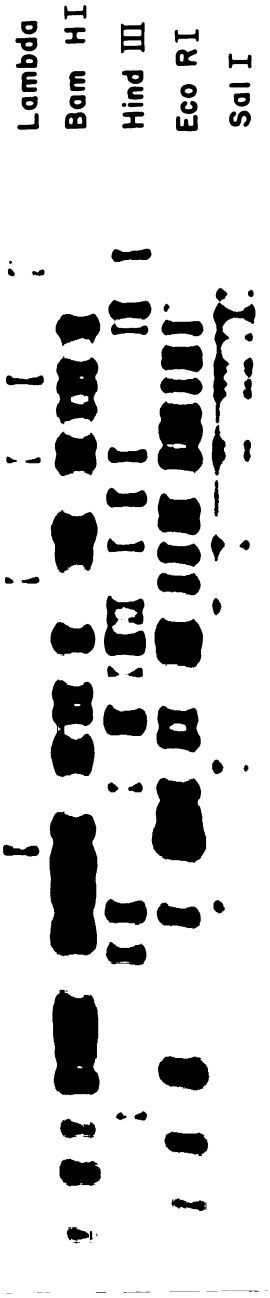

Figure 1

Figure 2. FHV-1 DNA restriction fragment molarity determination. Figure shows typical densitometer scans of autoradiograms of dried .8% agarose gels conmtaining digested and end-labeled FHV-1 DNA. Area under each peak was used to determine fragment molarity.

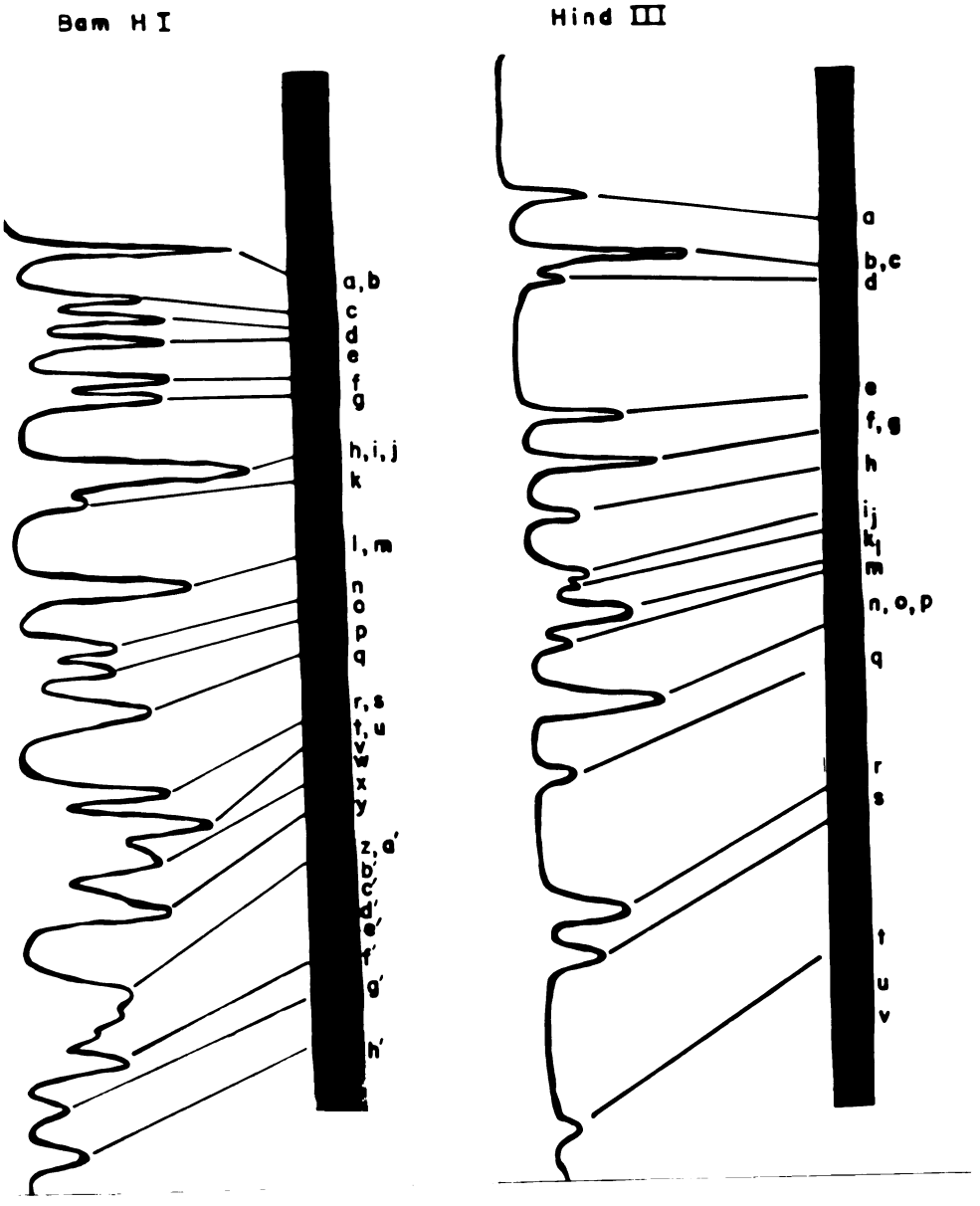

Figure 2 (continued on next page)

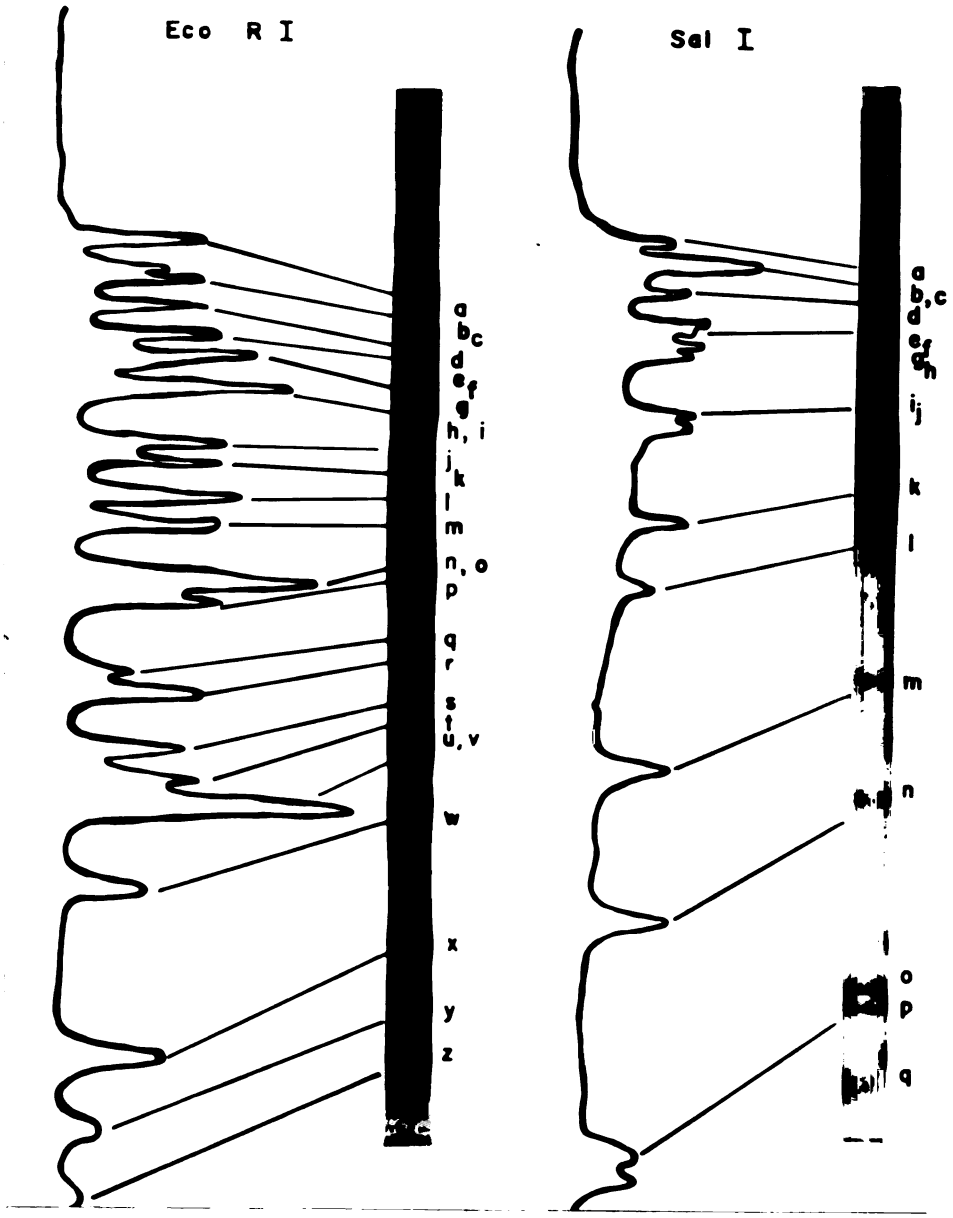

Figure 2 (continued)

Table 1. Restriction enzyme digestion patterns of FHV-1 DNA

Bam HI			Hind III			Eco R1			Sal I		
	mol. wt. (kb)	M		mol. wt. (kb)	M		mol. wt. (kb)	M		mol. wt. (kb)	M
A,B	14.5	2	A*	31.0	1	A	14.5	1	A	19.0	1
C	10.8	1	B,C	16.5	2	B*	12.0	1	B,C	16.3	2
D	9.5	1	D	14.2	1	C	10.7	1	D	13.0	1
E	8.3	1	E	7.0	1	D	9.5	1	E	10.8	1
F	7.1	1	F,G	5.8	2	E	8.2	1	F*	10.4	1
G	6.6	1	H	4.7	1	F	7.6	1	G	9.6	1
H,I,J	5.2	3	I	3.9	1	G	7.4	1	H	9.0	1
K*	4.8	1	J*	3.7	1	H,I	6.6	2	I	7.3	1
L,M	4.5	2	K	3.6	1	J	5.7	1	J	6.8	1
N	3.3	1	L	3.5	1	K	5.3	1	K	5.0	1
O	2.9	1	M	3.2	1	L	4.8	1	L*	3.9	1
P	2.7	1	N,O,P	2.6	3	M	4.3	1	M	2.4	1
Q	2.4	1	Q	2.2	1	N,O	3.7	2	N	1.6	1
R,S	2.1	2	R	1.7	1	P	3.3	1	O	.7	1
T,U	1.8	2	S	1.4	1	Q*	2.7	1	P	.6	1
V	1.7	1	T	.7	1	R	2.6	1	Q	.4	1
W	1.6	1	U	.5	1	S	2.2	1			
X	1.6	1	V	.4	1	T	2.1	1			
Y	1.5	1				U,V	2.1	2			
Z,A'	1.4	2				W	1.6	1			
B'*	1.0	1				X	.8	1			
C'	.9	1				Y	.5	1			
D'	.8	1				Z	.5	1			
E'	.8	1									
F'	.7	1									
G'	.5	1									
H'	.4	1									
Total:	133.3			134.1			134.4			133.1	

Average molecular weight: 133.7 $\pm$.6

* denotes end fragment, M = fragment molarity

To determine the size of the genome, the sizes of restriction fragments in each digest were totalled and adjustments were made for fragment molarity. Based on the average of the total fragment size for the four digestions, the size of the FHV-1 genome was determined to be 133.7 kb (Table 1).

Terminal fragment identification. FHV-1 DNA was digested with lambda 5' exonuclease for varying amounts of time and subsequently digested with either Bam HI, Eco Rl, Hind III or Sal I, end-labeled and electrophoresed with non-exonuclease-treated, digested DNA. Terminal fragments showed altered mobility compared to untreated controls. Figure 3 shows that Bam HI fragments K and B', Eco Rl B and Q, and Sal F and L have altered mobility after exonuclease treatment and presumably are located at the ends of the viral genome. Only one Hind III terminal fragment, Hind III A, was observed. Identification of Bam HI, Eco Rl and Sal I termini were confirmed using kinased synthetic linkers to label the ends of the genome (data not shown).

To determine which of the terminal fragments were colinear, Sal I termini F and L were purified from agarose gels and used to probe Eco Rl and Hind III cut FHV-1 DNA that had been transferred to nitrocellulose filters. Figure 4 shows that Sal I L hybridized to Eco Rl J, Q and U or V and to Hind III A. Sal I F hybridized to Eco Rl B and to Hind III E and J. The faint bands visible when Sal I F was used as a probe were due to contamination with Sal I E which migrates very close to Sal I F and is difficult to remove in electrophoretic separations (for the hybridization patterns of Sal I E to Hind III fragments of FHV-1 DNA, see Figure 8). Hybridization of the Sal I F probe to Sal I digest of FHV-1 DNA showed

Figure 3. Terminal restriction fragment identification. FHV-1 DNA was treated with lambda 5' exonuclease for 0, 10, 20, 30, or 40 minutes before being digested with restriction enzymes, end-labeled and subject to agarose gel electrophoresis on .7% or 1.4% gels. Fragments showing altered migration patterns after exonuclease treatment are noted.

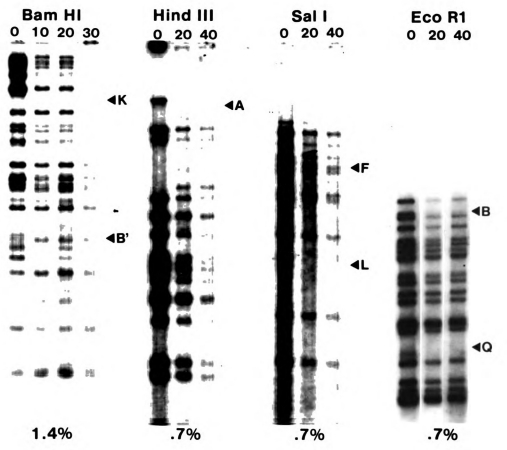
Bam HI
0 10 20 30
◀K
◀B'
1.4%
Hind III
0 20 40
◀A
.7%
Sal I
0 20 40
◀F
◀L
.7%
Eco R1
0 20 40
◀B
◀Q
.7%

Figure 3

Figure 4. Blot hybridization patterns of FHV-1 Sal I terminal fragments. Figure shows Southern blots of Hind III and Eco Rl digests of FHV-1 DNA after being hybridized to nick-translated FHV-1 Sal I fragments L or F.

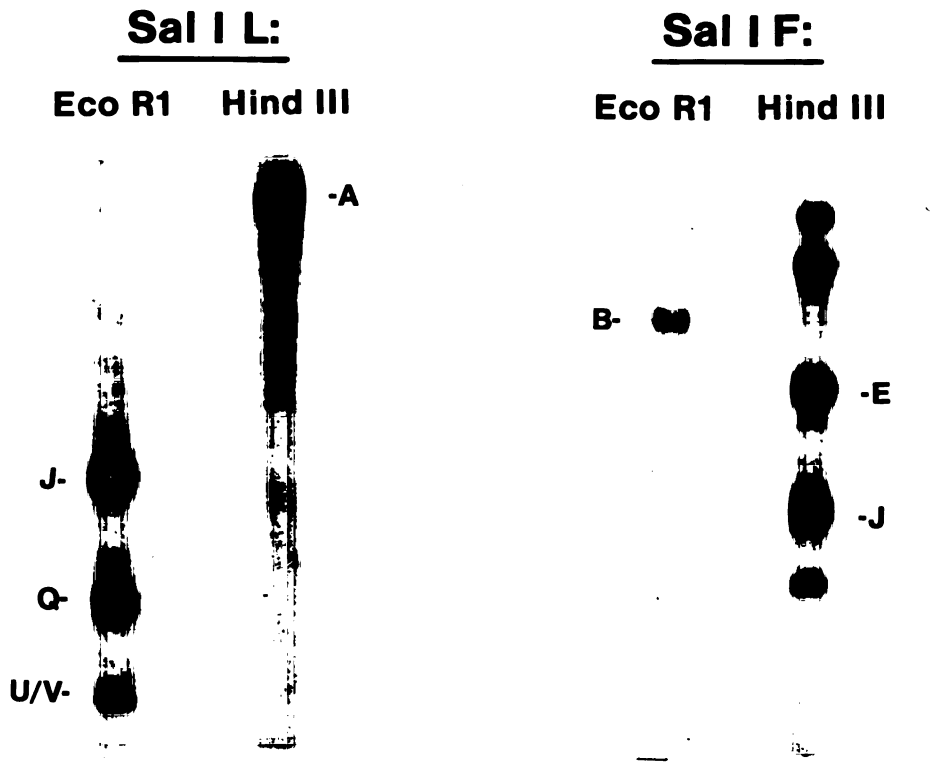

Figure 4

Table 2. Hybridization and restriction enzyme digestion patterns of FHV-1 Hind III fragment A.

Hybridization to FHV-1 Sal I and Eco Rl restriction digest patterns:

Sal I: B, H, J, L

Eco Rl: E, F, J, M, Q, U/V

Fragments (in kb) generated after digestion of FHV-1 Hind III A with:

Sal I: 16.0 (B), 6.8 (J), 4.0, 3.9 (L)

Eco Rl: 8.2 (E), 7.6 (F), 4.3 (M), 3.2, 2.7 (Q), 2.1 (U,V)

(corresponding Sal I or Eco Rl fragment given in parenthesis)

some hybridization to Sal I E as well as Sal I F (data not shown). Therefore, Sal I L, Eco R1 Q and Hind III A are located at the opposite end of the viral genome from Sal I F, Eco R1 B and Hind III E and J.

To confirm the positioning of Sal I L, Eco R1 Q, and Hind III A at one end of the genome, Hind III A DNA was electrophoretically purified, digested with both Sal I and Eco R1 and used to probe Sal I and Eco R1 digests of FHV-1 DNA. Results of these experiments (Table 2) confirmed that Hind III A contained both Sal I L and Eco R1 Q. These results also indicated that the non-terminal end of Hind III A contained only parts of both Eco R1 J and Sal I H. Sal I H and Eco R1 J therefore span the junction between Hind III A and the remainder of the genome.

Hybridization of Sal I L to the internal fragment Eco R1 J as well as to terminal fragment Eco R1 Q (Figure 4) suggested that Sal I L and Eco R1 J and Q shared common sequences. In addition, a bacteriophage clone (EM-7, see below) containing only Sal I H also hybridized to Sal I L (data not shown). Sal I F hybridized only to Eco R1 B, located at the opposite terminus from Sal I L. These results indicated that the FHV-1 genome contains terminal and internal repeat sequences located within approximately 30 kb of one end of the genome. This type of structure is consistent with a herpesvirus genome containing a single invertable short (S) region and suggests that FHV-1 has a two isomer genome.

Electron microscopic analysis of FHV-1 DNA. To obtain additional proof that FHV-1 DNA has two isomeric forms, viral DNA was denatured and allowed to reanneal and observed by electron microscopy. Figure 5 shows a representative molecule of reannealed FHV-1 DNA showing a single-stranded loop and double-stranded stem at one end of the viral DNA molecule. This

Figure 5. Electron microscopic analysis of FHV-1 DNA. Figure shows an electron micrograph of self-annealed FHV-1 DNA molecules. Arrows indicate the double stranded regions and fd phage single stranded size marker.

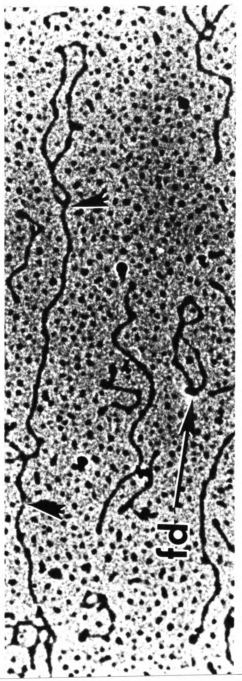

Figure 5

Figure 6. Electron microscopic analysis of the S region of FHV-1 DNA. Histogram shows the size distribution of the single-stranded loop region of self-annealed FHV-1 DNA molecules. The sizes (in megadaltons) are the double stranded molecular weights of the loops.

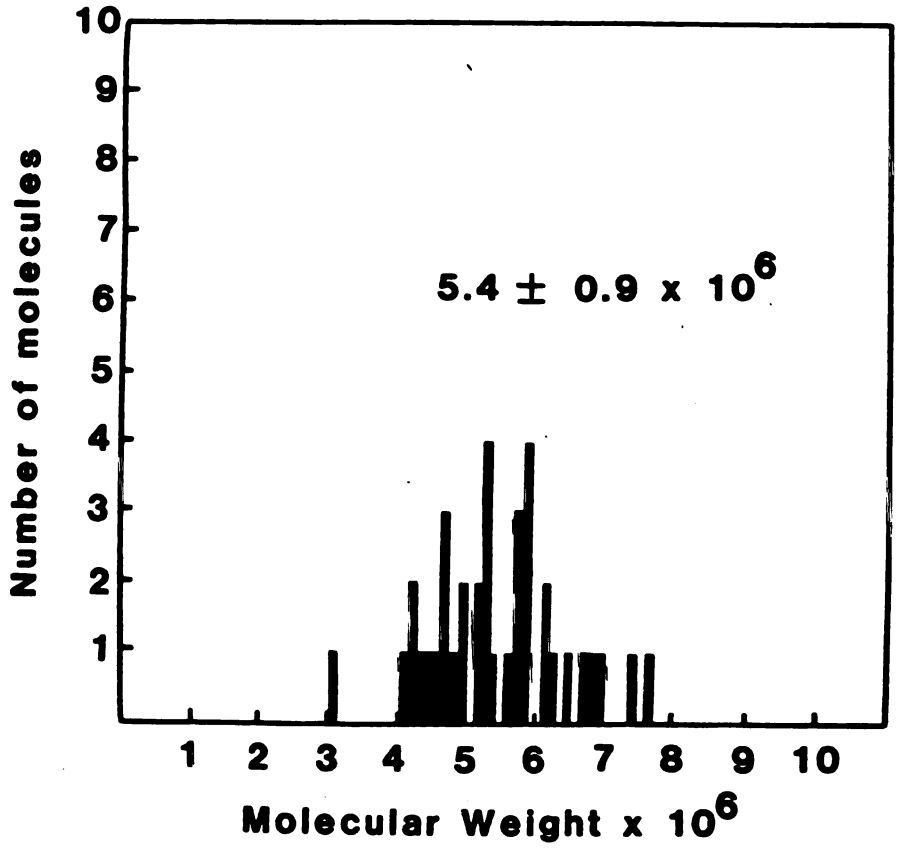
10
9
8
7
6
5
4
3
2
1
Number of molecules
5.4 ± 0.9 x 10^6
1 2 3 4 5 6 7 8 9 10
Molecular Weight x 10^6

Figure 6

confirms that inverted repeat sequences are present at one end of the DNA and suggests that FHV-1 has a two isomer genome. Measurement of the single-stranded loop region of the reannealed DNA indicated that the average length of the unique DNA of the S region was approximately 8 kb (5.4 Md, Figure 6). Contour length measurements also indicated that the FHV-1 genome is approximately 125 kb in size.

Cloning FHV-1 DNA. To facilitate further mapping studies on the genome of FHV-1, viral DNA was inserted into the bacteriophage vector, EMBL-3. EMBL-3 is a substitution vector capable of accepting fragments from 7 to 22 kb in size. Substitution requires the deletion of the lambda red and gamma genes. Therefore, recombinant phages will be spi- and can be selected for by plating on a bacterial host lysogenic for bacteriophage P2. The spi+ vector phages will not form plaques on Q359, but will grow on strain Q358 which does not contain a P2 lysogen (Karn et al., 1980, 1983).

Since Sal I digestion produced the greatest proportion of FHV-1 DNA fragments which were capable of being inserted into EMBL-3, Sal I cut FHV-1 DNA was ligated to Sal I cut EMBL-3. The phage library was titered on both Q358 and Q359. The initial library contained 10^5 recombinants and had 64% vector background contamination. Efficiency of spi selection was tested by *in situ* plaque hybridization. Filters containing plaque DNA from platings on both Q358 and Q359 were hybridized to nick-translated viral DNA. Only 33% of the plaques growing on Q358 hybridized to the FHV-1 probe while nearly 100% of the plaques on Q359 hybridized (data not shown).

The library was amplified twice by plating on Q359. The amplified library contained greater than 10^8 recombinants and less than 10%

Figure 7. Analysis of EMBL-3 clones containing FHV-1 DNA inserts. Panel A shows an ethidium bromide stained agarose gel containing Sal I digests of clones EM-1-9 (lanes 1-9 respectively). Sal I digests of EMBL-3 DNA (B) and FHV-1 (C) and a Hind III digest of lambda DNA (A) are shown as size markers. Panel B shows a Southern blot of the gel shown in panel A after being hybridized to nick-translated FHV-1 DNA.

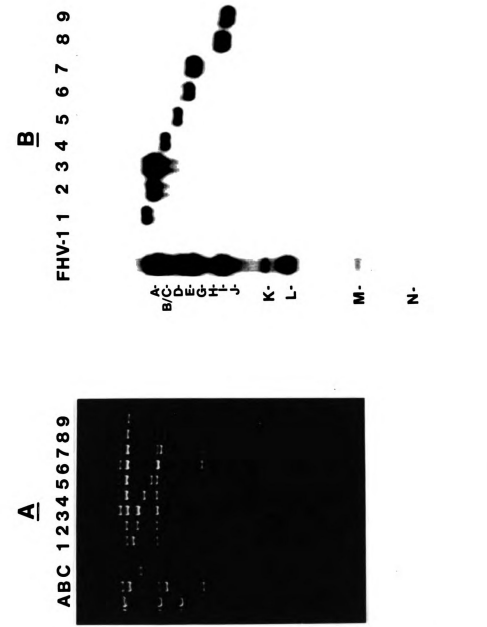

Figure 7

vector background. To screen the library, individual plaques growing on Q359 were picked and purified. Small scale DNA preparations were made from each plaque, digested with Sal I, and analyzed by agarose gel electrophoresis. DNA from over 400 plaques was examined by this method with the result that nine different clones containing approximately 85% of the viral genome were isolated. Figure 7A shows that seven clones containing fragments A, B, C, D, H, I, and J as single inserts (designated EM-1,2,3,4,7,8 and 9 respectively) were isolated. Two clones contained double inserts. Clone EM-5 contained fragment E and N and another fragment approximately 4 kb in size that was part of fragment E (data not shown). EM-6 contained fragments G and N. Figure 7B shows a Southern blot of the gel shown in 7A after being probed with FHV-1 DNA. This experiment confirmed that all of the clones contained viral inserts. Several clones contained small Sal I fragments from the internal region of EMBL-3. The lambda red and gamma genes are not contained on these fragments and give these phage a spi- phenotype.

Attempts were made to identify clones containing Sal I K and M. Plaques from both the original and amplified libraries were hybridized to nick-translated probes prepared from gel purified Sal I K and M DNA. No positive clones were found.

Genome mapping. To determine linkage relationships between Sal I and Hind III restriction fragments of FHV-1 DNA, Sal I fragments were nick translated and hybridized to nitrocellulose strips containing Hind III fragments. Clones containing single inserts were nick translated without further purification. Sal I fragments E, N, and G were electrophoretically purified from a digest of phage DNA. Sal I fragments M, and K were

Figure 8. Linkage relationships between FHV-1 Sal I and Hind III fragments. FHV-1 Sal I restriction fragments were nick translated and hybridized to filters containing Hind III restriction fragments. Identity of Hind III fragments is indicated at the left of each lane.

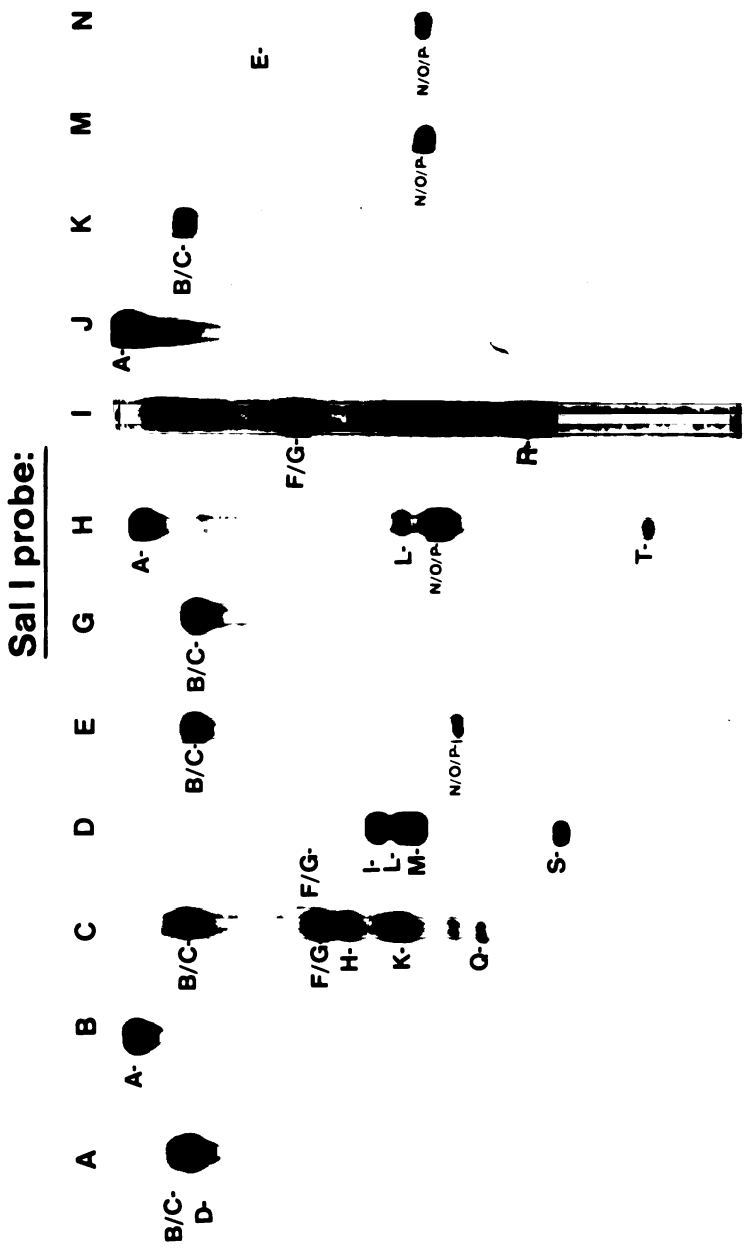
Sal I probe:
A
B
C
D
E
G
H
I
J
K
M
N
B/C-
D-
A-
B/C-
B/C-
F/G-
H-
F/G-
I-
L-
M-
K-
N/O/P-
Q-
B/C-
B/C-
N/O/P-
A-
F/G-
R-
A-
B/C-
L-
N/O/P-
N/O/P-
N/O/P-
E-
S-
T-

Figure 8

Table 3. Hybridization of FHV-1 Sal I fragments to FHV-1 Hind III fragments.

Sal I probe:	Hind III fragments detected:
A	B/C, D
B	A
C	B/C, F/G, H, K, Q,
D	F/G, I, M, L, S
E	B/C, N/O/P
F	E, J
G	B/C
H	A, L, T, N/O/P
I	F/G, R
J	A
K	B/C
L	A
M	N/O/P
N	E, N/O/P

Table 4. Hind III digestion patterns of FHV-1 Sal I fragments.

Sal fragment:	Hind III digestion products (kb):
A	14.5 (D), 3.0, 2.0
B	16.0
C	4.7, 4.7 (H), 3.6 (K), 2.5, 2.2 (Q)
D	3.9 (I), 3.2 (M), 2.5, 2.5, 1.4 (S)
E	8.6, 2.0
G	9.6
H	4.7, 2.6 (N), 1.0, .7 (T)
I	3.4, 3.2, 1.7 (R)
J	6.8
K	5.0
L	3.9
N	1.0, .6

corresponding Hind III fragments are shown in parenthesis

electrophorectically purified from a digest of viral DNA. These data are presented in Figure 8 and summarized in Table 3. DNA from the recombinant phage clones was digested with both Sal I and Hind III, and gel-purified Sal I fragments K, L, N were digested with Hind III to determine the number of internal Hind III sites contained in each Sal I fragment (Table 4). These data were used in combination to determine positions of the Sal I and Hind III fragments on the FHV-1 genome (Figure 10). Fragments in hypermolar bands were assigned locations in alphabetical order starting at the S region of the genome.

Sal I L, J, and B hybridized to terminal fragment Hind III A and none of these fragments contained internal Hind III sites. Sal H hybridized to to Hind III A, L, N/O/P and T, and contained fragments identical in size to Hind III N and T along with 4.7 kb of Hind III A and 1.0 kb of Hind III L. These results indicate that the order of Sal I fragments from the terminus of the S region must be: L, B or J, and H.

Sal H was linked to Sal I D based on common hybridization to Hind III L. Sal D was found to contain fragments corresponding to Hind III I, M, and S along with two 2.5 kb fragments. Since Sal I D hybridized to Hind III I, M, S, L, and F/G, one of these 2.5 kb fragments must be the part of Hind III L not contained in Sal I H. The other fragment was therefore part of Hind III F.

Sal I I contained Hind III R and two fragments 3.4 and 3.2 kb in size. Sal I I hybridized to Hind III F/G and R only. The 3.4 and 3.2 kb fragments were part of both Hind III F and G. Sal I I was placed adjacent to Sal I D since both fragments contained part of Hind III F. Sal I I was linked to Sal I C based on common hybridization to Hind III G. Sal I C hybridized to and contained internal Hind III fragments corresponding to

Hind III H, K, and Q. The 2.5 kb Hind III digest fragment of Sal I C was the remainder of Hind III G which was shared with Sal I I. The final Hind III fragment of Sal C was 4.7 kb in size and was presumably part of Hind III B.

At this point, examining the terminus of the L region genome and following the mapping strategy toward the center of the genome is appropriate. Sal I F was identified as the terminus of the L region of the genome. Sal I F hybridizes to Hind III J and E. Since Sal I N also hybridizes to Hind III E and no other Sal I fragments hybridize to Hind III J, Hind III J must lie completely within Sal I F.

Sal I N was cut into two fragments of approximately 1.0 and .6 kb by Hind III. Hybridization showed that Sal I N overlaped both Hind III E and N/O/P. Based on the distance from the terminus, Sal I N should share approximately .6 kb with E and 1.0 kb with Hind III P.

Sal I M was placed next to Sal I N based upon common hybridization to Hind III N/O/P. Sal I M overlaped the remaining 1.6 kb of Hind III P and approximately 1.0 kb of Hind III O.

Sal I E was placed adjacent to Sal M due to common hybridization to Hind III O. Sal I E was cut into two fragments of 2.0 kb and 8.6 kb. The smaller fragment therefore overlaped Hind III O leaving 8.6 kb of Sal E shared with Hind III C.

Sal I A was found to contain all of Hind III D and, in addition, to hybridize to Hind III B/C. Hind III digestion of Sal I A yielded a fragment corresponding to Hind III D and two fragments of 3.2 and 2.0 kb which must overlap both Hind III B and C.

Both Sal I G and K hybridized to Hind III B/C and neither fragment contained internal Hind III sites. Since it was already determined that

Sal I C contained approximately 4.7 kb of Hind III B and that Sal I E shared 8.6 kb with Hind III C, Sal G was placed adjacent to Sal C and Sal I K adjacent to Sal I E. This configuration was the only one that also satisfied the size requirements of the Sal I A overlaps into Hind III B and C. These assignments completed the Sal I map shown in Figure 10 and indicated the positions of most of the Hind III fragments.

To confirm the isomeric arrangement of the FHV-1 genome and to determine the order of the Sal I B and J fragments, a more complete map of the S region was necessary. Sal I fragments L, H, B, and J, which mapped to that region, were hybridized to Eco R1 fragments of FHV-1 DNA which had been transferred to nitrocellulose strips. Also, clones containing Sal I B, J, and H were digested simultaneously with Sal I and Eco R1. Results of these experiments are shown in Figure 9A and B.

Sal I terminal fragment L hybridized to Eco R1 terminal fragment Q and contained part of Eco R1 U. Sal I L was also contained within Sal I H and Eco R1 J which contain the internal repeats. Sal I H contained a fragment equivalent in size to Eco R1 J in addition to two smaller fragments. One of these smaller fragments contained part of Eco R1 V and the other Eco R1 T which was located in the L region. Eco R1 fragments U and V also span the junctions between Sal I L and J and Sal I H and B, since Sal I H, L, B, and J hybridized to these fragments.

Sal I B contained fragments equivalent to Eco R1 F and M and parts of Eco R1 E and U/V. Sal I J hybridized to Eco R1 E and F and must contain most of Eco R1 E since neither Sal I L or H hybridized to Eco R1 E. Hybridization of Sal I J to Eco R1 F can be accounted for by the presence of common repeat DNA on both bands. This hypothesis was confirmed by demonstrating that the electrophoretically purified 5.8 kb Eco R1/Sal

Figure 9. Blot hybridization analysis of the S region of the FHV-1 genome. Panel A shows hybridization of Sal I fragments B, J, H, and L to filters containing FHV-1 Eco R1 fragments. Panel B shows an ethidium bromide stained gel of a Sal I/Eco R1 digest of recombinant phage clones containg FHV-1 Sal I fragments H, B, and J. Sizes of fragments (kb) are noted. Panel C shows a Southern blot of the gel shown in Panel B after being hybridized with the nick-translated 5.8 kb Sal I/Eco R1 fragment of Sal I J.

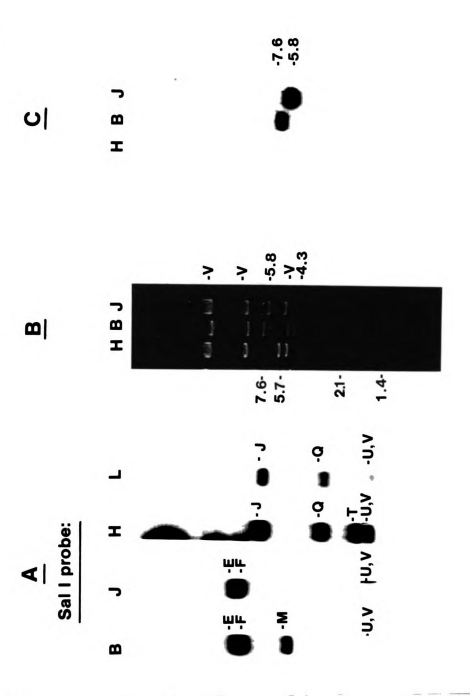
A
Sal I probe:
B J H L
-E
-F
-E
-F
-J
-J
-M
-Q
-Q
-T
-U,V
-U,V
-U,V
-U,V
B
H B J
-V
-V
-5.8
-V
-4.3
7.6-
5.7-
2.1-
1.4-
C
H B J
-7.6
-5.8

Figure 9

Figure 10. Physical map of the FHV-1 genome for Sal I, Hind III and Eco R1. Both isomers of the S region are shown. Vertical dashed line indicates inversion point. (Ul-unique long region, Us-unique short region, TRs-terminal repeat sequences, IRs-internal repeat sequences)

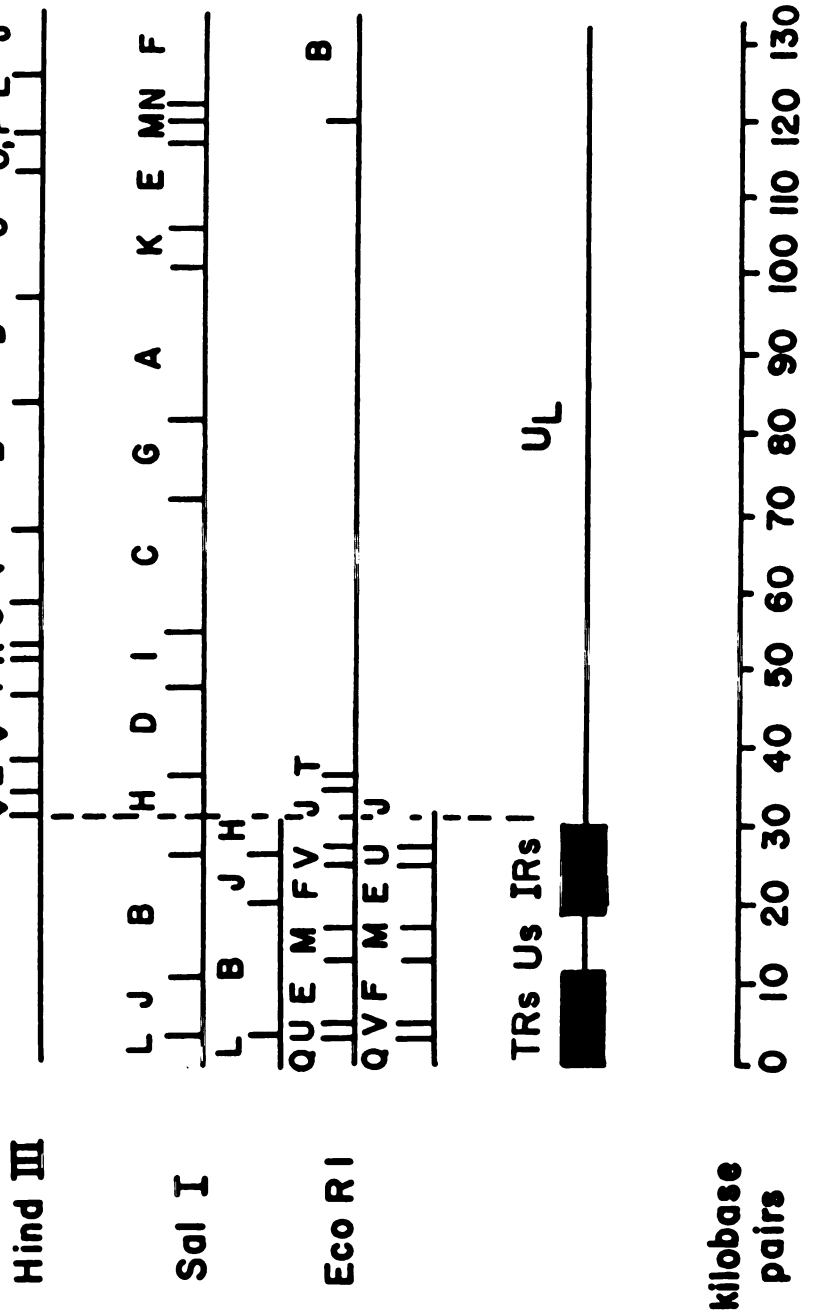
Hind III
Sal I
Eco R I
kilobase pairs
A
N,T
I,M,S
L
F
R
G
H,K,Q
B
D
C
O,P
E
J
L
J
B
H
D
I
C
G
A
K
E
M
N
F
L
B
J
H
Q
U
E
M
F
V
J
T
B
Q
V
F
M
E
U
J
TRs
Us
IRs
UL
0
10
20
30
40
50
60
70
80
90
100
110
120
130

Figure 10

fragment of Sal I J hybridized to the Eco Rl E fragment generated by Sal I/Eco Rl digestion of the clone containing Sal I B (Figure 9C). These data indicates that the inverted repeat sequences extend beyond the Eco Rl U and V fragments into Eco Rl E and F and therefore must be greater than 4.8 kb in length. The Eco Rl map of the S region of FHV-1 DNA is shown in Figure 10. Both orientations of the S region are shown. The orientation of the Sal I J and B fragments relative to the Eco Rl fragments is the only configuration that produced the appropriate Eco Rl digestion fragments of Sal I B and J.

Sizes of the fragments contained within the S region total approximately 31 kb. Based on the electron microscopic analysis, approximately 8 kb of the S region is composed of unique DNA. Therefore, the sizes of the repeats can be calculated to be approximately 11 kb. By subtracting the size of the S region from the size of the entire genome, the size of the L region was determined to be approximately 103 kb.

Discussion

This paper contains the initial description of the physical structure of the genome of the C-27 prototype strain of FHV-1. Based on restriction enzyme digestion analysis, the viral genome is 134 kb in size. This size estimate is based upon the average of the size total of the restriction fragments generated by Bam HI, Hind III, Eco R1 and Sal I digestions. Small variations in the size total calculated for each enzyme is due to the inherent error in determining the size of individual fragments from electrophoretic separations. Terminal restriction fragments were identified by both progressive digestion from the ends of the genome with lambda 5' exonuclease and by labeling the ends with kinased synthetic linkers. Two one molar terminal fragments in the Bam HI, Eco R1 and Sal I digests and one terminal fragment in the Hind III digest were identified. Hybridization patterns of Sal I terminal fragments identified the other Hind III terminal fragment and indicated that viral DNA contained both terminal and internal inverted repeat sequences at one end of the genome. The positions of these repeat sequences was confirmed by electron microscopic examination of reannealed FHV-1 DNA. The size of the genome based on electron microscopy was shorter than the size determined by restriction endonuclease digestion. This discrepency was probably caused by inevitable nicking of very large DNA molecules. Nicking could have caused an inversion of the L segment of the viral genome to go unnoticed, however hybridization patterns of the Sal I F terminal fragment indicate that a L segment inversion is unlikely.

In a previous study (Herrmann et al., 1984), the size of the FHV-1 genome was calculated to be about 10 kb smaller than the size determined

in this study. However, in that study, fragment molarity was not determined and some of the smaller restriction fragments might not have been detected since the fragments were visualized by ethidium bromide staining.

In this report we also describe the isolation of recombinant phage clones which contain approximately 85% of the FHV-1 genome as Sal I inserts. Clones containing viral termini were not represented in the library since they have only one terminus that was capable of ligating to the Sal I cohesive end on the vector DNA. Two clones were found to contain the 1.6 kb Sal I fragment N as a multiple insert. It was surprising that clones containing the smaller fragments Sal I M and K as multiple inserts were not identified even after screening a large number of plaques with probes prepared from these fragments. We are currently completing our clone collection of Sal I fragments by using additional cloning strategies to isolate clones containing the viral termini and the smaller fragments that were not represented in the existing clone collection.

The recombinant clones were used both as hybridization probes and as substrates for double digestion experiments to create a restriction map of the genome for Sal I and Hind III. The linear order of all Sal I fragments except the three smallest fragments (less than 1% of the genome) was determined. The approximate positions of 14 of 22 Hind III fragments were determined. Based on the data obtained, it was not possible to determine the order of multiple Hind III fragments lying completely within large Sal I fragments.

In addition, an Eco R1 map of the S region of the genome was also created. Lengths of the inverted repeat sequences were determined to be greater than 4.8 kb, but the exact length could not be determined from

the available data. Analysis of the S region with additional restriction enzymes will be necessary to determine the exact size of the repeats. The approximate size of the repeats, as determined by electron microscopic analysis of the S region, was approximately 11 kb.

Previous studies of the structure of herpesviruses genomes have traditionally involved electron microscopy followed by single and double restriction enzyme digestion analysis and Southern blot or cross-hybridization studies (Henry et al., 1981, Spector et al., 1982, Sullivan et al., 1984). Our strategy in mapping the genome of FHV-1 was basically similar. The availability of cloned DNA fragments for use in Southern blotting and double digestion experiments was very helpful since it made it unnecessary to have to electrophoretically purify all of the restriction fragments in a digest and also allowed us to separate two molar bands. We observed that individual gel-purified restriction fragments were frequently contaminated with other parts of the viral genome. Probes prepared from these fragments had to be tested by hybridizing them back to the appropriate genomic digest. Often, several rounds of electrophoretic purification were needed.

Based on the data presented in this report, the genome of FHV-1 is 134 kb in size and is composed of two segments. The longer segment (L) is composed of 103 kb of unique DNA (Ul) and is adjacent to a 31 kb short (S) segment. The short segment contains 8 kb of unique DNA (Us) bounded by 11 kb repeats. The short segment can invert relative to the Ul segment creating two possibe isomeric forms of the viral genome.

Therefore, FHV-1 has a group D genome based on the classification scheme proposed by Roizman (1980). Other herpesviruses in this group include pseudorabies virus (PRV) (Ben-Porat et al., 1979),

varicella-zoster virus (VZV) (Ecker and Hyman, 1982, Straus et al., 1981), equine herpesvirus-1 (EHV-1) (Henry et al., 1981), equine herpesvirus-3 (Sullivan et al., 1984) and bovine herpesvirus-1 (Mayfield et al., 1983). Of these, the size and arrangement of the FHV-1 genome is most similar to that of EHV-1. However, the biological properties of FHV-1 are closer to those of VZV which also has a limited host range. Thus far, no studies have been conducted to investigate sequence homology or cross reacting antigens between FHV-1 and any other herpesviruses with similar genomic structure. We have found that FHV-1 shares limited sequence homology and several cross reacting antigens with canine herpesvirus (Rota and Maes, unpublished observations).

Recent studies have indicated that a small number of VZV DNA molecules (Kinchington et al., 1985) and DNA from a strain of PRV (Lomniczi et al., 1984) contain inversions of the L region. It will be interesting to deterimine, in future studies, if such novel genomic arrangements exist in FHV-1.

In herpesviruses, the amount of interstrain genetic variability, as detected by restriction endonuclease analysis, varies considerably. For example, strains of herpes saimiri (Desrosiers and Falk, 1982) contained more restriction endonuclease fragment variability than strains of herpes simplex (Buchman et al., 1980), equine herpesvirus (Studdert et al., 1981) and PRV (Paul et al., 1982), while strains of VZV (Martin et al., 1982, Richards et al., 1979) showed far less interstrain variability. Recent studies have shown that, like VZV, various isolates of FHV-1 contain little restriction fragment size variability. This stability was initially observed by Herrmann et al (1984) in a study of both clinical isolates and vaccine strains of FHV-1. We have also examined the restriction digestion

patterns of a number of case isolates of FHV-1 (Rota et al., 1985) and found that the digestion patterns were very similar. The map that was constructed in this study for the C-27 prototype strain of FHV-1 should be applicable in the analysis of many FHV-1 isolates. Based on this map, most of the variation between the strains of FHV-1 occurred in fragments at the viral termini and within the internal inverted repeat sequences. This type of variation has been observed in other herpesvirus genomes (Hayward, et al., 1975, Paul et al, 1982).

The FHV-1 restriction map should prove extremely useful in future studies aimed at mapping genes on the viral genome and will allow us to explore herpesvirus infections in the natural host in greater detail. Also, comparing the relative map positions of important FHV-1 genes to the positions of those genes on other herpesviruses will be interesting especially in light of recent evidence for colinear genome organization (Davison and Wilkie, 1983).

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Chapter 3

The Immediate Early Genes and Polypeptides of Feline Herpesvirus-1

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Abstract

The immediate early (IE) genes and polypeptides of feline herpesvirus-1, a major upper respiratory tract pathogen of cats, were studied. RNA purified from cycloheximide-treated, FHV-1 infected cells was radiolabeled and hybridized to restriction fragments of FHV-1 virion DNA and to DNA from recombinant bacteriophage clones containing FHV-1 DNA inserts. The results indicated that IE RNA is transcribed primarily from the inverted repeat regions which bracket the unique short region of the viral genome. RNA from mock-infected cells did not hybridize to FHV-1 DNA. In Northern blot experiments, three species of immediate early RNA with approximate sizes of 5, 1.6 and .8 kb were detected by a FHV-1 DNA probe. The 5 and 1.8 kb RNAs hybridized to cloned FHV-1 DNA fragments containing the S region of the genome. Two polypeptides with approximate molecular weights of 155 and 120 kilodaltons were present in the *in vitro* translation products of IE RNA and were not present in the translation products of RNA from mock-infected cells. These polypeptides were also detected in infected cells at four hours post infection, in actinomycin D-treated, infected cells after reversal of a cycloheximide block, and in cells infected in the presence of azetidine.

Introduction

Feline herpesvirus-1 is a major viral pathogen of cats and its distribution is worldwide. Infections in adult cats result primarily in upper respiratory tract disease, while infections in kittens can cause generalized disease with high mortality rates (Gaskell and Povey, 1982, Povey, 1979). Preliminary evidence suggests that FHV-1 can establish latent infections in the trigeminal ganglia and the reactivation of these latent infections is an important mechanism of virus spread in the cat population (Gaskell et al., 1985).

Recent studies in our laboratory have focused on the physical structure of the genome of FHV-1. The viral DNA has two isomeric forms and is approximately 134 kb in size. Recombinant phage clones containing most of the genome have been isolated and have been used to construct restriction endonuclease cleavage maps of the FHV-1 genome (Rota et al., 1985). The goal of this research was to localize the immediate early gene region and to identify the immediate early polypeptides of FHV-1.

It is well established that during a lytic infection, herpesvirus gene expression is highly regulated (Feldman et al., 1979, Honess and Roizman, 1974). Immediate early genes are transcribed from a limited area of the viral genome immediately after infection. The immediate early gene products initiate the transcription of early genes. Early gene products stimulate the transcription of late genes and suppress the further production of immediate early peptides. The majority of late gene products are structural components of the virion. Several late genes are responsible for the suppression of early gene expression. In addition, a virion component functions to stimulate the expression of immediate early

genes at the beginning of the replication cycle (O'Hare and Hayward, 1985).

In the presence of cycloheximide, herpesvirus immediate early mRNA accumulates in infected cells (Ihara et al., 1983). RNA from cycloheximide-treated, infected cells has been radiolabeled in vitro and used in blot hybridization studies to identify the regions of the genomes of cytomegalovirus (Wathen and Stinski, 1982) and pseudorabies virus (Ihara et al., 1983) containing the immediate early genes. This IE RNA has been translated in vitro to identify the immediate early gene products (Ihara et al., 1983). When the cycloheximide block is removed from infected cells immediate early mRNA is rapidly translated into protein. Addition of actinomycin D to these cells prevents the expression of early genes and provides another means of identifying immediate early proteins (Fenwick et al., 1980, Fenwick and Clark, 1983, Fenwick and McMenamin, 1984).

Immediate early peptides of other herpesviruses have also been identified by treating infected cells with the proline analog, azetidine. Immediate early peptides accumulated in infected presumably because they had incorporated the analog and, as a result of this, were defective in their ability to activate early genes (Fenwick et al., 1980).

In this report, we identify the region of the FHV-1 genome containing the immediate early genes by hybridizing RNA from cycloheximide-treated, infected feline kidney (CRFK) cells to nitrocellulose filters containing both virion DNA and cloned viral DNA fragments. The major immediate early RNA species were identified by Northern blotting. In addition, the major immediate early peptides of FHV-1 were identified by in vitro translation of infected cell RNA and by SDS-PAGE analysis of polypeptides accumulating

in actinomycin D-treated, infected cells after release of a cycloheximide block or in infected cells treated with azetidine.

Methods and Materials

Virus and cells. The C-27 prototype strain of FHV-1 was obtained from the American Type Culture Collection, Rockville, MD (#VR 636). The virus was propagated in Crandell-Rees feline kidney (CRFK) cells maintained in Eagle's Minimum Essential Medium (EMEM) (K.C. Biologics, Lenexa, KS.) supplemented with 10% fetal bovine serum. (Hyclone defined, Sterile Systems, Logan, UT).

Immediate early (IE) RNA isolation and labeling. Confluent monolayers of CRFK cells were preincubated for 1 hour in EMEM with 2% fetal bovine serum and 100 ug/ml cycloheximide (Sigma, St. Louis, MO) (EMEM-cyc). The pretreated cells were infected with FHV-1 at a multiplicity of infection of approximately 5 $TCID_{50}$/cell. The medium overlaying infected cells was decanted after a one hour adsorption period and fresh EMEM-cyc was added to the culture flasks. At 4 hours post infection, the infected cells were scraped into the culture medium and washed twice in ice-cold PBS by low speed centrifugation (1000 x g, 10 min. 4°C). The washed cell pellet from approximately 10^8 cells was resuspended in 8 ml of 5.8 M guanidinium isothiocyanate, .1 M 2-mercaptoethanol, 1% (wt/vol) Sarkosyl, 50 mM Tris-HCl pH 7.6, and 10 mM EDTA. When the cells were completely dissolved, the suspension was layered onto a 3 ml cushion of 5.7 M CsCl and centrifuged for 20 hours at 25,000 rpm in a SW 41 rotor (Beckman Instruments, Palo Alto, CA) at 20°C. Following centrifugation, the supernatants were removed by aspiration and the RNA pellets were suspended in 200 ul of TE (10 mM Tris-HCL pH 7.6, 1 mM EDTA) with .1% SDS. Samples were extracted twice with phenol:chloroform and precipitated with 2.5 vol

of ethanol in the presence of .1M NaCl. RNA pellets were washed twice in 95% ethanol before being resuspended in RNase-free water.

Five to ten micrograms of total cellular immediate early or control RNA were hydrolized in 10 ul of 50 mM glycine pH 9.5, .1 mM EDTA, 1 mM spermidine at 90°C. for 15 minutes. After hydrolysis, the samples were chilled immediately on ice and 5 ul of cold kinase buffer (BRL) was added. 250 uCi of gamma ^{32}P-ATP (ICN, Irvine, CA, sp. act. 7,000 Ci/mmole) and 5 units of T4 polynucleotide kinase (BRL) were added to each sample and the volume was adjusted to 50 ul with water. Samples were incubated at 37°C. for 30 minutes. Reactions were stopped by addition of 4 ul of .5 M EDTA and unincorporated label was removed by centrifuging the samples through .9 ml Sephadex G-50 columns (Maniatis et al., 1982).

Hybridizations. FHV-1 virion DNA and cloned FHV-1 DNA were purified as previously described (Rota et al., 1985). DNA samples were digested with restriction enzymes using the reaction conditions recommended by the manufacturer (BRL) and the fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters using standard methods (Maniatis et al., 1982). Hybridization and wash conditions were similar to those described by Wathen and Stinski (1982). Filters were prehybridized for 24 hours at 68°C. in hybridization buffer (5X Denhardt's solution, 3X SSC, 150 ug/ml denatured salmon sperm DNA and 50 ug/ml yeast t-RNA. Labeled RNA (10^8 cpm) was heated to 100°C for 5 minutes before being added to the hybridization solution. Hybridizations were incubated for 48 hours at 68°C. Filters were washed twice at room temperature for 30 minutes in 2X SSC, treated with 30 ug/ml DNase-free (80°C., 10 min.) RNase (bovine pancreas) in 2X SSC for one hour at room

temperature, and washed in hybridization buffer at 68°C for one hour. Filters were given a final wash in 2X SSC before being exposed to Kodak X-AR film with intensifying screens (Dupont, Wilmington, DE).

Developed films were scanned with an Ortec 4310 densitometer (Oak Ridge, TN) equipped with an integrator.

Northern blotting. RNA samples from infected or mock-infected CRFK cells were denatured and separated on 1.2% agarose gels containing 7.5% formaldehyde and transferred to nitrocellulose filters using the methods described by Maniatis (Maniatis et al., 1982). Total FHV-1 virion DNA or DNA from bacteriophage clones were labeled with ^{32}P by nick-translation and filters were hybridized and washed as previously described (Rota et al., 1985).

In vitro translation. IE RNA was translated in vitro using a commercially avialable rabbit reticulocyte lysate kit (BRL) according to the protocol described by Jackson and Hunt (1983). Labeled translation products were separated by SDS-PAGE as described by Laemmli (1970). Gels were fixed for 30-60 minutes in 50% ethanol, 10% glacial acetic acid. The fixative was removed by soaking gels in a large volume of distilled water for 30 minutes. Gels were prepared for flourography by a 30 minute soak in 1M salicylic acid, dried and exposed to X-ray film. Molecular weight standards (SDS-6H, Sigma, St. Louis, MO) included myosin (205 kd), B-galactosidase (116 kd), phosphorylase B (97.4 kd), bovine plasma albumin (66 kd), egg albumin (45 kd), and carbonic anhydrase (29 kd). Marker lanes were cut from the gel and stained with Coomassie Brilliant Blue. Molecular

weights of the translation products were determined using the method of Weber and Osborn (1969).

Hybrid selection and hybrid arrest. For the hybrid selection experiments, the binding of denatured DNA to nitrocellulose filters was performed as described by Maniatis (1982). Hybridization of IE RNA to filters, washing of filters, and elution of bound RNA were performed as described by Belle Isle et al. (1981). The eluted RNA was precipitated with ethanol, washed twice with 95% ethanol and translated in vitro as described above. For hybrid arrest of translation experiments, 5 ug of total viral DNA was hybridized to 1 ug of IE RNA as described by Paterson et al. (1982) before being translated in vitro. The translation products were analyzed by SDS-PAGE as described above.

Production of IE proteins in infected cells. Two methods were used to identify IE proteins in infected CRFK cells. The first consisted of preincubating cells in EMEM-cyc for one hour before infecting them with FHV-1 at an m.o.i. of 5 $TCID_{50}$/cell. At 4 hours post-infection, EMEM-cyc was removed and replaced with EMEM containing 50 ug/ml of actinomycin D. Cells were harvested 4, 6 and 8 hours after release of the cycloheximide block. Prior to being harvested, the infected cells were in vivo labeled for 4 hours in labeling medium (EMEM containing 1/10 the normal concentration of methionine, plus 10 uCi/ml ^{35}S-methionine, Amersham, Arlington Hgts., Il, sp. act. 800 mCi/mmole) containing 50 ug/ml of actinomycin D. After the labeling period, the medium was decanted and the cells were washed three times in cold PBS containing 0.01 mM phenylmethylsulfonyl fluoride (PMSF) and resuspended in water. The washed

cells were lysed by three freeze-thaw cycles. An equal volume of 2X SDS-PAGE sample buffer (.125 M Tris-HCl pH 6.8, 20 % glycerol, 4% SDS, .01% bromphenol blue) was added to each sample and SDS-PAGE was done as described above.

The second approach consisted of infecting CRFK cells at an m.o.i. of 5 $TCID_{50}$/cell in medium containing 4 mM azetidine. Cells were harvested at 4 and 6 hours post-infection following a 4 hour labeling period in labeling medium (see above) with 4 mM azetidine. Cells were harvested and prepared for SDS-PAGE as described above.

Results

Identification of the immediate early gene region of FHV-1 DNA. The region of the FHV-1 genome containing the immediate early genes was initially identified by hybridizing labeled RNA from cycloheximide treated, infected CRFK cells (IE RNA) to nitrocellulose filters containing Sal I digested FHV-1 DNA. Figure 1A shows that IE RNA hybridized to Sal I fragments H, B/C, J, and L. The figure also shows that labeled RNA from mock infected CRFK cells did not hybridize to FHV-1 DNA. Films were scanned with a densitometer and the area under each peak was used to determine the proportion of labeled RNA binding to each restriction fragment. Data shown in Figure 1B are based on the average of measurements obtained by scanning three different gels. Labeled IE RNA hybridized extensively to Sal I fragments L and H and to a lesser extent to Sal I B/C and J. Sal I L is a terminal fragment located completely within the terminal inverted repeat sequences of the viral genome, and Sal I H maps partially within the internal inverted repeat region. These repeat regions flank the Us region of the viral genome (Rota et al., 1985) (Figure 4).

To determine which of the fragments in the Sal I B/C band hybridized to IE RNA, and to confirm the hybridization patterns of the other Sal I fragments, IE RNA was hybridized to to nitrocellulose filters containing Sal I digested DNA from a set of bacteriophage clones containing FHV-1 DNA as Sal I inserts (Figure 2). As expected, IE RNA hybridized to cloned Sal I fragments H and J and did not hybridize to any of the other cloned Sal I fragments which migrate close to Sal I H and J in digests of total viral DNA. Sal I B, but not C hybridized to IE RNA. It was not possible to confirm the hybridization of IE RNA to Sal I L since this fragment has not

Figure 1. Hybridization of IE RNA to FHV-1 DNA. Panel A autoradiograms of filters containing Sal I fragments of FHV-1 DNA that were hybridized to labeled viral DNA, IE RNA or RNA from mock-infected controls. A photograph of an ethidium bromide-stained agarose gel showing sizes (in kilobases) of Sal I fragments of FHV-1 DNA is shown at the left for reference. Panel B shows a typical densitometer scan of an autoradiogram of the filter hybridized to with labeled IE RNA (panel A). The numbers below each band indicate the percentage of labeled RNA binding to the individual fragments.

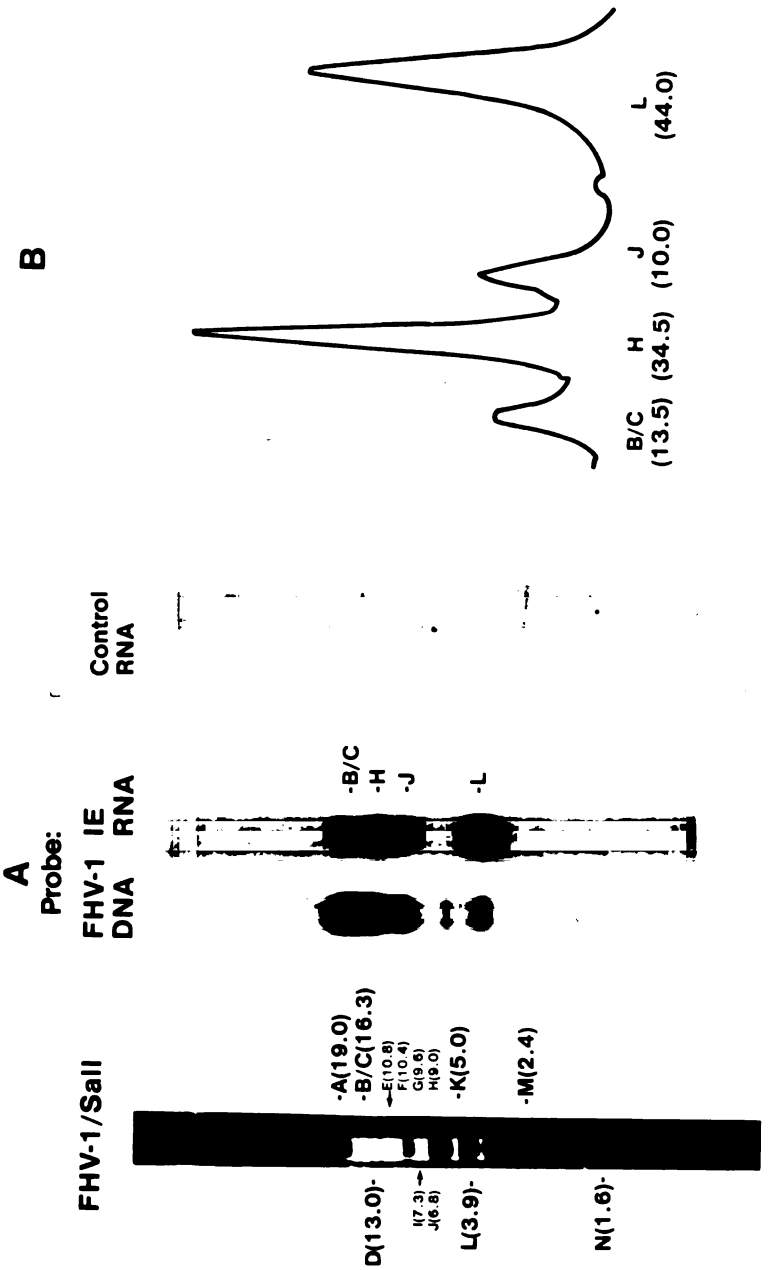
A
Probe:
FHV-1 DNA
IE RNA
Control RNA
FHV-1/SalI
-A(19.0)
-B/C(16.3)
E(10.8)
F(10.4)
G(9.6)
H(9.0)
-K(5.0)
-M(2.4)
D(13.0)-
I(7.3)
J(6.8)
L(3.9)-
N(1.6)-
-B/C
-H
-J
-L
B
B/C (13.5)
H (34.5)
J (10.0)
L (44.0)

Figure 1

Figure 2. Hybridization of IE RNA to DNA from recombinant clones containing FHV-1 DNA inserts. Sal I digested phage DNA was subject to agarose gel electrophoresis (a photograph of an ethidium bromide-stained gel is shown in panel A) and transferred to nitrocellulose filters and hybridized to labeled IE RNA (panel B). Letters above each lane designate the FHV-1 Sal I fragment contained in each clone. For reference a Sal I digest of FHV-1 DNA was hybridized to nick-translated FHV-1 DNA (at left).

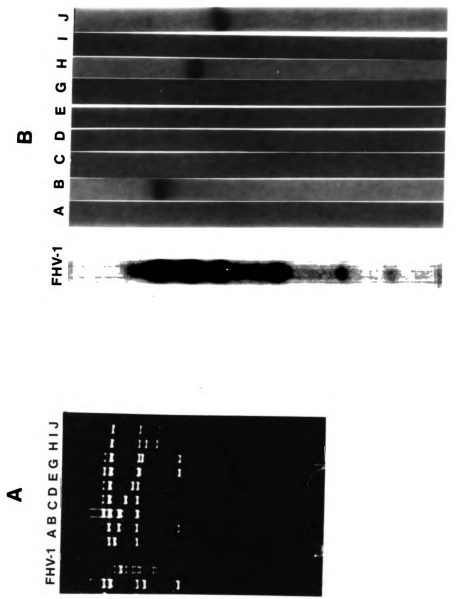

Figure 2

Figure 3. Hybridization of IE RNA to Sal I/Eco Rl digestion fragments of bacteriophage clones containing FHV-1 IE genes. Panel A shows an ethidium bromide stained stained gel containing Sal I/Eco Rl fragments of clones containing Sal I fragments H, B, and J which was transferred to nitrocellulose and hybridized to labeled IE RNA (panel B). The sizes (in kilobases) of the hybridizing fragments are shown.

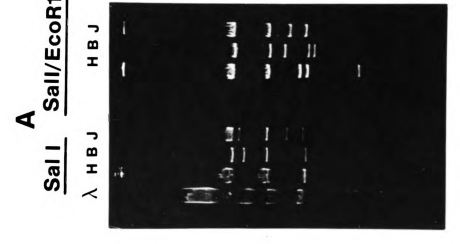
A
Sal I
Sall/EcoR1
λ H B J
H B J
B
Sal I
Sall/EcoR1
H B J
H B J
7.6
5.8
5.7
1.0
.5

Figure 3

been cloned. However, Sal I digestion of FHV-1 DNA does not produce any fragments which migrate close to Sal I L in electrophoretic separations. Therefore, the immediate early genes of FHV-1 are contained on Sal I fragments, H, B, J, and L which are located within the S region of the viral genome (Figure 4).

Clones containing Sal I fragments H, B, and J were digested with both Sal I and Eco R1 and the fragments were electrophoresed and transferred to nitrocellulose filters and hybridized to labeled IE RNA (Figure 3). IE RNA hybridized to the 5.7 kb Eco R1 J fragment and to the 1.2 kb portion of Eco R1 V which are contained on Sal I H. Eco R1 J and Sal I H both span the L-S junction of the viral genome (Figure 4). The 2.0 kb Eco R1 fragment of Sal I H which contains Eco R1 T and is located completely within the U1 region of the genome did not hybridize. The 7.6 kb Eco R1 fragment of Sal I B which corresponds to Eco R1 F and a small approximately .5 kb portion of Eco R1 V (barely visible on the 2 week exposure shown in Figure 3) hybridized to IE RNA. The Eco R1 M fragment within Sal I B and a 1.9 kb fragment that is part of Eco R1 E did not hybridize to IE RNA. These two fragments lie completely within the Us region of the viral genome. Both Eco R1 fragments of Sal I J hybridized to IE RNA. The .5 kb Eco R1 fargment of Sal I J is barely visible in Figure 3.

The limits of the IE gene region of the FHV-1 genome, based on the data obtained in this study, are shown in Figure 4. All of the fragments are at least partially contained within the inverted repeat regions of the viral genome. Some of the hybridizing fragments also contain portions of both the U1 and Us regions, but none were found that contained only unique DNA sequences.

Figure 4. The immediate early region of FHV-1 DNA. Figure shows a Sal I and Eco R1 map of the S region of the genome generated in a previous study (Rota, et al., 1985). The lower solid bar indicates restriction fragments hybridizing to IE RNA.

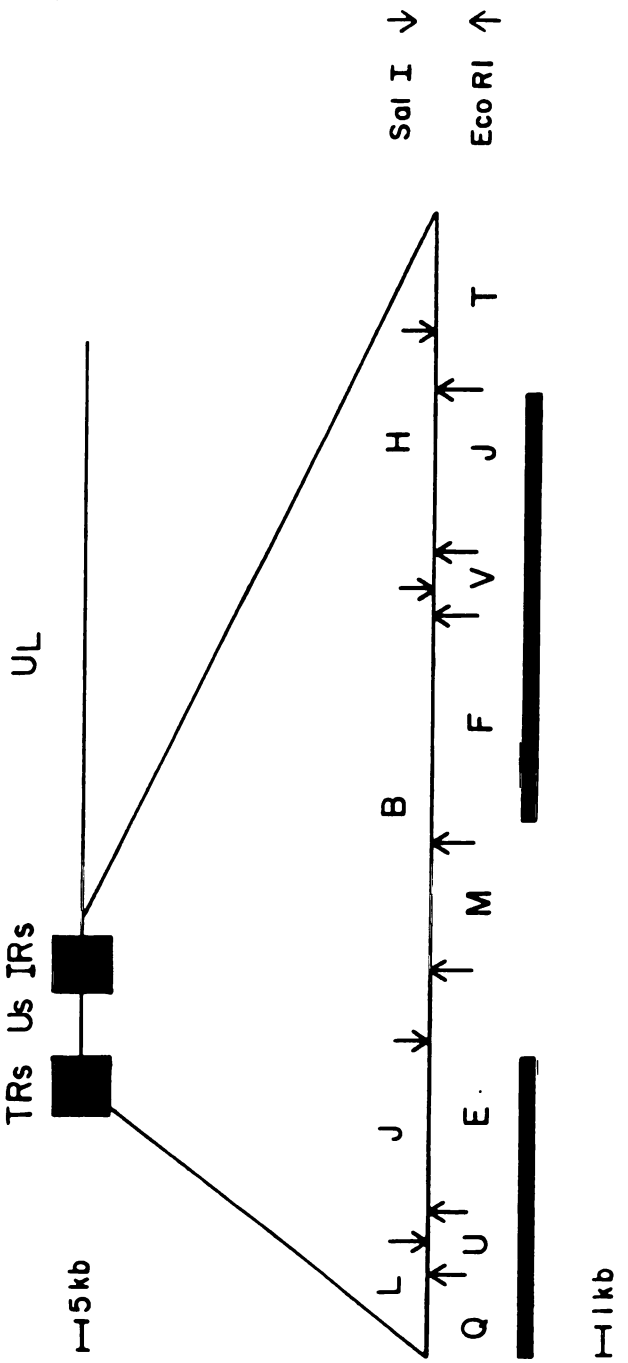

Figure 4

Northern blot analysis of IE RNA detected at least three RNA species. The largest migrated slightly slower than the 28S RNA marker (Figure 5). and had an approximate size of 5 kb. This RNA hybridized to a total viral DNA probe as well as to probes prepared from clones containing Sal I fragments H, B, and J which collectively contain the entire S region of FHV-1 DNA. Sal I L has not been cloned but contains only repeat DNA which should be present, in inverted orientation, on Sal I H. The hybridization signal of the Sal I H probe appeared to be more intense than that obtained with the other cloned fragment probes. Two additional IE RNA species were recognized by the FHV-1 DNA probe. The larger of these two migrated faster than the 18S RNA marker and its size was estimated to be 1.6 kb. This RNA was also detected by all of the the cloned fragment probes. A smaller RNA molecule with an estimated size of .8 kb was detected with the total viral DNA probe but not with any of the cloned fragments even at exposure times longer than the one shown in Figure 5.

Identification of FHV-1 immediate early peptides. The immediate early polypeptides induced by FHV-1 were identified by analysis of the *in vitro* and *in vivo* translation products of IE RNA. SDS-PAGE analysis of the *in vitro* translation products of IE RNA and RNA from mock-infected CRFK cells (Figure 6A) revealed that two polypeptides were present in the translation products of IE RNA that were not present in those of control RNA. The largest of these IE-specific polypeptides had an estimated molecular weight of 155 kilodaltons (kd). The second had an estimated molecular weight of 120 kd. Polypeptides which co-migrated with each of the IE-specific *in vitro* translation products were present in *in vivo*-labeled extracts of infected CRFK cells which were harvested at 4 hours post

Figure 5. Northern blot analysis of FHV-1 IE RNA. RNA purified from infected (lanes B-E) or mock-infected (lane A), cycloheximide-treated CRFK cells was separated by electrophoresis on a formaldehyde-agarose gel and transferred to nitrocellulose. Filter strips were hybridized to nick-translated FHV-1 DNA (lanes A, B) or recombinant phage DNA containing FHV-1 Sal I fragments H (C), B (D), or J (E). Positions of ribosomal RNA size markers are shown at left.

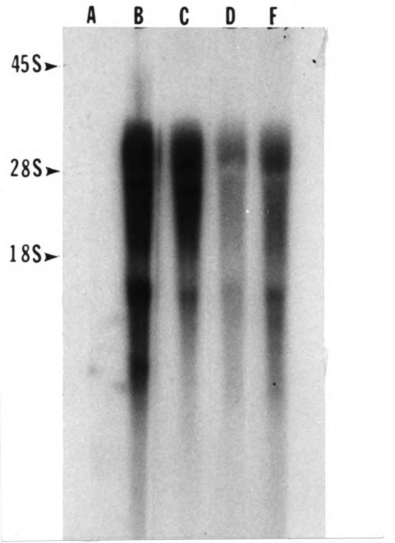

Figure 5

Figure 6. SDS-PAGE analysis of FHV-1 IE polypeptides. Panel A shows analysis of *in vitro* translation products of IE RNA (I) and RNA from mock-infected cells (C) compared to radiolabeled extracts from infected CRFK cells harvested at 4 hours post-infection (i). Panel B shows analysis of radiolabeled polypeptides accumulating in infected (I) or mock-infected (C) cells in the presence of actinomycin D and after reversal of a cycloheximide block. Panel C shows analysis of radiolabeled polypeptides from infected (I) or mock-infected (I) cells after incubation in 4 mM azetidine. The positions and sizes of the IE specific polypepties are marked with arrows.

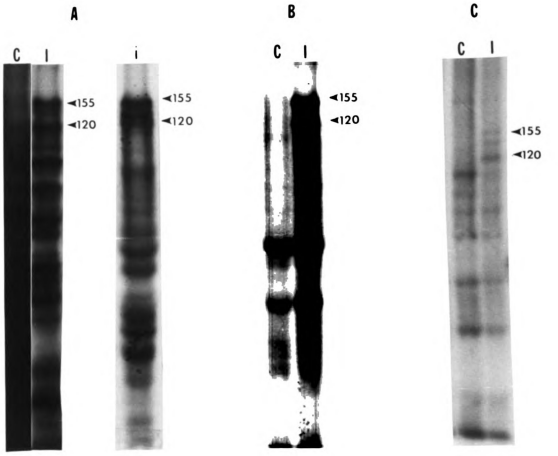

Figure 6

infection. This suggested that the IE specific polypeptides that were identified in the analysis of the in vitro translation products were not artifactual.

To specifically translate IE RNA in vivo, infected cells were released from a cycloheximide block and incubated in labeling medium in the presence of actinomycin D. The 155 and 120 kd polypeptides were detected by SDS-PAGE analysis of radiolabeled cell extracts 6 hours after cycloheximide reversal (Figure 6B). Cell extracts harvested at shorter and longer times after cycloheximide reversal were also analyzed by SDS-PAGE (not shown). The samples taken at later times showed essentially the same SDS-PAGE patterns as the ones shown in Figure 6B. In samples taken at earlier times, the IE-specific polypeptides were visible but appeared to be present in smaller amounts. We were not able to detect any differences in the rate of appearance of the two IE-specific polypeptides which could indicate a precursor product relationship.

In CRFK cells infected in the presence of azetidine, two IE-specific polypeptides were identified at 4 hours post infection which co-migrated with the 155 and 120 kd IE-specific polypeptides identified in the in vitro and in vivo translation studies (Fig. 6C). Additional FHV-1 induced peptides were present in the azetidine-treated cells. Presumably these represent either breakdown products of immediate early peptides or early gene products that were expressed in the presence of azetidine.

To demonstrate that the 155 and 120 kd IE-specific polypeptides were viral gene products, hybrid selection and hybrid arrest experiments were performed. In vitro translation of IE RNA hybrid-selected by filters containing FHV-1 DNA produced translation products that corresponded to

the 155 and 120 kd polypeptides produced by in vitro translation of non-selected IE RNA (data not shown). Control filters were unable to select for these translation products.

The hybrid arrest experiment provided additional evidence for the viral origin of the IE-specific polypeptides. Hybridization of FHV-1 DNA to IE RNA prior to in vitro translation resulted in elimination of only the 155 and 120 kd IE polypeptides from the translation products (not shown).

Discussion

This report contains the initial characterization of the immediate early genes and gene products of FHV-1. Immediate early RNA from FHV-1 infected cells was found to be transcribed from the 11 kb inverted repeat sequences which flank the unique short region on the viral genome. This region represents approximately 18% of the viral genome. Most of the IE RNA hybridized to restriction fragments within the inverted repeats that are nearest to the viral terminus or the L-S junction. IE RNA did not hybridize to any fragments which contained only DNA from the Us or Ul regions. Two HSV IE transcripts extend from the repeat regions into the Us region (Watson et al., 1981). The extent to which FHV-1 IE RNA may extend into the Us region, if at all, cannot be determined until further mapping studies to delineate the exact boundaries of the FHV-1 repeat regions are completed (Rota et al., 1985).

FHV-1 IE RNA was composed of at least three RNA species based on Northern blot analysis. The largest and apparently most abundant transcript was approximately 5 kb in size and a smaller less abundant transcript of 1.6 kb were detected by cloned FHV-1 DNA fragments which collectively contain all of the S region sequences of the FHV-1 genome. It is possible that more than one transcript of each size may be present. Interestingly, a FHV-1 DNA probe detected a very small RNA molecule that was not detected by the cloned fragment probes. This could possibly be a minor IE transcript that does not map within the S region of the genome or a transcript from an FHV-1 cycloheximide-resistent early gene that is being expressed in the absence of IE mediated activation. This RNA appears to be in relatively low abundance and it might not have been detected in

the initial hybridization experiments especially if it is coded for by a region of the genome that is not represented by our collection of cloned fragments.

At least two FHV-1 encoded IE polypeptides with molecular weights of 155 and 120 kd were identified by both in vivo and in vitro translation of IE RNA. These two polypeptides were also synthesized in infected cells analyzed at 4 hours post-infection and in cells infected in the presence of azetidine. Based on observations made for other herpesviruses (Feldman et al., 1979, Fenwick et al., 1980), it would be expected that FHV-1 IE polypeptides are phosphorylated. We did not observe any major differences in the SDS-PAGE migration patterns between FHV-1 IE peptides synthesized in vivo and in vitro. Small variations in molecular weight would not be detected by our elecrophoretic analysis. Further studies using in vivo radioactive phosphate incorporation will be needed to detect the presence and extent of phosphorylation in FHV-1 IE proteins. In addition, monoclonal and monospecific antibodies directed against these IE peptides would be very useful to study the kinetics of expression, post translational modification, and extent of interrelatedness between these proteins.

The organization of the immediate early genes of FHV-1 is generally similar to that of other alpha herpesviruses. IE genes are contained within the inverted repeat sequences which flank both the Us and Ul regions of HSV DNA (Wagner, 1985) and only the Us region of PRV DNA (Feldman et al., 1982, Ihara, et al., 1983). In HSV three IE transcripts with sizes of 4.2, 2.8, and 1.8 kb have been found to code for phosphoproteins that are 175, 110, 68, 64, and 12 kd in size (Wagner,

1985). PRV has a single 6 kb IE transcript which codes for a 180 kd polypeptide (Ihara et al., 1983). In varicella-zoster virus, a herpesvirus which is similar to FHV-1 in genome stability (Herman et al., 1984, Martin et al., 1982, Rota et al., 1985) and also in in vitro and in vivo growth characteristics (Roizman, 1980), a 4.3 kb IE transcript mapping within the inverted repeat regions has recently been identified (Felser et al., 1985).

The functions of herpesvirus immediate early genes are still poorly understood. HSV IE proteins have been shown to have DNA binding activity (Freeman and Powell, 1982) and to interact with host cell heat shock proteins (Notarianni and Preston, 1982). A recent study has shown that an HSV immediate early protein functions as a negative regulator of its own expression and a positive regulator of early gene expression, while other immediate early proteins were positive regulators of both immediate early and early gene expression (O'Hare amd Hayward, 1985). The 180 kd PRV IE polypeptide is a multifunctional molecule which is required for viral early and late gene expression and appears to be responsible for the inhibition of host cell protein synthesis (Ihara et al., 1983).

Various attempts have been made to find immediate early expression during latency. Expression of HSV immediate early genes has been detected in nervous tissue from latently infected experimental animals (Green et al., 1981) but not in human tissues (Galloway et al., 1982). The advantage of identifying the immediate early genes of FHV-1 is that this information can now be used to study the expression of immediate early genes in the natural host.

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Chapter 4

Mapping the Late Genes of Feline Herpesvirus-1

Paul A. Rota and Roger K. Maes

Abstract

In this study, the late genes of feline herpesvirus-1 (FHV-1) were mapped by hybrid-selection and *in vitro* translation of infected cell RNA. *In vitro* translation of non-selected infected cell RNA resulted in the synthesis of a number of polypeptides which reacted with a goat ant-FHV-1 serum. Several of the translation products comigrated with polypeptides immunoprecipiatated from FHV-1 virions. The SDS-PAGE migration patterns of two translation products which comigrated with proteins immunoprecipitated from enzymatically deglycosylated FHV-1 virions were altered by the addition of dog pancreas microsomes to the translation reactions. The genome locations of the genes coding for 21 of the translation products was determined by using a set of bacteriophage clones containing 85% of the viral genome to select infected cell RNA prior to *in vitro* translation. FHV-1 late genes map throughout the viral genome and most are contained within the unique long region. Sal I fragment E selected for 3 translation products which had sizes corresponding to the glycoprotein precursors identified by the enzymatic deglycosylation of FHV-1 virions. Two of these translation products were apparently converted to a higher molecular weight form in translation reactions performed in the presence of the microsome extracts.

Introduction

Feline herpesvirus-1 (FHV-1) is a major cause of upper respiratory tract disease in young and adult cats. Kittens and debilitated animals may suffer a more severe generalized infection with high mortality rates (14). Like other members of the herpesvirus family, FHV-1 can establish latent infections (3). These latent infections can be reactivated by a variety of stresses, though the biochemical mechanism involved in reactivation is not understood. The shedding of infectious virus by mostly asymptomatic cats in which viral reactivation takes place is a significant mechanism of FHV-1 spread in the cat population (2).

FHV-1 has been placed in the alpha herpesvirus subfamily (16). The virus has an extremely narrow in vivo and in vitro host range and appears to exist as a single serotype (14). FHV-1 DNA has been found to be less variable than the DNA of other herpesviruses when analyzed by restriction endonuclease analysis (5,18). The viral genome is 134 kilobase pairs in size and has two isomeric forms. The structure of FHV-1 DNA is therefore similar to other alpha herpesviruses such as pseudorabies virus, equine herpesvirus and varicella-zoster virus (17).

During a lytic infection, herpesvirus true late genes are expressed after viral DNA replication and most of these genes code for structural components of the virion (6). The virion of FHV-1 contains a minimum of 17 proteins. At least three antigenic glycoproteins with approximate molecular weights of 105, 68 and 60 kilodaltons have been identified on the envelope of FHV-1 virions by immunoprecipitation analyses. These glycoproteins are also present on the surfaces of infected cells and the 105 kd glycoprotein has been found in the supernatant medium of infected

cells. Several abundant non-glycosylated proteins present on the virion also reacted with the goat antiserum used in this study (10).

We have recently isolated a set of bacteriophage clones containing most of the FHV-1 genome as Sal I inserts (17). Current research is aimed at using these cloned restriction fragments to determine the map locations of FHV-1 genes which code for important viral antigens.

Herpesvirus genes have been mapped to specific locations on the viral genome by using cloned or gel-purified restriction fragments of viral DNA to select RNA from infected cells (4). The in vitro translation products of the selected RNA were analyzed either by direct SDS-PAGE or by SDS-PAGE following immunoprecipitation with monoclonal or monospecific antibody (9). The addition of dog pancreas microsome extracts to the in vitro translation reactions allowed the identification of specific translation products which required cotranslational processing such as glycosylation and/or signal peptide cleavage (12,15).

In this study, we have used a set of bacteriophage clones containing FHV-1 DNA inserts to select RNA from infected CRFK cells. RNA was translated in vitro in the presence or absence of dog pancreas microsome extracts and the translation products were compared to intact and enzymatically deglycosylated FHV-1 virions by SDS-PAGE. Translation products were also analyzed by SDS-PAGE following immunoprecipitation with the goat anti-FHV-1 serum that was used in the initial characterization of FHV-1 antigens (10). The results indicated that, like in other herpesviruses, FHV-1 late genes appear to be distributed throughout the viral genome. One fragment, Sal I E, selected for four translation products and at least two of these were identified as glycoprotein precursors.

Methods and Materials

Virus and cells. The C-27 prototype strain of FHV-1 was obtained from the American Type Culture Collection, Rockville, MD (#VR 636). The virus was propagated in Crandell-Rees feline kidney (CRFK) cells maintained in Eagle's Minimum Essential Medium (EMEM) (K.C. Biologics, Lenexa, KS.) supplemented with 10% fetal bovine serum. (Hyclone defined, Sterile Systems, Logan, UT).

FHV-1 virions or infected cell extracts were labeled with ^{35}S-methionine and prepared for subsequent analyses as described previously (10). Radiolabeled virions and infected cell extracts were enzymatically deglycosylated using two methods. Samples containing approximately 10^5 cpm were incubated for 5 hours at 37°C with either .5 units of Endo F (NEN, Boston, MA) in 100 mM sodium phosphate pH 6.1, 50 mM EDTA, and 1% NP-40 or with 10 units of Turbo cornutus mixed glycosidase (Miles, Naperville, Ill.) in .01 M phosphate buffered saline (PBS) pH 7.2, before being immunopecipitated and analyzed by SDS-PAGE as described below. All immunoprecipitations were performed using a goat anti-FHV-1 serum as previously described (10).

Infected cell RNA isolation. Confluent monolayers of CRFK cells were infected with FHV-1 at a multiplicity of infection of approximately 10 $TCID_{50}$/cell in EMEM with 2% FBS. Infection medium was decanted after a one hour adsorption period and fresh EMEM with 2% FBS was added to the culture vessel. After 15-17 hours of incubation, the cells were scraped from the vessel surface and washed twice in ice-cold PBS by low speed centrifugation (1000 x g, 10 min., 4°C). The washed cell pellet from

approximately 10^8 cells was resuspended in 8 ml of 5.8 M guanidinium isothiocyanate, .1 M 2-mercaptoethanol, 1% (wt/vol) Sarkosyl, 50 mM Tris-HCl pH 7.6, and 10 mM EDTA. When the cells were completely dissolved, the suspension was layered onto a 3 ml 5.7 M CsCl cushion and centrifuged for 20 hours at 25,000 rpm in a SW 41 rotor (Beckman Instruments, Palo Alto, CA) at 20° C. After centrifugation, the supernatants were removed by aspiration and the RNA pellets were suspended in 200 ul of TE (10 mM Tris-HCL pH 7.6, 1 mM EDTA) with .1% SDS. Samples were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 2.5 vol of ethanol in the presence of .1M NaCl. RNA pellets were washed twice in 95% ethanol before being resuspended in RNase-free water.

Hybridization selection. Ten micrograms of either FHV-1 virion DNA, recombinant phage clone DNA (individual clones contained Sal I fragments: A, B, C, D, E/N, G/N, H, I, and J), bacteriophage vector (EMBL-3) DNA or gel-purified restriction fragments prepared as previously described (17), were denatured and bound to nitrocellulose filters as described by Parnes (13). Fifty micrograms of infected cell RNA were hybridized to each filter in a 100 ul reaction and the subsequent washes and elution of specifically bound RNA were carried out using the procedure described by Maniatis (11). Eluted RNA was precipitated with 2.5 volumes of ethanol in the presence of 15 ug of carrier tRNA (bovine liver, Boehringer MannheiM, Indianapolis, IN) and washed twice with 95% ethanol before being dried under vacuum and resuspended in 10-15 ul of water.

In vitro translation. Selected RNA and 3-5 ug of control non-selected RNA were translated in vitro using a commercially available rabbit

reticulocyte lysate system (BRL, Gaithersburg, MD) according to the protocol described by Jackson and Hunt (7). All in vitro translation reactions were done in 30 ul volumes containing 10-20 uCi of ^{35}S-methionine (Amersham, Arlington Hgts., IL, sp. act. 800 mCi/mmole) and 1 unit/ul RNasin (Promega, Madison, WI) Incubation was for 90 minutes at 30°C. In some cases, a dog pancreas microsome extract (NEN, Boston MA) was added to the translation reactions to a final concentration of 3% prior to the start of incubation. Five ul aliquots of labeled translation products were diluted with 5 ul of water and incubated in 10 ul of 2x SDS-PAGE sample buffer (.125 M Tris pH 6.8, 20% glycerol, 4% SDS, .01% bromphenol blue) for 3 minutes at 100°C before being separated by SDS-PAGE as described by Laemmli (8). Gels were fixed for 30-60 minutes in 50% ethanol, 10% glacial acetic acid. Fixative was removed by soaking gels in a large volume of distilled water for 30 minutes. Gels were prepared for fluorography by a 30 minute soak in 1M salicylic acid, dried and exposed to X-ray film. Radiolabeled molecular weight markers (BRL) were run in adjacent lanes of the gel. The molecular weights of proteins were determined using the method of Weber and Osborn (20).

For immunoprecipitation, 5-10 ul of in vitro translation reactions were diluted with an equal volume of 2X SA buffer (.04 mM PBS, 2% NP-40. .2% SDS, 1% sodium deoxycholate) before being incubated with goat antiserum as previously described (10).

DNA purification, digestion and labeling. For mapping experiments, bacteriophage clone DNA was digested, separated by agarose gel electrophoresis and transferred to nitrocellulose filters as previously described (17). Restriction fragments were isolated from agarose gels by

electroelution onto nitrocellulose filters (NA-45, Schleicher and Schuell, Keene, NH) according to the protocol described by the manufacturer. Nick-translated probes were prepared as described by Maniatis (11) and blot hybridzations were carried out as described previously (17).

Northern blotting. RNA samples were denatured and separated on 1.2% agarose gels containing 7.5% formaldehyde and transferred to nitrocellulose filters using the methods described by Maniatis (11). Lanes containing RNA from uninfected cells were removed from the gel and stained with ethidium bromide to visualize 45S, 28S, and 18S RNA size markers. Total FHV-1 virion DNA or DNA from bacteriophage clones were labeled by nick-translation (11) and filters were hybridized, and washed as previously described (17).

Results

FHV-1 late RNA was routinely harvested from infected CRFK cells at 17-18 hours post infection (m.o.i. ~10 $TCID_{50}$/cell). At this time, nearly 100% of the cells in the monolayer were showing early signs of syncytia formation, but extensive degradation of the monolayer or cell lysis were not apparent. Radiolabeled extracts of whole cells, harvested at this time, were analyzed by immunoprecipiation analysis with the goat anti-FHV-1 serum and found to contain all of the major FHV-1 associated antigens that were identified in a previous study (10) (data not shown).

RNA from FHV-1 infected or mock infected cells was translated *in vitro* and the translation products were analyzed by SDS-PAGE (Figure 1). Both RNA samples produced a number of translation products with molecular weights which ranged from 130 kd to less than 25 kd. Most of the translation products synthesized from infected cell RNA reacted with the goat anti-FHV-1 serum, while none of the translation products from RNA from mock-infected cells were recognized by the goat antiserum. These results indicated that at 17-18 hours post-infection, most of the mRNA in infected cells is of viral origin and presumably most host cell mRNA production has been inhibited by the infection.

Several of the translation products from infected cell RNA comigrated with proteins immunoprecipitated from purified, radiolabeled FHV-1 virions (Fig. 1). The largest *in vitro* translation product was approximately 130 kd in size and comigrated with the largest virion-associated protein. This suggested that complete viral peptides were being synthesized *in vitro* and that extensive premature termination of translation was not occurring.

Figure 1. In vitro translation products of FHV-1 late RNA. RNA purified from infected (inf.) and mock-infected (con.) CRFK cells was translated in vitro in the presence (+) and absence (-) of dog pancreas microsomes. Translation products were analyzed by direct SDS-PAGE (d.a.) or by SDS-PAGE after immunoprecipitation with goat anti-FHV-1 serum (i.p.). Lanes at left show SDS-PAGE analysis of proteins immunoprecipitated from intact ^{35}S-methionine-labeled FHV-1 virions (V) or virions treated with either Endo F (F) or mixed glycosidase (MG). The sizes (in kilodaltons) of important bands are indicated at either left or right of the lane.

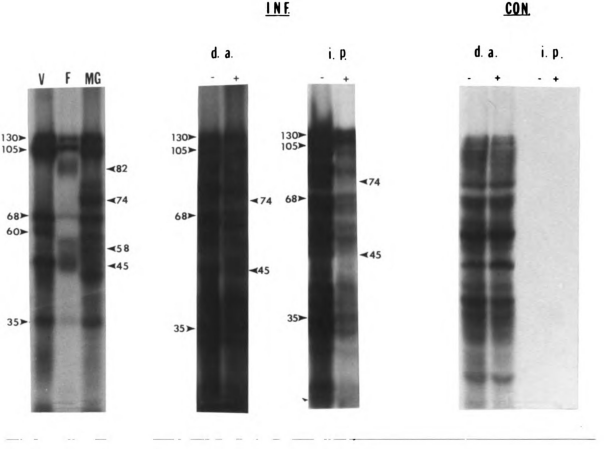

Figure 1

Figure 1 shows SDS-PAGE analysis of FHV-1 virions that were immunoprecipitated with the goat anti-FHV-1 serum. Three immunogenic glycoproteins with molecular weights of 105, 68, and 60 kd that are found on FHV-1 virions are labeled in this figure. Since we were especially interested in mapping the locations of the glycoprotein genes, it was necessary to attempt to identify the precursor molecules of these glycoproteins. The glycoprotein precursors were identified by two methods. First, to determine the approximate sizes of the glycoprotein precursors, FHV-1 virions were enzymatically deglycosylated with either Endo F or mixed glycosidases before being immunoprecipitated with the goat anti-FHV-1 serum. Endo F removes N-linked oligosaccharide chains and the mixed glycosidase preparation contains a number of exoglycosidase activities. Three possible glycoprotein precursors were identified by Endo F treatment of FHV-1 virions. These molecules had approximate molecular weights of 82, 58 and 45 kd. Mixed glycosidase treatment produced a potential glycoprotein precursor with a molecular weight of 74 kd.

Secondly, glycoprotein precursors were identified by comparing the SDS-PAGE migration patterns of translation products from reactions performed in the presence or absence of dog pancreas microsomes (Figure 1). Two <u>in vitro</u> translation products with sizes of 74 and 45 kd were apparently substrates for cotranslational processing since their migration was altered by the microsomes. Because of the number of bands on the gel it was difficult to find the higher molecular weight forms of these translation products, however, no additional bands were visible in the lower molecular weight ranges which might occur if only signal peptide cleavage was occurring. These translation products which were altered by the microsomes were virus specific since both were immunoprecipitated with

the goat antiserum and since no translation products were altered by microsome treatment of the in vitro translation reactions for mock-infected cell RNA. The 74 and 45 kd translation products of infected cell RNA were tentatively identified as glycoprotein precursors since 1) they comigrated with proteins immunoprecipitated from deglycosylated FHV-1 virions, 2) their migartion was altered by the addition of microsomes to the translation reaction, and 3) they were synthesized in large amounts which would be expected since glycoproteins comprise a significant amount of total virion protein. Though there were translation products which comigrated with the 82 and 58 kd potential precursors, identified by deglycosylation of virions, there was no evidence that these proteins were modified by the addition of microsomes.

To determine the regions of the FHV-1 genome which contained the genes for the various in vitro translation products, infected cell RNA was hybridized to filters containing total virion DNA or DNA from recombinant phage clones containing FHV-1 DNA restriction fragments. In vitro translation of the selected RNA revealed that virion DNA selected for a number of translation products which, with the exception of a 130 kd protein, were also present in the translation products of non-selected infected cell RNA (Figure 2). Most of the translation products that were selected for by virion DNA were also identified in the translation products selected for by the individual cloned restriction fragments. A filter control containing only bacteriophage vector DNA (EMBL-3) selected for products which were 92, 64, and 44 kd in size. These background contaminants can be seen in each lane of the gel shown in Figure 2.

The locations of the cloned fragments on the FHV-1 genome and the approximate molecular weights of the translation products that were

Figure 2. In vitro translation products selected for by FHV-1 Sal I fragments. Infected cell RNA which was selected for by filters containing cloned FHV-1 restriction fragments (A-J), FHV-1 virion DNA (V), or EMBL-3 DNA (fc) was translated in vitro and analyzed by SDS-PAGE. Sizes (in kilodaltons) of the translation products are indicated at right. Lanes A-J are labeled according to the Sal I fragment used in the hybrid selection. The clones containing Sal I G and E also contain Sal I N.

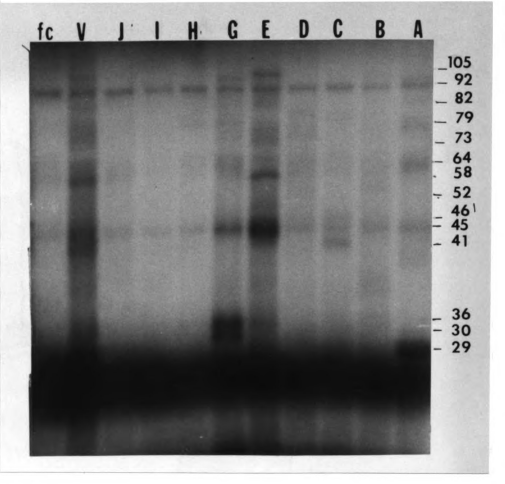

Figure 2

selected for are summarized in Figure 3. Immunoprecipitation experiments revealed that all of the translation products identified in this summary reacted with the goat anti-FHV-1 serum (not shown). It can been seen in this figure that FHV-1 late genes map throughout the genome but are mostly found in the unique long region. The clone containing Sal I E and N, which has been previously designated EM 5 (17), selected for six translation products. Because the molecular weights of some of these proteins corresponded to the sizes of the putative 74, 58, and 45 kd glycoprotein precursors identified above, and because several of these translation products were synthesized in large amounts, this clone was selected for further study.

Figure 4 shows a map of EM 5 that was generated by restriction digest and Southern blotting analysis. Bam HI/ Sal I digestion of DNA from this clone produced three restriction fragments designated E1 to E3. E1 and E2 are unimolar fragments of 4.5 and 2.1 kb in size, respectively. E3 contains three closely migrating bands 1.8 to 1.7 kb in size. E1, E2 and E3 were purified from an agarose gel and were 1) nick-translated and used as hybridization probes for Northern blot studies, and 2) bound to nitrocellulose for hybrid-selection studies.

Figure 5 shows the results of Northern blot analysis of FHV-1 late RNA using the Bam HI/Sal I subfragments of clone EM 5. Three major RNA species with sizes of 2.9, 1.5 and 1.0 kb were detected by total clone DNA. E1 showed strong hybridization to the 2.9 kb RNA and weak hybridization to a series of smaller, minor RNA species. E2 hybridized intensely to all three major RNA species while E3 showed strong hybridization to only the two smaller RNAs. The location of these RNA's on the EM 5 map are shown in Figure 4. These results suggested that these RNA

Figure 3. Map locations of FHV-1 late gene products. Figure shows location of FHV-1 Sal I restriction fragments on the genome and indicates the sizes (in kilodaltons) of the *in vitro* translation products selected for by each fragment. Sal I fragments L, K, M, and F have not been cloned and were not included in this analysis.

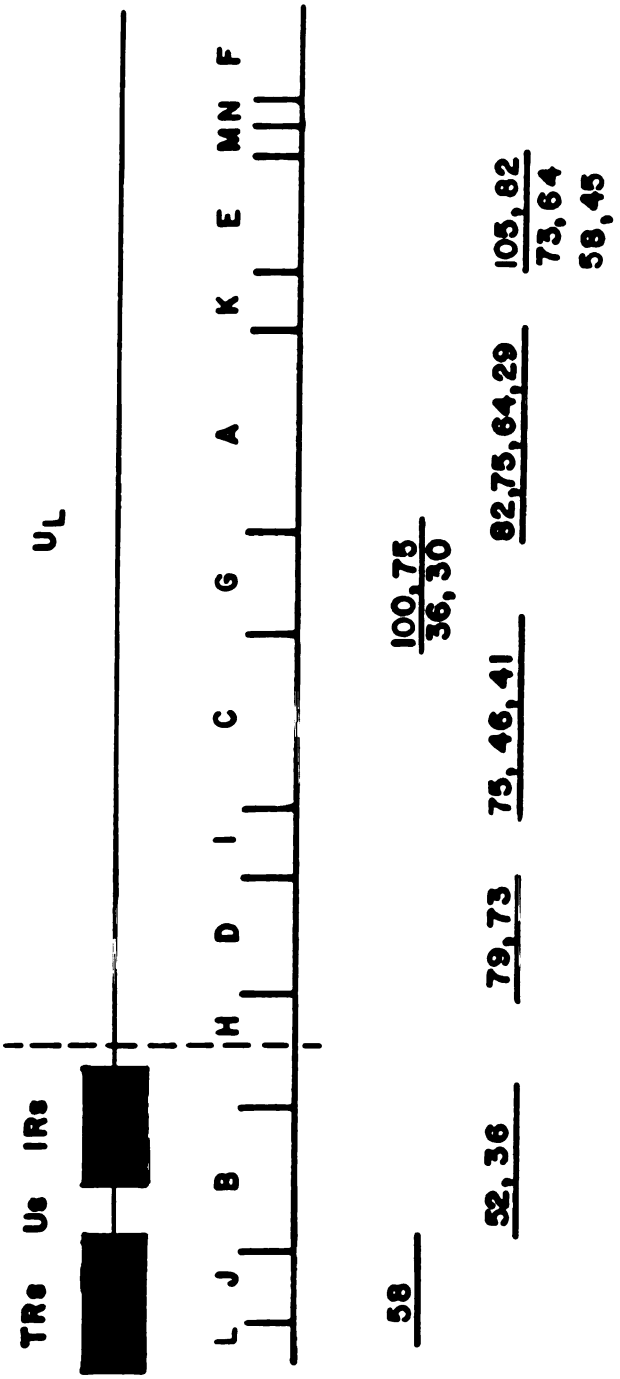
TRs
Us
IRs
L
J
B
H
D
I
C
G
A
K
E
MN
F
UL
58
52,36
79,73
75,46,41
100,75
36,30
82,75,64,29
105,82
73,64
58,45
10 kb

Figure 3

molecules may be transcribed from overlapping regions of the genome or contain common sequences.

Figure 6 shows SDS-PAGE analysis of the translation products selected for by the subfragments of clone EM 5. In this experiment, only four translation products were selected for by total clone DNA with sizes of 105, 73, 58, and 45 kd. It is possible that the 82 and 64 kd translation products identified in the previous hybrid selection experiment were either premature termination or breakdown products. Both E1 and E2 selected for the 105 and 73 kd peptides and E3 selected for the 58 and 45 kd peptides. It was unclear from these results whether the 58 and 45 kd translation products were being selected for by the E1 or E2 fragments since background bands are also visible in this region of the gel. The Northern blot analysis suggested that E2 should be able to select for the two smaller RNA species which presumably code for the 58 and 45 kd proteins. Given this assumption, there is good correlation between the size of the major RNA's and proteins which map to the individual Bam HI/Sal I subfragments (Figure 4).

RNA selected by clone EM 5 was also translated in the presence of dog pancreas microsomes (Figure 6). The migration of all of the translation products appeared to be altered by the addition of microsomes. Two bands were visible in the microsome-treated reactions that were slightly larger than the 45 and 58 kd bands found in the untreated reactions. These could represent partially glycosylated molecules and their sizes are approaching those of the 68 and 60 kd glycoproteins found on FHV-1 virions. These results confirmed the initial observation that the 45 kd translation product is a substrate for cotranslational modification and indicated that the 58 kd translation product is also processed. Though the 73 and 105 kd

Figure 4. Analysis of EM-5. Figure shows locations of Sal I/Bam HI subfragments E1 to E3. The line under each subfragment indicates sizes (in kilodaltons) of in vitro translation products selected for by each subfragment (above of line) and sizes (in kilobases) of late RNA species which hybridized to each subfragment (below line).

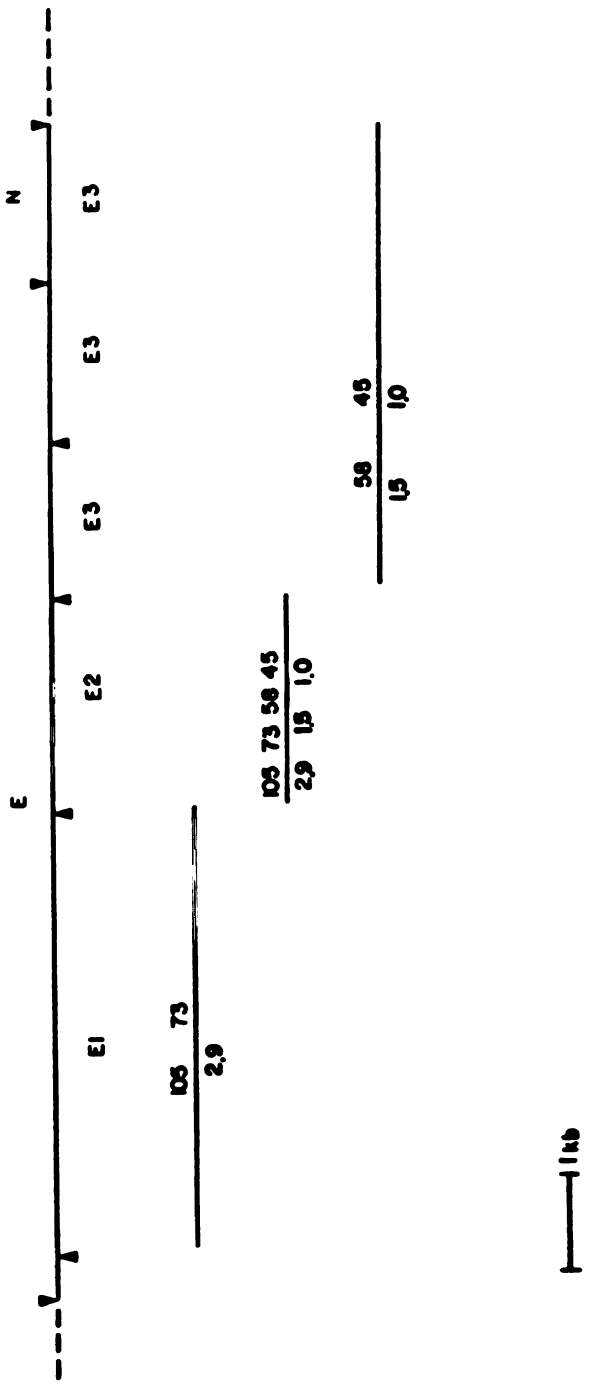

Figure 4

Figure 5. Northern blot analysis of FHV-1 late RNA. FHV-1 late RNA was subject to electrophoresis through formaldehyde-agarose gels and hybridized to nick-translated total EM 5 DNA (E) or EM-5 subfragments E1 to E3 (lanes 1-3). Sizes of the RNA species (in kb) are indicated at left.

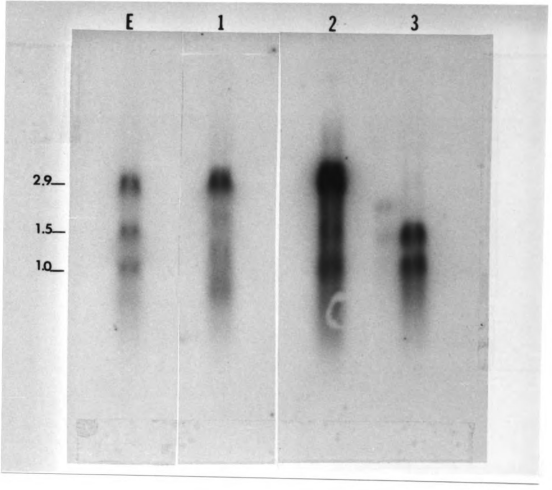

Figure 5

Figure 6. In vitro translation products selected for by subfragments of EM-5. DNA from EM-5 (E), from gel-purified Bam HI/Sal I subfragments E1-E3 (1-3), or from bacteriophage vector (EMBL-3) (fc) was denatured, bound to nitrocellulose and used to select infected cell RNA which was translated in the presence (E+) or absence (E-, 1-3) of dog pancreas microsomes. Translation products were analyzed by SDS-PAGE. Sizes (in kd) of the FHV-1 specific translation products are indicated.

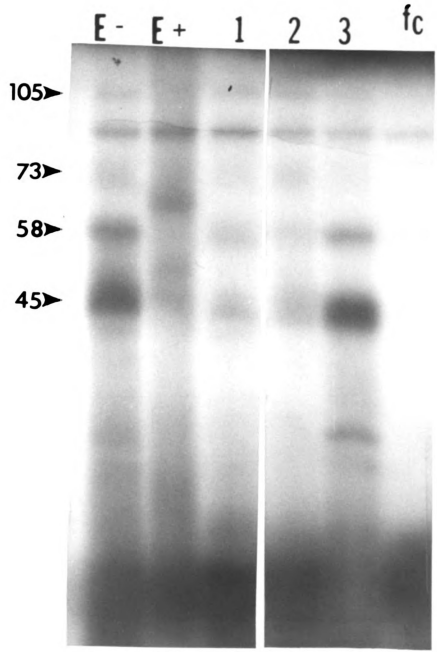

Figure 6

translation products were also altered by the addition of microsomes, no higher molecular weight forms of these molecules were visible on the gel. A higher molecular weight form of the 73 kd protein which may correspond to the 74 kd protein immunoprecipitated from deglycosylated virions could have been obscured by the 92 kd background band.

These results indicate that two and possibly three FHV-1 glycoprotein genes are clustered in a small region of the genome which is located near the end of the Ul region, and these proteins may be coded for by overlapping transcripts.

Discussion

This report decribes the initial attempts to map FHV-1 late gene products on the viral genome. In vitro translation of infected cell RNA produced a number of translation products which corresponded in size to proteins found on FHV-1 virions. Most of these translation products reacted with a goat anti-FHV-1 serum that was used in the initial characterization of the FHV-1 antigens (10). The migration of two of the translation products was clearly altered by the addition of dog pancreas microsome extracts to the translation reaction. These altered translation products comigrated with bands produced by the enzymatic deglycosylation of FHV-1 virions.

The purpose of deglycosylating virions was to attempt to determine the sizes of the glycoprotein precursors to provide a reference point for the analysis of the translation products. Without monoclonal or monospecific antibody against the individual glycoproteins it is difficult to study the events involved in the maturation and processing of these molecules. It is assumed that the 58 and 45 kd precursors are processed into the 68 and 60 kd glycoproteins found on FHV-1 virions. Deglycosylation of the 105 kd glycoprotein resulted in two different precursors depending on the enzyme used. Endo F treatment removes only N-linked carbohyrates while the mixed glycosidase degrades a variety of oligosaccharide side chains. Therefore, the 82 kd precursor observed in the Endo F-treated virions could be a partially glycosylated form which is further degraded to a 74 kd molecule by the mixed glycosidase. The 74 kd precursor corresponds with an abundant in vitro translation product that is altered by the addition of microsomes to the translation reaction. Of

course, it is possible that there may be more than one 105 kd glycoprotein and more than one precursor. In fact, other researchers (1) using gradient SDS-PAGE analysis have identified three glycoprotein bands with approximate sizes of 120-112 kd on FHV-1 virions. It is not clear if these three bands are glycosylation intermediates of the same glycoprotein or represent more than one glycoprotein.

Using recombinant clones to hybrid select RNA it was possible to determine the map locations of 21 of the in vitro translation products. FHV-1 late genes were found to map throughout the genome with the majority of them concentrated in the unique long region. Few late genes mapped in the S region. It is interesting that several neighboring fragments selected for proteins of the same approximate molecular weight suggesting that the genes which code for these proteins are contained on both fragments. This analysis is incomplete since several fragments of the genome were not represented in the clone collection. The results are sufficiently complete to show that the distribution of FHV-1 late genes is similar to the late gene arrangement of other herpesviruses (4,19).

An abundant protein of 130 kd was present in the in vitro translation products of infected cell RNA and comigrated with an immunoprecipitatble non-glycosylated protein on FHV-1 virions. This protein may be the major viral capsid protein because of its size, immunogenicity, relative abundance, and because it is not present in detergent extracts of FHV-1 virions (Rota and Maes, unpublished observations). Unfortunately, neither total virion DNA nor DNA from any of the cloned fragments was able to select for this protein under the conditions used in this study. Is is possible that RNA coding for larger proteins is less resistent to the manipulation required for hybrid selection experiments than RNA which

codes for smaller proteins. Other researchers have been able to hybrid select RNAs which code for large molecular weight proteins using conditions that were similar to those used in this study. However, in these studies the amount of high molecular weight translation products was reduced compared to the smaller translation products (4).

A region of the genome containing Sal I fragment E hybridized to three late RNA species which coded for four polypeptides. Further studies will be needed to determine to what extent these transcripts may overlap or whether they contain common sequences. Clusters of late genes which produce several overlapping transcripts have been identified on the genome of HSV (19).

Two of the polypeptides selected for by the Sal I E region of the FHV-1 genome were cotranslationally processed to higher molecular weight forms by the microsome extracts. Complete glycosylation usually does not occur in vitro (12) so it would be difficult, without specific antibody, to determine if these higher molecular weight forms of the translation products correspond to any of the glycoproteins found on intact virions. Rea et al (15) have used a similar approach to identify a restriction fragment of pseudorabies virus which selected for a translation product that was modified by the addition of microsomes. This protein reacted with monoclonal antibody directed against the viral glycoprotein that is found in the medium of infected cells.

The migration of the other two peptides selected for by Sal I E was altered by the microsomes. Since no higher molecular weight forms were apparent, the possibility that the translation of these proteins was inhibited by the microsomes cannot be ruled out. Therefore, while there is good evidence that the 58 and 45 kd translation products are glycoprotein

precursors, the identity of the 73 kd translation product is unclear. These initial studies indicate that further analysis of this region of the genome are justified.

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Chapter 5

Biochemical and Antigenic Characterization of Feline Herpesvirus-1-Like Isolates from Dogs.

Paul A. Rota and Roger K. Maes

Summary

The DNA and polypeptide patterns of feline herpesvirus-1 (FHV-1), a virus usually associated with feline respiratory infections, were compared to those of five herpesvirus isolates from dogs. These canine isolates had been shown to be antigenically similar to FHV-1 by cross neutralization tests. DNA from FHV-1 (C-27 strain) and each canine isolate was digested with either Bam HI, Eco R1 or Sal I and analyzed on .8% agarose gels. The restriction digest patterns of the canine isolates were nearly identical to C-27 for all three restriction enzymes. Interestingly, all of the canine isolates showed a small extension of the largest Bam HI fragment (14.5 kb) that was not present in the C-27 strain. Bam HI digested FHV-1 DNA from clinical cases in cats had digest patterns that were very similar to the canine isolates and also showed an extension of the 14.5 kb fragment. Southern blotting experiments revealed that DNA from the canine isolates has extensive homology to C-27 DNA. SDS-PAGE analysis of radiolabeled polypeptides from C-27 and the canine isolates showed identical virion-associated polypeptide profiles. In addition, a goat anti-FHV-1 antiserum precipitated three glycoprotein antigens from the canine isolates with migration patterns that were identical to the three major antigenic glycoproteins found on C-27. Hind III and Eco R1 digestion patterns of canine herpesvirus DNA showed no similarity to C-27 DNA. In addition, canine herpesvirus DNA had no homology to C-27 DNA under the stringent conditions used.

Introduction

Feline herpesvirus-1 (FHV-1) is a major upper respiratory tract pathogen in cats. The virus can also cause more severe generalized disease in kittens, abortions in pregnant cats as well as latent infections (17). The virus has been classified as a member of the alphaherpesvirinae and has been found to have a limited in vivo and in vitro host range infecting only Felidae or cells of feline origin (18).

Herpesviruses which were shown to be antigenically similar to feline herpesvirus-1 (FHV-1) have recently been isolated from dogs. The primary clinical sign in each of the cases examined was diarrhea. The age, vaccination history and antibody status of the dogs at the time of virus isolation were not consistent from case to case. Virus isolates from these dogs grew only in Crandell-Rees feline kidney (CRFK) cells and not in monkey kidney (Vero) cells or Madin Darby canine kidney (MDCK) cells. The isolates were chloroform sensitive, showed basophilic intranuclear inclusion bodies and were neutralized by antiserum to FHV-1 but not by antiserum to canine herpesvirus (CHV) (4). It was concluded that these infections were caused by a virus that was similar to FHV-1.

Thus far, CHV and pseudorabies virus have been the only recognized herpesvirus infections in dogs (1, 5, 9, 16), but they have not been associated with enteric infections in dogs over two weeks old. Also, previous studies have indicated that FHV-1 was incapable of infecting dogs (7). Current research in our laboratory has focused on the physical and antigenic characterization of FHV-1. The report on the canine isolates was therefore very interesting to us. We obtained five canine isolates in

order to do a more detailed biochemical and antigenic comparision of these canine herpesvirus isolates to the C-27 prototype strain of FHV-1.

We report here that the five canine isolates have genomes which show restriction enzyme digestion patterns that are nearly identical to those of C-27 and to those of FHV-1 isolates of feline origin. Also, these canine isolates appear to contain all of the major antigens and virion-associated polypeptides of C-27 including the three major glycoproteins.

Methods and Materials

Virus and Cells. Five previously described (4) canine FHV-1-like isolates and five FHV-1 strains of feline origin, isolated at the Washington Animal Disease Laboratory, Pullman WA, were used in this study. The C-27 prototype strain of FHV-1 was obtained from the American Type Culture Collection, Rockville, MD (#VR 636). The viruses were propagated in Crandell-Rees Feline Kidney (CRFK) cells which were maintained in Eagle's Minimum Essential Medium (EMEM) (K.C. Biologics, Lenexa, KS.) with 10% fetal bovine serum (Hyclone defined, Sterile Systems, Logan, UT). Canine herpes virus was obtained from the A.T.C.C. (#VR-552) and grown in Madin Darby canine kidney (MDCK) cells using the same medium.

Plaque Assay Procedure. Confluent monolayers of CRFK cells growing in tissue culture plates (60 mm, Corning) were infected with serial dilutions of the virus sample in EMEM. After a one hour adsorbtion period, the inoculation medium was removed and EMEM containing 1% agarose was layered over the cells. Plaques were visualized 48 hours later by staining the viable cells with 2% neutral red in Hank's Balanced Salt Solution. The plaques were picked with a sterile Pasteur pipette, diluted into EMEM, and allowed to incubate at 4°C. for 4 hours before being used to infect CRFK cells.

Isolation of Viral DNA. Confluent monolayers of CRFK cells were infected with virus at an multiplicity of infection of .01. When the CPE was advanced, the infected cells were shaken from the culture vessel surface and pelleted by low speed centrifugation (500 rpm, 10 min). The washed

cells were resuspended in 10 ml of TE (10mM Tris-HCl pH 7.5, 1 mM EDTA) and left on ice for 15 minutes. NP-40 (Sigma, St. Louis, MO.) was added to a final concentration of .5% and the cells were incubated for another 15 minutes on ice. Cell nuclei were pelleted by centrifugation at 2,000 x g for 10 minutes and the supernatant containing the viral nucleocapsids was adjusted to 1% SDS and 25mM EDTA. Pre-digested (80°C., 15 min.) pronase was added to a final concentration of 1 mg/ml and the supernatant was incubated overnight at 37°C. Saturated sodium iodide (1.5 volumes) containing 1 ug/ml ethidium bromide was added to the supernatant. The mixture was centrifuged for 48 hours at 44,000 rpm at 20°C. in an SW 50.1 Rotor (Beckman Instruments, Palo Alto, CA.). The viral DNA bands were visualized by u.v. illumination and harvested by side-puncturing the tube with a 20 ga. needle. The DNA-containing samples were extracted three times with isoamyl alcohol (5 volumes) before being dialyzed extensively against TE.

Restriction Enzyme Analysis. One microgram amounts of viral DNA were incubated with 10 units of either Bam HI, Eco R1, Sal I or Hind III (Bethesda Research Laboratories, Gaithersburg, MD.) using the reaction conditions recommended by the manufacturer. When the digestion was complete, 1 uCi of the appropriate α^{32}P-labeled trinucleotide (Bam HI: dGTP, Eco R1 and Hind III: dATP, Sal I: dTTP, Amersham, Arlington Heights, Ill., sp. act. 800 Ci/mmole) was added to the reaction along with 1 unit of the Klenow fragment of DNA polymerase (BRL). The samples were incubated at room temperature for 45 minutes, precipitated twice with ethanol and loaded onto .8% agarose gels and electrophoresed (20 V, 20

hrs). When electrophoresis was complete, the gel was dried on a gel dryer without heat and exposed to Kodak X-AR film.

Southern Blotting. After electrophoresis, restriction fragments were transferred to nitrocellulose filters by the method of Southern (21). ^{32}P-labeled DNA probes were prepared by nick translation using standard methods (13). Filters were prehybridized for 4 hrs at 42°C. in 20% formamide, .6M NaCl, .06M sodium citrate, .01M EDTA, .1% SDS, 5X Denhardt's solution (.1% ficoll, .1% polyvinylpryollidone, .1% bovine serum albumin), and 50 ug/ml denatured salmon sperm DNA. Hybridizations were performed at 42°C. in 50% formamide, 1X Denhardt's, 4X SSC (1X SSC: .15M NaCl. .015M sodium citrate, pH 7.0), .1M EDTA, .1% SDS, 50 ug/ml denatured salmon sperm DNA. After Hybridization, the filters were washed twice for 15 min with 2X SSC at room temperature followed by two 1 hr washes with .1X SSC at 68°C. The filters were allowed to dry and exposed to X-ray film.

Immunoprecipitation Analysis. ^{14}C-glucosamine or ^{35}S-methionine labeled virions were prepared as previuosly described (14) The virions were partially purified from the supernatant medium by centrifugation through cushions of 25% potassium tartarate in TE at 100,000 x g for 1 hour at 4°C. in a Beckman SW-27 rotor.

Following preincubation with normal goat serum, the samples were incubated overnight with goat anti-FHV-1 serum. The complexes were pelleted by addition of 10 volumes of a protein-A producing strain of Staphylococcus aureus prepared by the method of Kessler (10). The

immunoprecipates were then analyzed by SDS-PAGE (11). The gels were fixed and dried (12) and exposed to X-ray film.

Results

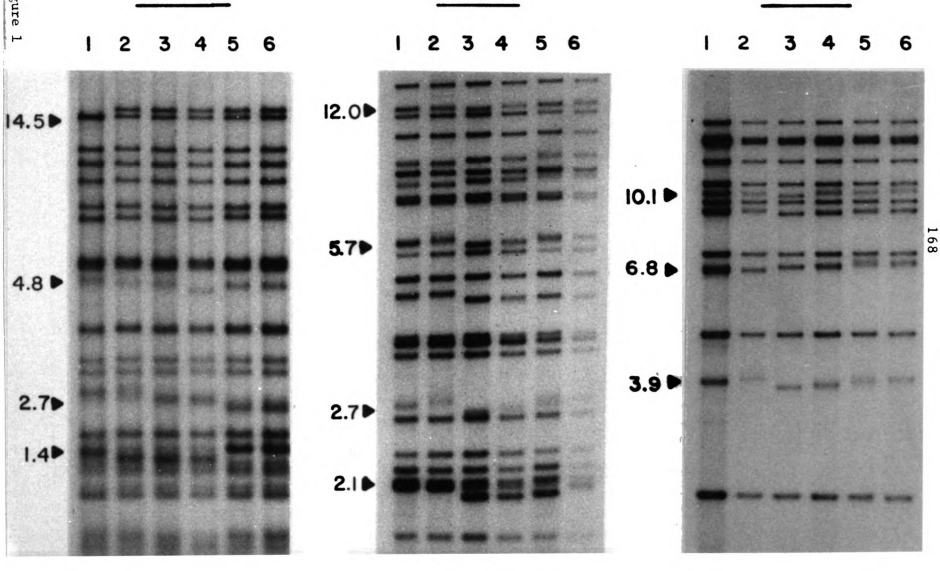
Figure 1 shows a comparison of the Bam HI, Eco R1 and Sal I digestion patterns of DNA from the C-27 strain of FHV-1 and DNA from five plaque-purified canine isolates. The sizes of the Bam HI, Eco R1 and Sal I restriction fragments of C-27 DNA and the locations of some of these fragments on the viral genome have been determined in a concurrent study (19). In the Bam HI digest of C-27 DNA, the largest two fragments migrate as a single two molar band of 14.5 kb. In all of the canine isolates, one of these fragments is slightly larger and two one molar bands are observed. The canine isolates also show some variation in the Bam HI 4.8, 2.7 and 1.4 kb fragments. However, these variations are not consistent among the individual isolates. The 4.8 and 1.4 kb Bam HI fragments have been identified as terminal fragments (19) and the size differences may be due to variations in the size of the terminal repeat DNA.

Consistent differences were not observed between the canine isolates and C-27 in both the Eco R1 and Sal I digests (Fig.1). The Eco R1 12.0, 5.7, 2.7 and 2.1 kb fragments and Sal I 10.1, 6.8 and 3.9 kb fragments show size variation from isolate to isolate. The Eco R1 12.0 and 2.7 kb and Sal I 10.1 and 3.9 kb fragments are located at the viral termini. The Eco R1 5.7 and 2.1 kb and Sal I 6.8 kb fragments are located within the internal inverted repeat regions of the viral genome (19). Size variation was apparent in other fragments whose locations have not yet been determined.

For each canine isolate, DNA was purified from a number of individual plaque isolates and compared by restriction endonuclease digestion (data not shown). The restriction digest patterns were consistent for the

Figure 1. Restriction enzyme digest patterns of C-27 DNA (lane 1) and DNA from five canine isolates (lanes 2-6). Restriction fragments were end-labeled with ^{32}P and separated on .8% agarose gels. Figure shows autoradiogram of dried gels. The molecular weights (kb) of certain bands are shown on the left.

Figure 1


Bam HI
1 2 3 4 5 6
14.5
4.8
2.7
1.4
Eco R1
1 2 3 4 5 6
12.0
5.7
2.7
2.1
Sal I
1 2 3 4 5 6
10.1
6.8
3.9

individual isolates suggesting that the variations observed in Figure 1 are the result of interstrain and not intrastrain variation.

To determine if there were any consistent differences between the canine isolates and field strains of FHV-1, DNA was also purified from five plaque-purified FHV-1 clinical isolates of feline origin. Figure 2 shows a comparison of the Bam HI digestion patterns of these isolates to C-27. All of the feline isolates showed the same extension of one of the largest Bam HI fragments that was observed in the digests of DNA from the canine isolates. The feline isolates also showed the same general variation in the size of Bam HI terminal fragments.

Southern blotting experiments were done to examine the extent of similarity between the genomes of the canine isolates and C-27. DNA from the canine isolates was digested with Bam HI, electrophoresed, transferred to nitrocellulose, and probed with nick-translated C-27 DNA (Figure 3). All of the Bam HI fragments of the canine isolates were detected by the C-27 probe indicating that the canine isolates share extensive sequence homology to C-27 throughout their entire genomes.

Purified virus stocks of the C-27 strain and the canine isolates, labeled with ^{14}C-glucosamine or ^{35}S-methionine, were used to compare the patterns of the virion-associated and major antigenic structural polypeptides. Figure 4A shows a direct SDS-PAGE comparison of one of the canine isolates with C-27. The polypeptide patterns based upon one dimensional SDS-PAGE appear to be identical suggesting that the canine isolates do not differ significantly from C-27 with respect to the structural components of the virion.

Figure 4 also shows a comparison of the immunoprecipitation analysis of a canine isolate and C-27 obtained with a goat anti-FHV-1 antiserum

Figure 2. Bam HI digestion patterns of C-27 DNA (lane 1) and five isolates of FHV-1 of feline origin (lanes 2-6). Restriction fragments were end-labeled with ^{32}P and separated on .8% agarose gels. Figure shows autoradiogram of a dried gel.

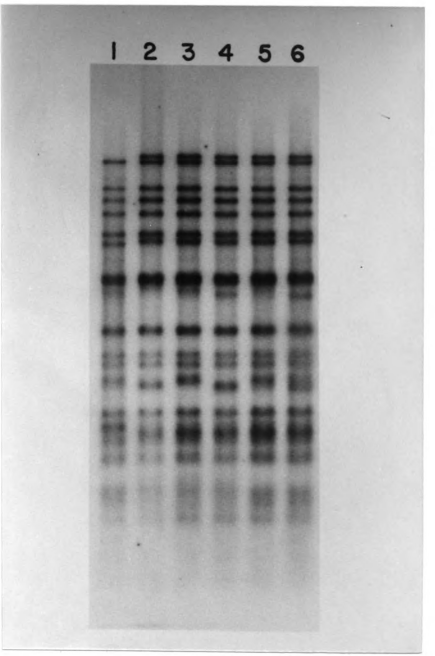


Figure 2

Figure 3. Southern blot analysis of C-27 DNA (lane 1) and DNA from five canine isolates (lanes 2-6). DNA samples were digested with Bam HI, separated on .8% agarose gels and transferred to a nitrocellulose filter. The filter was probed with nick-translated C-27 DNA and washed as described in Methods and Materials.

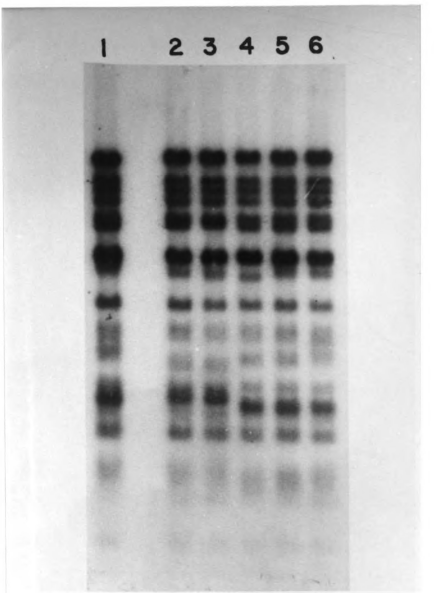


Figure 3

Figure 4. SDS-PAGE and immunoprecipitation analysis of C-27 and canine isolate purified virions. Panel A shows direct SDS-PAGE analysis of ^{35}S-methionine-labeled C-27 virions (lane 1) and canine isolate virions (lane 2). ^{14}C-glucosamine-labeled C-27 virions are shown (lane 3) as size markers. Panels B and C show SDS-PAGE analysis of immunoprecipitates formed after the incubation of radiolabeled C-27 virions (lanes B2, C1) and canine isolate virions (lanes B3, C2) with goat anti-FHV-1 antiserum. Panel B shows ^{35}S-methionine labeled virions, panel C shows ^{14}C-glucosamine labeled virions. ^{14}C-glucosamine labeled virions are shown in panel B (lane 1) as size markers.

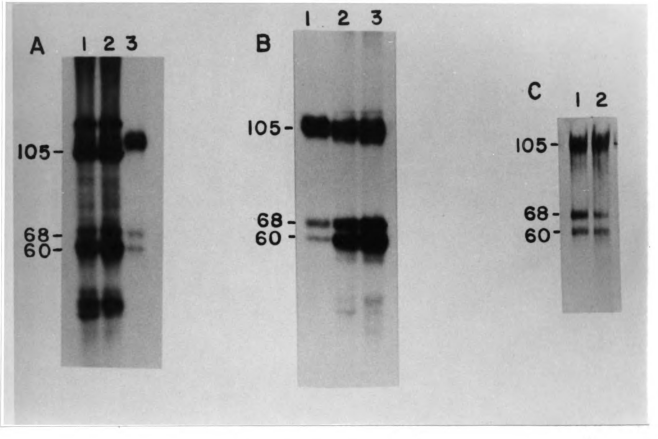


Figure 4

(14). SDS-PAGE analysis of the immunoprecipitates reveals that both viruses appear to be antigenically equivalent (Fig. 4B). Three of the common antigens also labeled with ^{14}C-glucosamine indicating that both viruses contain the same major antigenic glycoproteins (Fig. 4C). Radiolabeled virion preparations from the other four canine isolates reacted with the goat serum in the same manner as the isolate shown in Figure 4 (data not shown).

Figure 5 shows a comparison of the Hind III and Eco R1 digestion patterns of CHV and FHV-1 DNA. It is obvious from the data that the digestion patterns are completely different . Also, in Southern blotting experiments C-27 DNA did not hybridize to CHV DNA under the conditions used in Figure 3 (data not shown). There appears to be no homology between the genomes of feline and canine herpesviruses using the hybridization and wash conditions described above.

Figure 5. Restriction enzyme digest patterns of C-27 DNA (lanes 1,3) and canine herpesvirus DNA (lanes 2,4). Lanes 1 and 2 show an Eco R1 digest, lanes 3 and 4 a Hind III digest. DNA fragments were end-labeled with ^{32}P and separated on .8% agarose gels. Figure shows an autoradiogram of a dried gel.

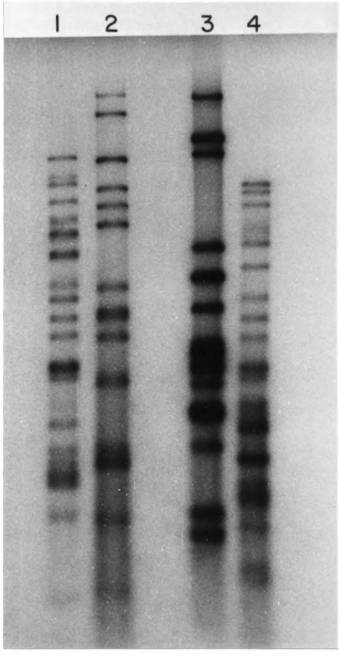


Figure 5

Discussion

Five herpesvirus isolates from dogs were compared to the C-27 prototype strain of feline herpesvirus-1 by restriction endonuclease digestion, Southern blotting, SDS-PAGE and immunoprecipitation. DNA from the five canine isolates showed Bam HI, Eco R1 and Sal I digestion patterns that were nearly identical to those of the prototype strain of FHV-1 and to DNA purified from clinical isolates of FHV-1. The majority of the variation observed in the digest patterns between individual virus isolates occurred in fragments located at the viral termini or within the inverted repeat regions of the viral genome. Therefore, some of the heterogeneity presumably is due to variations in the DNA of terminal or internal repeat sequences. The feline and canine case isolates all had an extension of one of the two 14.5 kb Bam HI fragments observed in C-27 DNA. It is of molecular epidemiological importance that the restriction patterns generated by a number of clinical isolates from both dogs and cats were very similar. Generally, only minor variations are observable in restriction digestion patterns of a number of case isolates. This stability of the genome of FHV-1 has been noted in a previous study which examined the restriction endonuclease digestion patterns of DNA from a number of FHV-1 isolates from Europe, the United Kingdom and the United States (6). In this study, some variation was found in the sizes of terminal restriction fragments. Another interesting aspect is the extension of the 14.5 kb Bam HI fragment in all of the clinical isolates but not in the C-27 strain. The role of this extension is unclear and the location of this fragment on the viral genome has not yet been determined. It may code for viral functions that are lost on repeated cell culture.

Both the canine isolates and the clinical isolates of FHV-1 were studied after a low number of passages in cell culture, while the C-27 strain had been passed repeatedly in culture in our lab before this study. We did not notice any difference in plaque morphology between C-27 and the canine isolates, however.

Southern blotting experiments confirmed and extended the restriction analysis data in the sense that a C-27 DNA probe was able to detect all of the Bam HI fragments of the canine isolates.

The canine isolates and the C-27 strain of FHV-1 showed identical virion-associated polypeptide patterns by SDS-PAGE analysis. Also, a goat anti-FHV-1 antiserum recognized the same major virion-associated antigens from purified virions of both the canine isolates and the C-27 strain. In addition, both the canine isolates and the C-27 strain appear to share at least three glycoprotein antigens. These three glycoproteins have been previously identified as the major glycoprotein antigens of FHV-1 (14). It is theorectically possible that the canine isolates contained unique antigens that were not recognized by the goat antiserum. These antigens would most likely be minor since they could not be visualized in the direct SDS-PAGE analysis of the viral structural proteins. It would be necessary to use more rigorous methods of analysis to determine if such minor differences exist between the protein antigens of these virus isolates. It will be especially important to further characterize the common glycoproteins because herpesvirus glycoproteins are extremely important in viral infectivity and in host range specificity (2,15). These molecules are also important targets of the host's immune response (20). We conclude from the results of the restriction digestion, Southern

blotting, SDS-PAGE and immunoprecipitation that the canine isolates we examined were very similar to the C-27 strain of FHV-1.

The isolation of feline-related viruses from dogs is not without precedent. Previous studies have reported the isolation of feline calicivirus (3) and a parvovirus related to feline panleucpaenia virus (8) from dogs.

The disease potential of the canine FHV-1-like isolates remains unclear. The initial isolations were made from dogs showing readily observable clinical signs. Since these isolates had no HA activity and grew only in CRFK cells, is seems unlikely that the reported clinical signs were due to simultaneous infection with other viruses, especially canine parvovirus. In a small scale experiment in our laboratory, dogs orally and intranasally exposed to the canine isolates developed mild clinical signs. These included a rise in temperature, mild leucopenia and in some dogs, soft stools. Postinfection, but not preinfection, sera from these dogs were able to immunoprecipitate several antigens from FHV-1 virions (Maes and Rota, unpublished observations).

Data from this study show that the canine isolates are clearly different from CHV, a recognized herpesvirus pathogen of dogs. CHV DNA gave restriction digest patterns that were markedly different from FHV-1 and did not hybridize to a FHV-1 probe under the stringent hybridization and wash conditions used. Southern blotting studies using varying stringency conditions are currently underway. This will allow us to obtain a more precise definition of the extent of homology between the DNA's of CHV and FHV-1. Furthermore, antiserum against both CHV and FHV-1 is being used in immunoprecipitation studies to determine if there are common antigens shared by these two viruses.

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SUMMARY

While the description of the FHV-1 genome provided by this report is far from complete, it is sufficient to indicate that the structure and general organization of the FHV-1 genome is similar to the structure and organization of other alpha herpesviruses. These experiments provide the first step toward understanding the molecular biology of FHV-1 and should provide the basis for future studies in several areas.

The cloned FHV-1 DNA fragments should be useful in the study of FHV-1 latency. These clones could initially be used to construct hybridization probes of high specific activity for use in Southern blotting and in situ hybridization studies. Once the tissue or tissues of latency have been identified, a rapid and sensitive method could be developed to determine if cats are FHV-1 carriers. This assay would be needed to fully evaluate the efficacy of current and proposed FHV-1 vaccines. Also, these clones could be used as probes in Northern blotting experiments to measure gene expression from various regions of the FHV-1 genome during latency in the natural host.

When monoclonal and monospecific antibodies to FHV-1 antigens become available, the gene mapping studies described in Chapter 4 could be continued to isolate genes for individual FHV-1 antigens. These antigens could be expressed via recombinant DNA techniques and assessed for their ability to prevent clinical disease and the establishment of latent infections in the natural host.

Finally as more information on the biochemical structure of FHV-1 is obtained, a more complete comparison could be made between FHV-1 and other herpesviruses. It will be interesting to compare the genome arrangements of different herpesviruses with similar biological properties.