INVESTIGATIONS INTO THE BIOLOGY AND MOLECULAR BIOLOGY OF ALPHAHERPESVIRUS SAIMIRI

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Investigations into the Biology and Molecular Biology of Alphaherpesvirus Saimiri. By Sally Patricia Mossman.

Abstract

Previous Studies:
Two isolates of alphaherpesvirus saimiri (αHVS) have been shown to exhibit significant differences in neurovirulence for rabbits. One strain, KM322, produces a stable latent infection in sensory ganglia while the second, KM91, causes an acute encephalitis which is invariably fatal. Inoculation of rabbits with KM322 is able to protect them from a simultaneous challenge with KM91. Analysis of several *in vitro* characteristics of these two strains has revealed only the larger plaque size of KM91 as a possible neurovirulence marker.

The Present Study:
KM322 was found to be replication-deficient relative to KM91 in both Vero and Neuro 2a cell lines. Variable plaque size in the two strains was shown by plaque-purification not to be due to mixed populations.

KM322 reactivated from latently infected explanted rabbit dorsal root ganglion fragments at an increased rate in the presence of a DNA hypomethylating agent, 5-azacytidine. This suggests methylation of viral and/or host DNA may participate in the control of αHVS latency. This agent significantly improved the detection of latent virus in explant cultures. A variety of other agents tested did not affect KM322 reactivation rates.

Twenty six virion-specific polypeptides were detected from SDS PAGE analysis of KM322 and KM91, these ranged from 14K to 155K in molecular weight. Highly immunogenic polypeptides, within the range 37K to 47K, were found to vary between KM91 and KM322. A high degree of similarity between αHVS and HSV-1 was detected from polypeptide profiles and antigenic cross-reactivity. Three αHVS-specific monoclonal antibodies were identified, two of which bind to post-translationally modified proteins with electrophoretic mobilities of major glycoproteins.

Restrictions endonuclease analysis of αHVS DNA with single and paired enzymes suggest the genome has two isomeric forms due to inversion of the short region. The genome was found to be 155kb in length with a 34kb short region and a 121kb long region. Three restriction enzymes were shown to identify stable genetic differences between KM91 and KM322. These were HpaI, SalI and BglII.
The αHVS genome did not hybridise to HSV-1 DNA fragments at high stringencies suggesting a lack of significant nucleotide sequence homology between the two. At low stringencies hybridisation was found between αHVS long segment sequences and HSV-1 fragments encoding a number of genes known to be conserved amongst herpesviruses.

CONTENTS

CHAPTER 1: INTRODUCTION

1.1 THE HERPESVIRIDAE

The Herpesviridae exhibit an extensive degree of diversity in their biological properties and genome structure. Members of the family have been identified which infect hosts as evolutionarily diverse as fungi, oysters, iguanas, marsupials and primates (Roizman, 1982). However, despite this variation two properties are common to all members of the family, the ability to become latent and the gross virion structure.

1.1.1 Virus Structure

Members of the herpesvirus family are so similar in morphology that they cannot be differentiated on this basis. The double stranded DNA genome is wrapped in a toroid around a protein spindle in the capsid core (Furlong et al, 1972). The icosahedral protein capsid is approximately 100nm in diameter and consists of 162 subunits or capsomeres, 150 of which are hexameric and 12 are pentameric. The proteins constituting the capsid appear to be arranged in outer, intermediate and inner structural layers (Schrag et al, 1989; Newcombe and Brown, 1989). Also present within the capsid core are DNA binding proteins.

The capsid is enclosed by a lipid-containing membrane,

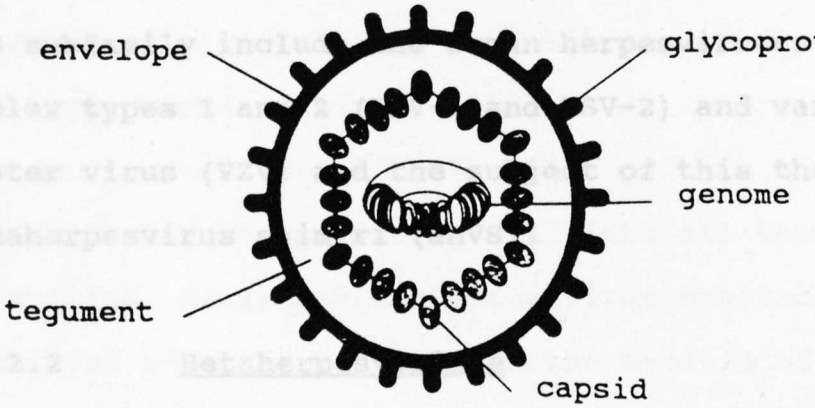
called the envelope, which is derived from the modified inner nuclear membrane of the host cell. Cellular proteins are not detectable within the envelope (Corey and Spear, 1986). Embedded in the bilayer are the virus glycoproteins which mediate attachment to and penetration into the host cell. Between the capsid and the envelope is an ill-defined layer, known as the tegument, which contains a number of proteins of unknown functions. The regulatory protein of HSV, termed the alpha transinducing factor (α - TIF), is also present in the virus tegument. This layer is variable in width, thus intact virions are between 120nm and 300nm in diameter (Corey and Spear, 1986; Roizman and Batterson, 1986). The structure of the herpesvirus virion is illustrated in fig 1.1. The genome structure of herpesviruses is reviewed in the introduction to chapter 6.

1.1.2 Classification

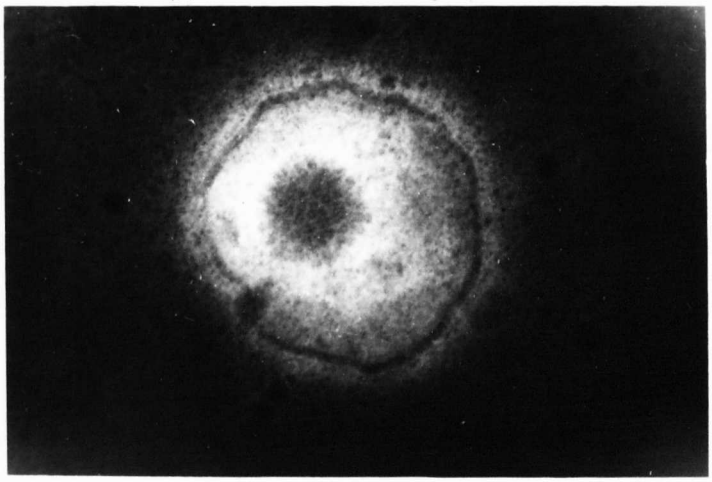
The herpesviruses have been classified into three groups according to biological characteristics; these subfamilies are termed alpha-,beta- and gammaherpesvirinae (Roizman and Batterson, 1986).

Fig 1.1 Schematic diagram of the herpesvirus virion structure (a) (from Corey and Spear, 1986) and an electron micrograph of a virus particle (x 175,000) (b).

a



b



1.1.2.1 Alphaherpesvirinae

Herpesviruses within this class generally exhibit a variable host range, a relatively short reproductive cycle and rapid spread in cell culture with efficient cytolysis of infected cells. Latency within the alphaherpesviruses is for the most part established within ganglia. Examples of this subfamily include the human herpesviruses herpes simplex types 1 and 2 (HSV-1 and HSV-2) and varicella -zoster virus (VZV) and the subject of this thesis, alphaherpesvirus saimiri (αHVS).

1.1.2.2 Betaherpesvirinae

Some members of this group have a restricted host range. Other characteristics include a long reproductive cycle, slow progression of infection in culture and infected cells enlarging to produce cytomegala. Latent virus is harboured in secretory glands, lymphoreticular cells and kidneys among other sites. Examples of this class include the human and murine cytomegaloviruses (HCMV and MCMV)

1.1.2.3 Gammaherpesvirinae

The hosts of these viruses are limited to the family or order of the natural host. Replication *in vitro* occurs in

lymphoblastoid cells and possibly also lytic infection in epithelial and fibroblast cells. Members of this subfamily are specific for either B or T lymphocytes. Within the lymphocyte infection is often arrested at a prelytic or lytic stage without production of progeny virions. Latency frequently occurs in lymphoid tissue. Examples of gammaherpesviruses include the human herpesvirus Epstein-Barr virus and the squirrel monkey virus gammaherpesvirus saimiri.

Thus the human herpesviruses fall into all three subfamilies. A sixth human herpesvirus has recently been identified after isolation from the B-cells of AIDS patients (Salahuddin et al, 1986). Characterisation of this novel herpesvirus has since shown it to possess T-cell as well as B-cell tropism and the virus has been named human herpesvirus-6 (HHV-6) (Lusso et al, 1987; Downing et al, 1987; Tedder et al, 1987). The biological characteristics of this virus suggest classification as a gammaherpesvirus (Lopez et al, 1988). However, analysis of the HHV-6 genome by hybridisation (Efstathiou et al, 1988) and nucleotide sequence comparisons (Lawrence et al, 1990) imply a closer relation to HCMV, a betaherpesvirus. Attempts to identify a disease caused by HHV-6 have yielded only a common but mild disease of infancy, exanthem subitum (Yamanishi et al, 1988). Antibodies to this virus appear

to be widely prevalent throughout the adult population (Tedder et al, 1987).

This thesis is concerned with an alphaherpesvirus, αHVS. Therefore the remainder of this introduction will be concerned only with properties of this subfamily and will refer in particular to the herpes simplex viruses. HSV-1 and HSV-2 represent prototype members of the alphaherpesvirinae and have been studied more extensively than any other member of the family.

1.2 THE ALPHAHERPESVIRUS - HOST INTERACTION

1.2.1 The Replication Cycle

Upon infection of permissive cells alphaherpesviruses undergo a lytic cycle which produces as many as 50,000 to 200,000 virions per cell and results in cell death (reviewed in Corey and Spear, 1986; Roizman and Batterson, 1986). This is due in part to inhibition of the cell's macromolecular synthesis or host shut-off.

1.2.1.1 Infection of the Cell

The identity of the receptors on host cells and the anti-receptors on virus particles is not known, but the

broad host range of alphaherpesviruses suggests that receptors must be widely distributed. Few mammalian cells have been found to lack HSV receptors. WuDunn and Spear (1989) have provided evidence to suggest that heparan sulphate molecules on the cell surface might serve as initial receptors for the attachment of HSV-1 and HSV-2, since heparin blocks virus adsorption. However, evidence that HSV-1 and HSV-2 recognise different receptors and the involvement of the envelope glycoproteins, in particular gD (Johnson and Ligas, 1988; Johnson and Spear, 1989), suggest that virus adsorption is a multi-step process or occurs by more than one pathway.

The virion enters the cell cytoplasm by fusion of the envelope with the cell membrane. This process requires gB in HSV (Cai et al, 1988). The capsid is then transported to the nuclear pore (Puvion-Dutilleul et al, 1989) where controlled nucleocapsid disassembly results in release of the DNA into the nucleus, accompanied by specific virion components. Studies with virus mutants suggest that this process is mediated by a viral function. Once released into the nucleus the virus genome circularises and a virion protein within the nucleus transactivates the transcription of immediate early viral genes. Further virion components are involved in the early stage of host shut-off.

1.2.1.2. Host Shut-Off

The inhibition of host protein synthesis, and the subsequent diversion of cellular resources towards viral replication, is a two stage process. The early or virion-induced host shut-off is induced by virus structural proteins, requires a threshold input number of virions per cell and does not require de novo protein synthesis. The host shut-off is effected by disaggregation of host polyribosomes by decreasing the stability of host mRNA.

Virus mutants defective in virion host shut-off (vhs mutants) are defective in the ability to degrade host mRNA (Kwong and Frenkel, 1989; Oroskar and Read, 1987). In one such HSV-1 mutant, vhs-1, the defect has been mapped to the UL41 gene. This mutant can block the wild-type vhs function leading to the suggestion that the vhs protein interacts with a cellular factor controlling the half life of mRNA. This wild-type interaction results in destabilisation of host mRNA, but the mutant protein interacts to effect an increase in the half life of mRNAs. These vhs mutants are not defective in the late host shut-off function which requires de novo viral protein synthesis.

This destabilisation of mRNA applies to host and virus

molecules (Strom and Frenkel, 1987; Kwong and Frenkel, 1987; Kwong et al, 1988). It has been suggested that this allows a more rapid switch between expression of temporal classes of viral genes (Kwong and Frenkel, 1989).

1.2.1.3 Regulation of HSV-1 Gene Expression

Transcription of viral genes occurs in the nucleus using host cell RNA polymerase II with the participation of virus-encoded factors. Protein synthesis occurs in the cytoplasm.

The expression of alphaherpesvirus genes is coordinately regulated at the transcriptional and to a lesser extent at the post-transcriptional level in a sequential cascade fashion. The viral genes are divided into classes according to the kinetics of their expression and requirements for the presence of other polypeptides and DNA synthesis. The immediate early (IE) or alpha proteins are produced first, peaking at 2 to 4 hours post-infection, and have no requirement for viral protein synthesis. There are five IE proteins; infected cell protein 0 (ICP0), ICP4, ICP22, ICP27 and ICP47.

The early (E) polypeptides are divided into two classes, $beta_1$ and $beta_2$. The former are synthesised very early in

infection but are distinguished from IE proteins by a requirement for ICP4 for their synthesis. E protein synthesis peaks at 5 to 7 hours post-infection and genes expressed within this class include those coding for components of the DNA replication machinery and nucleotide metabolism.

The late (L) or gamma proteins are characterised by a requirement for viral DNA synthesis. This requirement is more stringent for $gamma_2$ than for $gamma_1$ proteins. Genes expressed in this kinetic class are mostly virion structural components.

The IE proteins regulate their own expression and the expression of both E and L genes, and E gene products are also responsible for regulation of L gene expression. There is some evidence that L proteins may repress IE gene transcription (Everett, 1987; Roizman and Batterson, 1986).

1.2.1.3.1 Transactivation of IE Gene Transcription

A protein within the tegument of HSV-1 virions, present at about 400-600 molecules per particle, is able to induce transcription from IE gene promoters. This protein, known as Vmw65 or α-TIF, also has a structural role within the virion. The induction of IE gene expression by α-TIF

requires the consensus sequence TAATGARATTC, where R is a purine, which is present in one or more copies upstream of IE genes (reviewed by Goding and O'Hare, 1989; Roizman et al, 1988). The α-TIF protein has no DNA binding properties and transactivation by it requires the formation of a complex with a cellular DNA binding protein OCT-1. This host factor is important in the expression of a variety of cellular genes containing an octamer binding consensus, ATGCAAAT (Gelman and Silverstein, 1987; McKnight et al, 1987; O'Hare et al, 1988; Preston et al, 1988).

OCT-1 binds to the TAAT element of the IE promoter sequence and mutations within the GARAT region prevent complex formation and subsequent transactivation by α-TIF. Analysis of the Vmw65 protein has revealed the existence of a domain involved in transcriptional activation, possibly by allowing strong protein-protein interaction with components of the transcriptional machinery (Werstuck and Capone, 1989).

1.2.1.3.2 Transcriptional Regulation by IE Proteins

The HSV-1 IE protein ICP4 is required for the transcription of later classes of viral genes (Shapira et al, 1987; Arsenakis et al, 1986; Sekulovich et al, 1988) and for autoregulation of IE gene transcription (reviewed by

Everett, 1987; Roizman et al, 1988). ICP4 represses transcription of its own gene by binding to the transcription start site (Muller, 1987; Faber and Wilcox, 1988). Low doses of ICP4 stimulate ICP4 and ICP4 7/22 promoters but increased doses produce repression. Thus, ICP4 can regulate transcription positively and negatively (Arsenakis et al, 1988). The mechanism by which this occurs is unknown but it has been suggested to be mediated by differences in the binding site and its position relative to those of other transcriptional factors (Faber and Wilcox, 1988). Production of a functional ICP4 is essential for growth of HSV-1 in tissue culture (Smith and Schaffer, 1987).

ICPO also has a role in transcriptional transactivation but it is not as important as ICP4. Mutants in this gene do grow in tissue culture but with reduced efficiency, particularly at low multiplicities of infection (Stow and Stow, 1986; Sandri-Goldin et al, 1987; Sacks and Schaffer, 1987). Transactivation by ICPO and ICP4 is increased by a synergistic effect when both proteins are together.

ICP27 is an essential regulatory protein of HSV-1. Mutants do grow in tissue culture (Maclean and Brown, 1987) but are deficient in the production of many later gene products and over produce IE proteins. The transactivation of certain E

and L genes by ICP27 requires the presence of both ICP4 and ICPO (Everett, 1986; Sekulovich et al, 1988; Rice and Knipe, 1988). ICP27 also down-regulates the transcription of IE genes (McCarthy et al, 1989).

ICP22 mutants have been shown to grow poorly in some cell lines (Sears et al, 1985) but neither this protein nor ICP47 have been shown to play a significant role in transactivation of HSV-1 genes. ICP47 is not essential for growth in vitro. ICP4, O, 27 and 22 are all phosphorylated and associated with the nucleus while ICP47 is not phosphorylated and is cytoplasmic. Domains of ICPO and ICP4 have been identified which code for nuclear localisation (Everett, 1988; Paterson and Everett, 1988).

Gene regulation in HSV-1 occurs mainly at the level of transcription (Smith and Sandri-Goldin, 1988) with kinetics of expression being determined by upstream sequences of IE, E or L genes (Weir and Narayanan, 1990). There is also some evidence for regulation of protein synthesis at the post-transcriptional level by control of the level of accumulation of viral mRNA (Weinheimer and McKnight, 1987)

1.2.1.4 Replication of Viral DNA

Herpesviruses specify a large number of enzymes involved in

the synthesis of viral DNA. Large molecular weight head-to-tail DNA concatamers have been detected in infected cells suggesting that the bulk of DNA synthesis occurs by rolling-circle replication (Roizman and Batterson, 1986). These concatamers are then cleaved at specific sequences, known as "a" sequences, to produce unit length DNA. Replication is initiated at cis acting sequences of DNA termed the origin (Ori). HSV-1 contains three origins of replication; one within U_L, Ori_L, and two within the short repeat sequences, Ori_S. Viable mutants without Ori_L have been isolated (Polvino-Bodnar et al, 1987) and without one copy of Ori_S, but so far none have been identified to lack both copies of Ori_S. Ori_S and Ori_L show high sequence homology and consist of palindromes with a central AT-rich region which is essential for normal function (Lockshon and Galloway, 1988).

Experiments investigating replication of plasmids containing Ori sequences and analysis of mutants have identified seven HSV-1 genes whose products are essential for viral DNA replication (Wu et al, 1988; Weller et al, 1988; Olivo et al, 1988; Carmichael and Weller, 1989; Elias and Lehman, 1988; Goldstein and Weller, 1988). These are UL9, an origin binding protein which probably unwinds DNA; UL30, the DNA polymerase; UL29, the major DNA binding protein which binds in a non-specific manner to single

stranded DNA; UL42, a double stranded non-specific DNA binding protein and UL5, UL8 and UL52, whose functions are unknown. Possible roles for these include a helicase and a primase (Holmes et al, 1988).

1.2.1.5 Virion Assembly and Release

Transcription, DNA replication and capsid assembly all occur within the nucleus and the replication process from beginning to end takes approximately 18 hours in HSV.

Empty capsids are assembled within the nucleus and DNA is processed and packaged into them. Envelopment occurs by attachment of nucleocapsids to patches of modified inner nuclear membrane and subsequent budding through into the perinuclear space. These patches of modified lamella contain viral glycoproteins and tegument proteins are associated with the inner surface.

Egress from the host cell occurs by reverse phagocytosis, transport via the Golgi apparatus or by direct release into an adjacent cell by fusion of cell membranes. This latter process is mediated by modification of the infected cell membrane and is thought to involve gB (Cai et al, 1988). Alphaherpesviruses differ from each other with respect to effects on the "social behaviour" of infected cells.

Ejercito et al (1968) showed this property to vary within the herpes simplex viruses. A number of regions of the HSV-1 genome have been identified as coding for syncytium formation and not all map within glycoprotein genes (Spear, 1985).

Virus modification of the host cell membrane renders the infected cell a target for the immune response (reviewed in chapter 5). Other structural changes in the infected cell include fragmentation of the nucleolus and chromosome margination and fragmentation (Peat and Stanley, 1986). The host cell does not survive productive alphaherpesvirus infection.

1.2.2 Latency and Reactiviation

Alphaherpesviruses enter the host via mucous membranes; oral, genital or ocular in the case of herpes simplex viruses. Local replication ensues and virus particles enter sensory neurons supplying the infected area. Internalisation into the neuron occurs by fusion of the viral envelope and the plasma membrane (Lycke et al, 1988). Nucleocapsids travel centripetally by axoplasmic flow at a rate of 2 to 10 mm/hour (Wildy, 1986) towards the neuron cell body. The host immune response matures after the virus reaches the nervous system and contributes towards

elimination of the local mucous membrane infection, but it cannot eliminate virus from the sensory ganglia. Once virus reaches the ganglion an acute ganglionic infection is initiated and progeny virus may return down axons to the dermatome and initiate further replication at the periphery. Several round trips may thus occur during the initial acute stage of the infection.

Latency is established within the ganglion coincident with the appearance of local interferon and an inflammatory response within the ganglion. Approximately 0.1% of neurons within an infected ganglion have been estimated to harbour latent virus (Stroop, 1986).

Evidence suggests that cells of neuronal origin are selectively restrictive for HSV replication and are thus more prone to the establishment of virus in the latent state (Gerdes et al, 1979; Cook et al, 1986).

The mechanisms behind the latency of herpesviruses are not well understood and a great deal of research is concentrated on elucidating the regulation of establishment, maintenance of and reactiviation from the latent state. Much interest was recently aroused on the discovery of limited transcription from the HSV genome during latency (Stevens et al, 1987). It was widely

proposed that the latency associated transcript (LAT) might be involved in controlling latency. However, from studies with LAT-deficient mutants it is now apparent that LAT is not essential for the establishment or maintenance of latency and LAT-negative mutants do reactivate but with a reduced frequency (Javier et al, 1988b; Leib et al, 1989b; Steiner et al, 1989; Sedarati et al, 1989). The finer points of latency and reactivation are reviewed in chapter 4 and so will not be considered here.

On reactivation the virus is transported along the periphery of the axon in vesicles (Wildy, 1986) towards the dermatome where a recrudescent lesion or asymptomatic shedding may occur, depending on local conditions at the periphery. Evidence suggests that reactivation results in death of the infected neuron (McLennan and Darby, 1980).

However, latent virus persists for the life of the host by recurrent peripheral infections sending more virus up axons to establish latency.

1.3 THE CLINICAL SIGNIFICANCE OF ALPHAHERPESVIRAL INFECTIONS

Herpetic syndromes have been recognised in man for centuries. References to mucocutaneous herpetic lesions

have been found in the writings of Hippocrates, Celsus and Galen among others (Cumston, 1926). Distinction between HSV and VZV was made as far back as 1814, but only in the 1960s were the two types of herpes simplex virus recognised as being distinct (Sunstrum, 1989). The infectious nature of the herpes agent was demonstrated by Löwenstein in 1919 who produced lesions in the rabbit eye from vesicular fluid of oral and genital sores (Stroop, 1986).

Orofacial lesions are most commonly due to HSV-1. Infection is usually acquired asymptomatically during childhood and reactivation is relatively common producing cold sores, often at the vermillion mucocutaneous border of the lips. Approximately 80% of adults have antibodies to HSV-1 while only 20-40% suffer from recurrences (Sunstrum, 1989). The most serious consequence of HSV-1 infection is produced by replication within the brain. HSV-1 is the most common cause of fatal sporadic encephalitis. This may result from a primary or reactivated infection and causes serious and often permanent neurological damage. Usually the temporal lobes of the brain only are affected rather than widespread infection. A good prognosis depends on rapid diagnosis and intravenous acyclovir treatment. HSV-1 encephalitis will be dealt with in more detail in the following section.

HSV-2 is the primary cause of genital herpes. Occasional cases are due to HSV-1 but genital recurrences are more common with HSV-2. In the same way HSV-1 orolabial infections reactivate with greater frequency than those of HSV-2. Generalised neonatal HSV-2 infections occur when the neonate contacts the mother's infected birth canal. These infections often involve a widespread brain infection with frequent long-term neurological impairment. The use of acyclovir has significantly reduced mortality from HSV-2 and HSV-1 encephalitis. Generally neonates with HSV-1 infection fare better than HSV-2 infected patients due to the increased neurotropism and possibly an increased immunopathology induced by the latter virus type. This may also be due to an increased interferon induction by HSV-1 (Corey et al, 1988).

Other herpes simplex virus diseases include herpetic whitlow or infection of the finger, normally observed in dental practitioners, keratitis and aseptic meningitis.

The increased use of immunosuppressive treatment and the advent of AIDS has produced a significant increase in the numbers of reactivated herpes infections. However, the number of cases of encephalitis have not been significantly increased. Indeed, most patients with HSV encephalitis do not have any immune impairment.

Varicella-zoster virus primary infection occurs in most people before adulthood as the highly infectious chickenpox. However, only 10 to 20% of people who have experienced varicella develop the recurrent infection of zoster or shingles. This demonstrates how much less likely VZV is to recur than HSV. When it does occur zoster affects large portions of a single dermatome and produces severe and prolonged symptoms due to extensive tissue damage within the ganglion. Asymptomatic shedding of VZV has never been reported.

Straus (1989) has suggested these differences in VZV and HSV infections to be due to differences in the control of latency for each virus. Latent VZV DNA has been found to reside within satellite cells in the ganglia, rather than neurons as is the case with HSV. It may be that such cells receive less reactivating stimuli than neurons. In addition, Croen et al (1988) have shown that transcription of the latent VZV genome occurs from at least three genes and is therefore more extensive than that described for HSV. Straus (1989) also suggested that VZV is more readily controlled by the host immune response than HSV. Thus, as antibody levels decline later in life zoster is more likely to occur.

Serious alphaherpesvirus infections of humans have also

included encephalitis produced by B-virus or Herpesvirus simiae. This agent has been transmitted to humans from infected macaque monkeys on a number of occasions, usually from bites or scratches and often proves to be fatal (reviewed by Palmer, 1987).

1.3.1 HSV-1 Encephalitis

The majority of the adult population has serological evidence of HSV infection ,but only a small number of cases of acute necrotising HSV encephalitis occur, approximately one in one million of the population (reviewed by Stroop, 1986; Whitley, 1988). This relative rarity leads to the question of why these cases occur at all? What factors are involved in the establishment of HSV-1 encephalitis? As stated earlier encephalitis patients do not generally exhibit immune defects.

The main source of information on the pathogenesis of HSV-1 encephalitis is through the use of animal models. However, the majority of these models produce widespread lesions within the brain, involving many structures such as the mid-brain, brainstem and cerebral hemispheres. This situation resembles HSV-2 encephalitis in neonates rather than the HSV-1 restriction of infection to the temporal lobes. Animal models which do produce such a limited

infection of the brain are achieved by olfactory inoculation of rabbits (Schlitt et al, 1986; Stroop and Schaefer, 1986). This led to the suggestion that the olfactory bulb may represent the route of inoculation required for the establishment of human encephalitis (Stroop, 1986), possibly by inadvertant autoinoculation from orolabial lesions.

HSV-1 can produce a latent brain infection in mice and humans (Sequiera et al, 1979; Fraser et al, 1981) as a normal consequence of peripheral inoculation. Encephalitis may result from reactivation of a latent ganglion infection which subsequently infects the brain, or from reactivation of a latent temporal lobe infection. Nahmias et al (1982) analysed sera from HSV-1 encephalitis patients and found that 30% of cases were as a result of primary infection while 70% were produced by reactivation of latent virus.

There is the possibility that exogenous reinfection with a second HSV strain might lead to encephalitis. However, secondary colonisation of rabbit and guinea-pig ganglia has been shown to be inhibited following reinfection with a different HSV strain (Centifanto-Fitzgerald et al, 1982a; Richardson et al, 1987; Gerdes and Smith, 1983). If a primary infection were established within the sensory ganglia the nasal route would represent a potential portal

of entry for a second strain. Thus, Whitley et al (1982) analysed encephalitic HSV-1 isolates with restriction endonucleases. Identical strains were isolated from individuals for 65% of cases while 35% showed evidence of two strains being present.

In the case of a reactivation or a reinfection an already primed immune response would contribute towards immunopathologic necrosis in the infected brain.

Stroop (1986) suggested that the restricted nature of HSV-1 encephalitis is due to inoculation by the olfactory route and possibly due to the localising effect of the host immune response in the brain. A number of groups have provided evidence to suggest that the localised nature of HSV encephalitis is due to selective vulnerability of various brain structures to HSV replication. McFarland and Hotchin (1987) inoculated HSV-1 strains into the murine hippocampus and found extensive virus spread within this area, but inoculation into the cerebellum produced only a few scattered antigen positive cells and minor pathological effects. Javier et al (1988a) have localised a function in an HSV intertypic recombinant which conferred a neurovirulent phenotype, but this neurovirulence property functioned independently of high titre replication in the brain. A mutant defective in a region of the genome (0.079

to 0.143 map units) replicated to high titre in mouse brains but was 10,000 fold less neurovirulent than wild-type virus. They explained this paradoxical phenotype as an altered cellular tropism in which the mutant was restricted in specific brain cells essential for survival of the host. Stroop and Schaefer (1989) used two clonally related strains of HSV-1 to investigate virus tropism for specific CNS structures. Both strains invaded and replicated within the brain but one produced fatal infection while the second was asymptomatic. This difference was correlated with a differential ability to replicate within specific brain centres. Thus it is possible that the temporal lobe in humans is more permissive to HSV-1 replication than other CNS structures.

Infections that establish latency within CNS structures have proved to require stronger stimuli for reactivation than latent virus within ganglia. Cabrera et al (1980) failed to reactivate latent CNS infections from explanted fragments of murine brain, yet HSV DNA sequences were shown to be present in the tissue by hybridisation. Stroop and Schaefer (1986, 1987) have shown differing abilities of HSV-1 strains to reactivate from latent brain infection and produce encephalitis in their rabbit model. Thus, ability to reactivate was correlated with encephalitogenicity.

For neurovirulence there is a requirement for efficient replication at the site of inoculation and productive infection within the CNS. Thus, genes essential for virus multiplication will also be important for pathogenicity (Sedarati and Stevens, 1987). In addition, HSV has been shown to possess genes whose expression in culture is not required for multiplication but are important in the host. Thus, in addition to the ability to reactivate from the CNS other virus properties have been shown to determine whether encephalitis is produced in animal models (Stroop, 1986; Heard et al, 1987). Such properties include thymidine kinase production (Field and Wildy, 1978; Ben-Hur et al, 1983; Chrisp et al, 1989), a wild-type DNA polymerase (Larder et al, 1986; Day et al, 1988), gG (Weber et al, 1987), ribonucleotide reductase (Cameron et al, 1988) and the IE protein ICP22 (Sears et al, 1985).Other as yet unidentified "virulence genes" have also been detected (Chaney et al, 1983; Dix et al, 1983; Stroop and Schaefer, 1986). In some cases these functions have been localised to short regions of viral DNA (Centifanto-Fitzgerald et al, 1982b; Rösen et al, 1986; Taha et al, 1989a and b; Thompson et al 1989; Javier et al, 1988a). However, the fact that viral factors alone do not control the outcome of HSV-1 infection is illustrated by the findings of Dix et al (1983). This group analysed isolates from encephalitis patients and compared them with other isolates, they found

that encephalitic strains were not especially neurovirulent.

It is likely that a number of factors contribute towards the occurrence of HSV-1 encephalitis. Route of inoculation is likely to be important, in particular the olfactory route may produce a greater likelihood of encephalitis, a significant stimulus may be necessary to reactivate latent brain infection and a neurovirulent strain of HSV-1 may be required for encephalitogenesis. These factors perhaps explain the relative rarity of encephalitis produced by HSV-1 together with the fact that most people are likely to be protected from infection with a virulent strain by an established latent infection.

The identification of HSV genes contributing towards neurovirulence will provide useful information for understanding why encephalitis does sometimes occur. It is also possible that such genes will be found to play a role in the switch from latent to productive infection within nervous tissue.

1.4 ALPHAHERPESVIRUS SAIMIRI AND THE AIMS OF THIS THESIS

αHVS, a virus of New World monkeys, was originally isolated

from fatal infections in white-lipped and cotton-topped marmosets (Tamarinus nigricollis and Oedipomidas oedipus) (Melnick et al, 1964; Holmes et al, 1964). This led to the original name of the virus being designated Herpesvirus tamarinus. However, serological studies later suggested that the virus was in fact indigenous to squirrel, spider and cinnamon ringtail monkeys and produced fatal infections when marmosets or owl monkeys became infected (Holmes et al, 1966; Sheldon and Ross, 1966). Infection of marmosets, owl monkeys and squirrel monkeys with αHVS was further characterised by a number of groups (Emmons et al, 1968; Burkholder and Soave, 1970; Morita et al, 1979; Barahona et al, 1974).

The squirrel monkey is the only natural host of αHVS exhibiting occasional disease (Hunt and Melendez, 1969; Daniel et al, 1967). Primary infection usually occurs in young animals and virus is spread by direct contact and aerosols. When rare occurrences of disease are observed they are characterised by oral and labial ulcers. Epizootic infection of non-natural host monkeys produces a generalised infection with characteristic lesions in most organs of the body and death in 2 to 3 days after a 7 to 10 day incubation period.

Barahona et al (1974) suggested that αHVS infection of its

natural host, the squirrel monkey, was analogous to HSV infection in man, while αHVS infection of marmosets or owl monkeys produced a generalised disease similar to that seen with B-virus in humans or HSV in monkeys, and occasionally in man.

αHVS has been referred to as H.tamarinus, Herpes-T, H.platyrrhinae and marmoset herpesvirus in the past. The name H.saimiri-1 was suggested to reflect the natural host (Saimiri sciureus) (Roizman,1982) and the symbol α is used to differentiate the virus from the lymphotropic gammaherpesvirus saimiri.

The strains of αHVS used in this thesis, KM91 and KM322, were isolated from lethal infections in owl monkeys in Liverpool between 1968 and 1971 (Tosolini and McCarthy, 1975; McCarthy and Tosolini, 1975b).

The most notable characteristic of these two virus strains was their differing pathogenicity for rabbits. Inoculation into the rabbit flank with KM322 produced a stable latent infection within the dorsal root ganglia, while KM91 produced a rapid and fatal encephalitis (McCarthy and Tosolini, 1975a; Tosolini and McCarthy, 1975; Tosolini et al, 1981; Tosolini et al, 1982; Leib et al, 1987a; Leib et al, 1988). Thus, KM322 infection in rabbits provided a

model for alphaherpesvirus latency. The stable and prolonged nature of this latency also suggests that KM322 infection in rabbits provides a suitable model for VZV infection in humans, particularly since VZV lacks its own animal model (McCarthy, 1972). KM91 infection of rabbits produces a widespread necrotic encephalitis involving the brainstem, cerebellum and cerebral hemispheres (Leib et al, 1988). This pattern does not mirror that seen in HSV-1 human encephalitis, but comparison of KM91 with KM322 will produce information on the functions coding for neurovirulence in αHVS and possibly in alphaherpesviruses in general.

Studies by Leib (1986) initiated characterisation of these αHVS isolates and confirmed their identity by restriction endonuclease (RE) analysis and SDS PAGE comparisons with a standard strain. The viruses were shown to be thymidine kinase producing and grew rapidly in a wide range of cell lines to produce characteristic syncytial cytopathic effect. The plaque size of KM91 was found to be larger than KM322, but also with a larger range of sizes. Analysis of the αHVS genome revealed a G+C content of 67%, a genome size of 153kb and the existence of four isomeric forms (see chapter 6). Co-inoculation and superinfection studies with KM91 and KM322 in rabbits showed that KM322 was able to induce a rapid protective immune response in the host which

prevented KM91 encephalitis and allowed survival of the animal.

There are precedents for protection by an apathogenic herpes strain against pathogenic virus inoculation into animals. Daniel et al (1978) used a small plaque variant of αHVS to protect owl monkeys and marmosets against lethal challenge with a large plaque variant. Kit et al (1983) used TK^- mutants of αHVS to protect mice against a potentially lethal injection of wild-type virus. Such protective studies have also been performed with HSV strains in mice (Schröder et al, 1983; Yamada et al, 1986a). Comparisons between KM91 and KM322 by in vitro replication, RE analysis , SDS PAGE immunoprecipitation and TK production revealed only plaque size to be significantly different between the two strains (Leib, 1986). Thus, the two strains appeared to be very similar in a number of genetic and in vitro biological characteristics but exhibited significant pathogenic differences in vivo.

This thesis was therefore concerned with further comparing the two strains in order to determine factors which might be responsible for differential neurovirulence. Plaque size was investigated since this represented a potential neurovirulence marker. The range of plaque size was analysed with both virus strains after plaque -

purification to investigate the possibility that KM91 stocks consisted of a mixed population of variants. The replication of KM91 and KM322 was investigated in Vero cells and neural cells in order to determine whether KM322 might be replication-deficient with respect to KM91 and whether this might be restricted to cells of a neuronal origin. The polypeptides of the two strains of αHVS were compared to each other and to HSV-1 by SDS PAGE analysis. Their comparative immunogenicity was investigated using immunoblots with hyperimmune rabbit sera and monoclonal antibodies raised against KM322. It was anticipated that such experiments would reveal an antigenic difference between the two strains to explain their differing immunogenicity in the rabbit.

The genomes of KM91 and KM322 were compared with an extensive range of restriction endonucleases and the αHVS genome was further characterised by double RE digests and hybridisation studies with HSV-1 probes. Comparisons between KM91 and KM322 genomes were performed in order to identify a genetic difference which might determine pathogenic differences between the two strains. It was hoped to resolve whether the αHVS genome was in four isomeric forms, as reported by Leib et al (1987b), or two isomers, as reported by Kit et al (1980) and Desrosiers and Falk (1981)

The KM322 latency model in rabbits was utilised to investigate the effects of virus stimulating agents on reactivation of latent αHVS (KM322). It was hoped that such experiments might improve the sensitivity of detection of latent virus by explant culture and prove informative as to mechanisms of control of latency in αHVS.

Special precautions were taken at all times when handling live αHVS (McCarthy and Tosolini, 1975a), since one report has suggested that this virus may have produced encephalitis in an infected human (Melendez, 1968).

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Tissue Culture

2.1.1 Vero Cells

Vero cells, African green monkey kidney fibroblasts, were grown in Medium 199 with Earle's salts (Gibco, Paisley, U.K.) containing 5% fetal calf serum (FCS), 2% v/v of 7.5% sodium bicarbonate (Gibco), 100 units/ml penicillin (Glaxo, Greenford, U.K.) and 100μg/ml streptomycin (Evans, Greenford, U.K.). Once confluent Vero cells were maintained in 199 containing 2% FCS, 2% sodium bicarbonate, 100μ/ml penicillin and 100μ/ml streptomycin.

Veros were subcultured by discarding the medium, washing monolayers with 0.02% v/v versene in phosphate buffered saline (PBS) followed by incubation with 0.25% trypsin (Gibco) and 0.02% versene in PBS. Once cells were seen to be detaching from the surface the trypsin was discarded and cells were stripped from flasks and resuspended in fresh growth medium using a bent Pasteur pipette.

One confluent 12oz bottle of cells was used to seed 4 12oz bottles, in 30mls growth medium per bottle, or 100 16mm diameter stoppered test tubes at 1ml medium per tube. Where large scale culture was required Roux bottles were used; 3 12oz bottles of confluent cells being subcultured into 4

Roux bottles in 100mls medium per bottle. Cell cultures were incubated at 37°C in 5% CO_2 and confluency was generally reached within 48 hrs.

2.1.2 Other Cell Lines

Rabbit kidney epithelial cells, RK-13s, were cultured in Minimum Essential Medium (MEM) with Earle's salts (Gibco) containing 5% normal rabbit serum, 2mM L-glutamine (Gibco), 1% non-essential amino acids (Gibco), 100u/ml penicillin and 100µg/ml streptomycin. Maintenance medium was as above but with 2% rabbit serum.

Cells were trypsinised as for Veros and one 12oz bottle was used to produce four equivalent sub-cultures.

Murine neuroblastoma cells, Neuro 2a, derived from clone C1300, were grown in MEM with Earle's salts containing 10% FCS, 2mM L-glutamine, 1% non-essential amino acids, 2% sodium bicarbonate, 100u/ml penicillin and 100µg/ml streptomycin. Neuro 2a s were maintained in medium containing 5% FCS.

Cells were trypsinised as for Veros and the split ratio was 1:3.

Both the above cell lines were incubated at 37°C in 5% CO_2.

2.2 Viruses and Virus Stocks

2.2.1 Virus Strains

Strains of alphaherpesvirus saimiri (αHVS), KM91 and KM322, were isolated from separate lethal infections in owl monkeys (Aotus trivirgatus) between 1968 and 1971 (McCarthy and Clarkson, unpublished). A standard strain of αHVS isolated in Boston, U.S.A. (Melendez et al, 1966) was also used for certain experiments, and is designated αHVS (Boston).

The standard strain of herpes simplex virus (HSV) used was type 1 strain HFEM, except in Southern blotting experiments in which case HSV-1 (strain KOS) was used.

2.2.2 Preparation of Virus Stocks

Virus stocks were prepared from infected Vero cells. Confluent monolayers in 12oz bottles were infected at a multiplicity of infection (moi) = 0.01 to 0.1 pfu/cell in 5mls maintenance medium per bottle. The virus was left to adsorb for 1 hr on a rocking platform at 37°C after which time 25mls of pre-warmed maintenance medium was added to

each bottle. Cultures were then incubated stationary at 37°C for 48 hrs, or until 100% cytopathic effect (cpe) was achieved.

Once monolayers were completely destroyed cultures were centrifuged at 3000 RPM for 15 mins in a Centaur 1 bench centrifuge (MSE, Crawley, U.K.) to pellet cellular debris. The supernatant was kept on ice while the cell pellet was resuspended in PBS and frozen-thawed 3 times to disrupt cells and release cell-bound virus. The cellular debris was repelleted by centrifugation at 3000 RPM for 15 mins and the supernatant combined with the first and distributed into ampoules for storage at -70°C.

2.3 Plaque Purification

Confluent Vero monolayers in 3cm diameter plastic petri dishes (Sterilin Ltd., Feltham, U.K.) were infected with 300µl of virus suspension at moi $\leq 1 \times 10^{-4}$ to obtain well separated plaques. After adsorption for 1 hr at 37°C the medium was removed and the infected monolayers washed with PBS and overlayed with 5% molten low gelling temperature agarose (Sigma, Poole, U.K.), cooled to about 37°C, added to an equal volume of pre-warmed 2 x maintenance medium. Once the overlay was set petri dishes were incubated in 5% CO_2 at 37°C, inverted to prevent condensation running over

the agarose surface.

After 48 hrs discrete plaques were picked off by removing the cylinder of agarose above with a bent Pasteur pipette. Each cylinder was placed in 300μl maintenance medium and freeze-thawed to produce a suspension for subsequent inoculations. Three successive cycles of plaque picking were used to produce a plaque-purified stock.

2.4 Virus Titration

Virus suspensions were titrated on confluent Vero monolayers in 16mm diameter stoppered test tubes. Ten-fold serial dilutions of the stock to be quantified were made in maintenance medium ranging from 10^{0} through to 10^{-6}. 0.1ml of virus was inoculated onto 5 tubes per dilution and left to adsorb for 1 hr at 37^{o}C. After this time 0.9ml of pre-warmed maintenance medium was added to each tube. The tubes were incubated for 40 hrs at 37^{o}c before plaque counts were made using dark ground and phase contrast microscopy (McCarthy, 1960; McCarthy and Taylor-Robinson, 1967).

It was not considered necessary to overlay infected monolayers since previous studies have shown that secondary plaque formation does not occur within the 40 hrs

incubation time (Tosolini et al, 1982).

2.5 Purification of Virus Particles

Virus was purified by centrifugation on pre-formed Nycodenz gradients (Nyegaard and Co., Oslo, Norway). A 40% w/v solution of Nycodenz was prepared by dissolving Nycodenz powder in a 0.75% w/v solution of sodium chloride in buffered medium. The buffered medium consisted of 5mM tris-HCl pH7.5, 3mM potassium chloride and 0.3mM ethylene diamine tetraacetic acid (disodium-calcium salt). Gradients were pre-formed by freeze-thawing 4ml of 40% w/v Nycodenz in 5ml ultra-clear centrifuge tubes (Beckman, High Wycombe, U.K.) at least 3 times.

Virus was concentrated from tissue culture fluids by ultracentrifugation for 1 hr at 50,000g in a MSE 8x50ml aluminium angle rotor with a Superspeed 75 ultracentrifuge (MSE). Virus pellets were re-suspended in PBS and 0.75ml was overlayed onto each pre-formed gradient. Gradients were centrifuged for 3 hrs at 70,000g in a Beckman Sw65 Ti swinging bucket rotor on a L5-65 Beckman centrifuge.

The resultant band of virus particles was visible to the eye and was removed with a needle and syringe from above. The purity of the fraction was confirmed by electron

microscopy. The purified virus was then dialysed for 2 days at 4°C against PBS with frequent changes of buffer.

2.6 Inoculation of Rabbits with Live αHVS

The flanks of male New Zealand White rabbits were shaved and 3 sites of inoculation were mapped out in marker pen on each flank. 500pfu KM322 in 0.2ml were injected intradermally into each site as described by Tosolini *et al*, (1982). The inoculation sites were on the lateral aspect of each flank within the 3 dermatomes T_{12} to L_2, on a paravertebral line joining the costal margin to the iliac crest, 10-12cm from the midline. Intradermal injections were performed under ketamine hydrochloride anaesthesia (Parke-Davis, Gwent, U.K.). 1ml 50mg/ml anaesthetic per 4-5kg rabbit was injected into the ear vein. The intravenous injection was rendered painless by first injecting 0.1ml of 1% w/v lignocaine hydrochloride (Antigen Ltd., Roscrea, Ireland) into the adjacent lymphatic vessel.

As a safety precaution when injecting live virus a clear plastic box was placed over the needle and syringe as a shield. Since the virulence of αHVS to humans is unknown it was treated as a high-risk pathogen. Infected animals were kept in airlock-protected and filtered isolation rooms and operators wore gloves, gowns, masks and boots.

In order to prevent rabbits biting and scratching virus lesions at the inoculation sites they were fitted with a 30cm diameter collar made of soft vinyl floor-covering. The collars were removed approximately 3 weeks later after the lesions had healed.

2.7 Explant Culture of Rabbit Dorsal Root Ganglia

2.7.1 Dissection of Rabbits

Rabbits were killed and exsanguinated at least one month after inoculation to ensure that latency was established and that the acute infection was resolved. Dissection was performed immediately.

The rabbit was fixed to a dissection board ventral surface down and the fur of the back swabbed with Videne surgical scrub (Beta Medical Products Ltd., Runcorn, U.K.). An incision was made through the skin along the dorsal surface of the rabbit from the rib cage to the hind legs. The muscle tissue was then cut away to expose the spine. Bone forceps were used to clip off the inferior articular, superior articular and the spinous processes along the length of exposed spine. The spinal cord was then exposed by removal of the dorsal arch by processive clipping through each wall.

Dorsal root ganglia (DRGs) were removed from both sides from T_{11} through to L_6 using springbow scissors (Mercian Surgical Supply Co. Ltd., Birmingham, U.K.) and fine forceps. Care was taken to avoid excising spinal cord or nerve fibres with the ganglia.

2.7.2 Explant Cultures

The ganglia were placed in sterile petri dishes containing 0.5ml PBS with 2% FCS. The tissue was minced into approximately $0.5mm^3$ fragments with a pair of scalpels. The fragments were divided equally into the various test groups and inoculated onto Vero monolayers in test tubes at 3 tubes per ganglion. The explants were cultured in Earle's 199 medium containing 20% FCS, 2% sodium bicarbonate, 100u/ml penicillin, 100μg/ml streptomycin and either 50μM 5-azacytidine (Sigma), 5ng/ml 12-o-tetradecanoylphorbol 13-acetate (Sigma), 2mM sodium-n-butyrate (BDH, Poole, U.K.) or no drug.

The tubes were incubated at 37°C and left undisturbed for 3 days. After this time the medium in each tube was removed, using separate Pasteur pipettes to prevent cross-contamination, and the cultures were re-fed with drug-free medium containing 20% FCS. This was repeated subsequently every 7 days. The cultures were maintained

for 8 weeks and examined every day for cpe associated with reactivating virus.

In addition a limited number of explant cultures were incubated in 200mM dimethylsulphoxide and 5mM hexamethylenebisacetamide (Sigma). In these cases cultures were re-fed with medium containing drug at each medium change.

All isolates were confirmed as the input strain by restriction endonuclease analysis of viral DNA (section 2.13.2).

2.8 Production of Virus Antigen for Immunisation of Mice and Rabbits

Virus for hyperimmunising rabbits was passaged twice in RK-13s in 5% rabbit serum and titrated on Vero cells. In order to produce antigen for the inoculation of mice for monoclonal antibody (McAb) production virus was cultivated in Vero cells and titrated on Veros. In each case virus was purified on Nycodenz gradients (section 2.5) and inactivated with β-propiolactone (BPL) (Sigma).

2.8.1 Inactivation of Virus with BPL

A 1% w/v solution of BPL was prepared in PBS and stored in 1ml aliquots at -70°C. In order to inactivate virus BPL was added to suspensions to a final concentration of 0.05% w/v. Bottle caps were tightly closed and sealed with Parafilm (American Can Co., Greenwich, USA) and the virus suspensions shaken for 10 mins at 4°C. The mixture was then left at 37°C for 2 hrs and shaken every 10 mins, and was subsequently left to stand at 4°C overnight to allow complete hydrolysis of the BPL.

Each suspension was tested for infectivity by inoculation onto Vero cells in tubes. If any residual virus activity was detected the procedure was repeated on the same suspension.

Killed virus suspensions were dialysed against PBS at 4°C for 2 days with frequent changes of buffer.

2.9 Hyperimmune Serum Production

Male New Zealand White rabbits were used for the production of hyperimmune sera. A small sample of blood was taken from the ear vein before 1ml intramuscular (im) inoculations were made into each hindquarter. The im

inoculations consisted of BPL-inactivated virus suspension mixed with an equal volume of Freund's Complete Adjuvant (Gibco) to produce $1.5x10^8$ particles/ml.

At 2 weeks a second blood sample was taken from the ear vein and at 4 weeks a further im inoculation was performed. At 6 weeks each rabbit was bled as before and at 8 weeks they were given an intravenous booster injection of 2ml BPL-inactivated virus. A final blood sample was taken at 9 weeks after which rabbits were boosted and bled as required.

Hyperimmune sera were tested by indirect immunofluorescence.

2.10 Indirect Immunofluorescence (IF)

2.10.1 Preparation of Slides

Vero monolayers in 12oz bottles were infected with αHVS at an moi = 1.0 and were harvested 24 hrs later using a rubber policeman, since at this stage cells were largely still intact. Uninfected Vero cells were harvested in the same way. Infected and uninfected cells were resuspended in PBS and centrifuged at 3000 RPM in a Centaur 1 bench centrifuge for 15 mins. The cell pellets were washed and centrifuged

twice more before being resuspended in PBS at a final volume of 1-2ml per 12oz bottle of cells ie. $1x10^7$ to $5x10^6$ cells/ml.

Microscope slides were cleaned with alcohol and then 8x10µl drops of cell suspension were placed in 2 rows of 4 along the slide. It was found most convenient to place 1 row of infected cells in parallel with uninfected controls on the same slide.

The wells were air-dried before being fixed in acetone at room temperature for 10mins. Fixed slides were stored in air-tight containers at -20°C for up to 2 months.

2.10.2 IF Staining

Test sera were heat inactivated for 30 mins at 56°C before dilution in PBS. Hyperimmune rabbit sera were generally tested at 1/50, 1/100, 1/500 and 1/1000 dilutions. McAb supernatants were screened in bulk at a dilution of 1/10 in PBS. Positives were subsequently titrated out as for sera.

Slides were equilibrated to room temperature and 20µl of diluted sera or McAb supernatants were added to infected Vero and control wells. The slides were incubated in humidified boxes at 37°C for 1 hr, and then washed 3 times

for 10 mins in PBS, with stirring, at room temperature. The slides were then air-dried before 10μl conjugate was added to each well. In the case of rabbit sera goat anti-rabbit 1gG (whole molecule) conjugated to fluorescein isothiocyanate (FITC) (Sigma) was used diluted to 1/80 in PBS containing 0.0005% w/v Evans blue (Sigma). McAb supernatants were stained using rabbit anti-mouse 1gG FITC (whole molecule) (Sigma) diluted to 1/40 in PBS with 0.0005% w/v Evans blue.

The slides were incubated with the conjugate for 1 hr at 37^{o}C in humidified boxes and then washed for 3x10 mins in PBS. After air-drying slides were mounted with 9:1 glycerol: PBS and viewed the same day under a Laborlux K fluorescence microscope (Leitz, Wetzlar, Germany).

Rabbit sera were controlled with normal rabbit serum and McAb supernatants were tested alongside NS-1 supernatant as a negative control and heat inactivated polyclonal mouse serum, removed from the immunised animal, as a positive control.

A positive result was indicated by apple-green fluorescence covering most of the infected cell.

2.11 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

2.11.1 Preparation of Antigen

Confluent Vero cells were infected with αHVS, KM91 and KM322, and HSV-1 at an moi = 1.0 and incubated at 37°C to 100% cpe. Adherent cells were washed into the medium and cellular debris was pelleted by centrifugation in an MSE bench centrifuge at 3000 RPM for 15 mins. The supernatants were kept on ice while cell pellets were resuspended in PBS, freeze-thawed 3 times and re-pelleted for 15 mins at 3000 RPM. The supernatants were combined with the first and the cell pellets discarded. Virus was concentrated by centrifugation for 1 hr at 50000g in an 8x50ml fixed angle rotor on a MSE Superspeed 75 ultracentrifuge. Virus pellets were resuspended in PBS at a final volume of 80μl per 12oz bottle (approximately $4x10^9$ pfu/ml).

Uninfected Vero cells were harvested and washed as described for IF slide production (section 2.10.1) and resuspended in a final volume of 1ml per 12oz bottle (approximately $1x10^7$ cells/ml).

2.11.2 SDS PAGE

SDS PAGE was used to separate viral proteins according to molecular weight.

A 30% acrylamide stock solution was prepared from 29.2g acrylamide (BDH) and 0.8g N'N'-bis methylene acrylamide (BDH) made up to 100mls with distilled water. The stock was filtered and kept in the dark at 4^{o}C for up to 1 month. Gloves were worn at all times while handling acrylamide.

Separating gels contained 10% acrylamide, 0.56M tris pH 8.8 and 0.1% SDS. Gel solutions were de-gassed using a vacuum pump as oxygen inhibits polymerisation. Chemical polymerisation was initiated by the addition of 0.03% ammonium persulphate and 0.0007% tetramethylethylenediamine (TEMED) (Sigma). 12cm gels were poured in a vertical electrophoresis system (Biorad, Watford, U.K.), overlayed with water-saturated butanol and left to polymerise for at least 1 hr or overnight.

3cm stacking gels were poured on top of separating gels and contained 5% acrylamide, 0.06M tris pH 6.8, 0.1% SDS and were polymerised as above. A comb placed in the stacking gel produced wells for sample loading. After polymerisation the comb was removed and wells washed 3

times with distilled water and then filled with running buffer (0.025M tris, 0.192M glycine, 0.1% SDS, pH 8.3).

Each sample was mixed with an equal volume of sample buffer (0.06M tris pH 6.8, 4% SDS, 0.2% glycerol, 0.2M dithiothreitol (DTT) (Sigma) and 0.01% bromophenol blue (Sigma)) and heated at 100^{o}C for 5 mins. Non-reducing gels were run with DTT omitted from the sample buffer.

40μl of sample/sample buffer mix was loaded into each well of the stacking gel alongside molecular weight markers which were bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor and α lactalbumin (Sigma).

Gels were electrophoresed in running buffer at 25mA through the stacking gel and then at 40mA through the separating gel in 5 hrs or at 8mA overnight.

2.11.3 Staining Acrylamide Gels

Separated proteins were stained with 1% Coomassie Brilliant Blue (Biorad) in 45% v/v methanol and 10% v/v acetic acid. Gels were stained overnight and were diffusion-destained in several changes of destain (25% v/v methanol, 9% v/v acetic

acid).

Gels for use in Western blotting were not stained in Coomassie Blue as this process fixes the protein.

2.12 Western Blotting

2.12.1 Electro-Blotting

After SDS PAGE gels were removed from the apparatus and equilibrated in transfer buffer (20mM tris, 100mM glycine) to eliminate swelling at a later stage, the distance from origin to dye front was then noted for each gel. A piece of nitrocellulose paper (NCP) (Schleicher-Schuell, Kingston-U-Thames, U.K.) cut slightly larger than the gel was also soaked in transfer buffer before formation of the blot sandwich.

The sandwich was assembled with a scotch-brite pad soaked in transfer buffer, followed by a soaked piece of Whatman 3 paper (Whatman Ltd., Maidstone, U.K.). The gel was placed on top of this followed by the NCP. Care was taken to exclude air-bubbles from between the gel and the NCP. The sandwich was completed with the second piece of Whatman 3 followed by the second scotch-brite pad.

The electro-blotting was run overnight in transfer buffer at 100mA in a Biorad Transblot unit. The gel was oriented cathodic to the NCP since protein-SDS complexes are strongly negatively charged.

2.12.2 Immunoblotting

After electrophoretic transfer was complete the gel was stained in Coomassie Blue to confirm protein transfer. The NCP was stained for protein in 0.2% Ponceau S (Sigma) in 3% trichloroacetic acid and lanes containing molecular weight markers were cut from the NCP and distances of bands from the origin noted. The stain was removed from the sample blots by rinsing with distilled water.

The NCP was quenched in 2% bovine serum albumin (Sigma) in PBS for 1 hr at room temperature, in order to remove any non-specific protein binding, and then washed for 2x30 mins in washing buffer (0.2% TWEEN 20 (Sigma) in PBS).

Rabbit sera for immunoblotting were diluted 1/30 in 0.2% TWEEN 20, 10% FCS, 1M glucose and 10% glycerol in PBS. McAb supernatants were used neat. The NCP was incubated with the appropriate antibody for 90 mins at room temperature on a rocking platform and then washed at least 4 times for 20 mins each in washing buffer.

The strips of NCP were probed using an ^{125}I-conjugated second antibody. ^{125}I-labelled sheep anti-mouse Ig (whole molecule) (Amersham, U.K.) was used against McAb immunoblots and ^{125}I-labelled donkey anti-rabbit Ig (whole molecule) (Amersham, U.K.) was used to probe rabbit sera blots. Approximately $1.5x10^6$ counts per minute (cpm) of radiolabelled conjugate were diluted in 20mls of washing buffer and incubated with each blot for 90 mins on a rocking platform at room temperature.

Strips of NCP were washed at least 4 times in washing buffer, dried, mounted onto cardboard, with radioactive ink marking the origin, and then covered in cling film.

2.12.3 Autoradiography

Blots were exposed to X-Omat S X-ray film (Kodak, Hemel Hempstead, U.K.) in X-Omatic intensifying screens (Kodak) at $-70^{o}C$ for 3 to 28 days.

Films were developed under safe-light illumination for 2 mins in 1 in 5 Phenisol (Ilford, Mobberley, U.K.), washed for 30 seconds in 2% glacial acetic acid, fixed for 2 mins in 1 in 5 Hypam (Ilford), rinsed extensively in water and then air-dried.

2.13 Restriction Endonuclease (RE) Analysis of Viral DNA

2.13.1 Extraction of ^{32}P-labelled DNA

The following method was adapted from Darville (1983) and was used for the extraction of small amounts of viral DNA.

2.13.1.1 Labelling of Viral DNA

A 24-well tissue culture plate (Beckton Dickinson and Co., New Jersey, USA) was seeded with Vero cells at $1x10^5$ cells/ml in 1ml medium per well and incubated at 37°C in 5% CO_2. On reaching confluency the medium was removed from each well using a vacuum pump fitted to a sterile Pasteur pipette. The cells were then starved of phosphate overnight by incubation in Minimum Essential Medium Eagle (modified), (Flow, Irvine, Scotland) supplemented with 2mM glutamine, 2% sodium bicarbonate, 10mM Hepes buffer (Gibco), 2% FCS, 100u/ml penicillin and 100µg/ml streptomycin.

The phosphate-free medium was then removed and 100µl of virus added to the wells at an moi = 0.01-0.1 (approximately $3x10^3$-$3x10^4$ pfu). The virus was left to adsorb for 1 hr at 37°C before 400µl of warmed

phosphate-free medium was added and the plate was incubated for a further 2 hrs. After this time the medium was removed from the wells and replaced with 500μl of phosphate-free medium containing 0.05μCi/μl ^{32}P-orthophosphate (Amersham, U.K.). The plate was then incubated for approximately 48 hrs by which time confluent infection had occurred.

2.13.1.2 **Harvest of Viral DNA**

250μl of 5% w/v SDS was added to each well of the 24-well plate and the contents transferred to 1.5ml microcentrifuge tubes (Eppendorf, Hamburg, Germany). This was then extracted with an equal volume of phenol (BDH) saturated with 10mM tris pH 8.0. The phenolic layer was discarded and the extraction repeated until the interface between the two layers was clear.

The DNA was precipitated by adding 2 volumes of absolute ethanol to the aqueous layer and standing at -20^{o}C overnight. The tubes were then centrifuged for 5 mins at 11600g in a microcentrifuge (Microcentaur, MSE). The ethanol was removed and the pellets dried under vacuum before redissolving the DNA in 150μl of distilled water containing 100μg/ml RNaseA (Sigma). The tubes were incubated at 37^{o}C for 1 hr before storage at 4^{o}C.

30μl DNA was used for each RE digest (section 2.13.4).

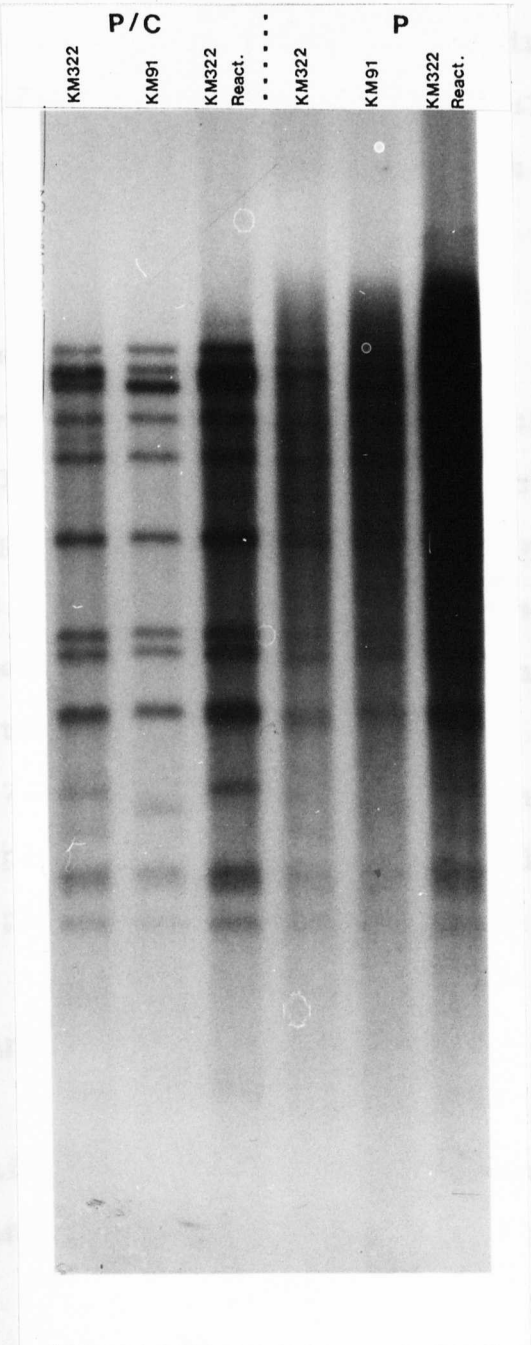
2.13.1.3 A Comparison of Two DNA Extraction Methods

The above method for DNA extraction was found to be improved by the use of phenol dissolved in 24:1 chloroform:isoamylalcohol and by the addition of 8-hydroxyquinoline (Sigma) to inhibit the oxidation of phenols and remove divalent cations. 100g phenol and 0.1g 8-hydroxyquinoline were dissolved in 100ml chloroform/isoamylalcohol saturated with 10mM tris pH 8.0 and used for extractions as before. Finally the preparations were extracted with chloroform/isoamylalcohol to remove traces of phenol before alcohol precipitation of the DNA (fig.2.1).

2.13.2 Second Method for Extraction of Viral DNA

As in the ^{32}P method above this method was used to extract small amounts of DNA. It replaced the ^{32}P method because of its increased reliability and it negated the need for use of a radioactive isotope as gels were stained with ethidium bromide (EtBr) (Sigma).

Figure 2.1: A comparison of methods for extracting αHVS DNA using phenol (P) and phenol-chloroform (PC). DNA from KM91, KM322 and an isolate of KM322 reactivated from DRG explant cultures was digested with HindIII.



Vero cells in 3cm petri dishes were infected with virus at an moi = 0.01-0.1 and incubated to 100% cpe. The infected cells were then harvested, transferred into microcentrifuge tubes and freeze-thawed 3 times before centrifugation for 30 mins at 4°C in a microcentrifuge. The supernatant was discarded and the pellets resuspended in 180µl TE buffer (10mM tris-HCL, pH 8.0, and 1mM ethylenediamine tetraacetic acid, EDTA). To this 10µl of 10% SDS and 10µl of 10mg/ml proteinase K (Sigma) were added and the tubes were incubated at 37°C for at least 4 hrs.

The samples were extracted with phenol-chloroform-isoamylalcohol and then with chloroform-isoamylalcohol as described in section 2.13.1.3. The DNA was precipitated at -20°C overnight by the addition of 1/10 volume 3M sodium acetate and 2 volumes of absolute ethanol. The tubes were centrifuged for 15 mins in a microcentrifuge, the supernatant removed and the pellets washed gently in 70% ethanol and then dried under vacuum. The DNA was resuspended in 30µl TE buffer and 10µl of each preparation used per RE digest (section 2.13.4).

2.13.3 Batch Extraction of Herpesviral DNA

Where large quantities of viral DNA were required ie. for RE site mapping and for Southern blotting experiments the

following method was used for DNA extraction.

Vero cells in 2 Roux bottles per virus strain were infected with αHVS at an moi of 0.01 to 0.1 and incubated to 100% cpe. Adherent cells were scraped into the medium and the cell debris was pelleted by centrifugation at 5000 RPM for 15mins at 4^{o}C in a Beckman J-6 centrifuge. The supernatant was kept on ice and the cells were resuspended in 10ml TE buffer and centrifuged at 3000 RPM for 10 mins in an MSE Centaur 1 centrifuge. The supernatant was combined with the first and the cell pellet kept on ice.

The supernatants were centrifuged for 1 hr at 50,000g in an 8x50ml fixed angle rotor on an MSE Superspeed 75 ultracentrifuge. The supernatant was then discarded and the virus pellet washed gently in TE buffer before resuspending in 5ml TE. The cell pellet was combined with the virus suspension, vortexed and the volume carefully measured. The volume was made up to 9.3 ml using TE buffer and then 0.6ml 10% SDS and 0.1ml 20mg/ml proteinase K were added, the mixture swirled gently and then incubated at 37^{o}C for at least 4 hrs.

A 57% w/v caesium chloride solution (CsCl) (Sigma) was prepared by adding 13.26g CsCl to 10ml lysate and dissolving by gentle agitation. The solution was then

centrifuged at 2000 RPM in an MSE Centaur 1 for 20 mins. The precipitated SDS formed a plug at the top of the tube which was removed with a Pasteur pipette. The clear solution was added to a 13ml polyallomer centrifuge tube (Beckman) and centrifuged at 20°C for 68 hrs at 80000g in a SW41 rotor on an L5-65 ultracentrifuge (Beckman).

The gradient was collected in 10 drop fractions using a Varioperpex II pump (LKB, Milton Keynes, U.K.) and the refractive index of every 5th tube was determined using an Abbé refractometer (Bellingham and Stanley Ltd., London, U.K.). Fractions in the refractive index range 1.403 to 1.401 were stained with EtBr to test for the presence of viral DNA. 5μl spots of each fraction were mixed with 5μl of 1μg/ml EtBr on a piece of cling film stretched over a ring frame and viewed under ultra-violet (UV) illumination. Fractions showing fluorescence were dialysed against TE for 2 days at 4°C with frequent changes of buffer.

The concentration and purity of each preparation was assessed by measuring the optical densities (O.D.) at 260nm and at 280nm. A ratio of $OD_{260}:OD_{280}$ = 1.8 to 2.0 signifies purity, less than this usually implies protein or phenol contamination. At 260nm an OD=1 is equivalent to 50μg/ml double stranded DNA, and this was used to calculate the concentration of DNA in each sample.

Samples were digested with enzymes and run on agarose gels to confirm purity (section 2.13.4). All DNA preparations were stored at 4°C.

2.13.4. Restriction Endonuclease Digestion of Herpesviral DNA

1μg of DNA was digested with 10 units of restriction endonuclease in 1x incubation buffer as supplied by the manufacturer (International Biotechnologies Inc., Cambridge, U.K.) in a total volume of 40μl. Lambda DNA (Sigma) digested with HindIII was included on each gel as a molecular weight marker. After 2 hrs incubation at 37°C 10μl stop buffer (0.1M EDTA, 0.06% bromophenol blue (Sigma), 30% glycerol) was added to each tube and the DNA fragments were loaded into the wells of 0.6% agarose gels (ICN, High Wycombe, U.K.). Electrophoresis was performed at 60V overnight at 4°C for full size gels and at 100V for 3 hrs at room temperature for minigels. The electrophoresis buffer consisted of 36mM tris, 30mM sodium dihydrogen orthophosphate and 10mM EDTA (pH 8.4) using a horizontal immersion electrophoresis unit (Bethesda Research Laboratories, Paisley, Scotland).

Radioactive gels were dried onto glass plates at 80°C and autoradiographed overnight (section 2.12.3). Other gels

were stained for 30 mins in 1μg/ml EtBr and photographed under UV illumination.

2.14 Southern Blotting

2.14.1 Preparation of Probes

Plasmids containing fragments of HSV-1 (strain KOS) DNA were kindly supplied by D.A. Leib, Dana-Farber Cancer Institute, Boston, U.S.A.

In order to separate probe DNA from vector DNA approximately 5μg aliquots of each plasmid were incubated for 2 hrs at 37°C with the appropriate REs in the recommended incubation buffer. Fragments were separated on 0.8% low melting point agarose (Sigma) minigels for 3 hrs at 100V.

The gel was examined on an ultra-violet transilluminator and the required bands were excised with separate scalpels and placed into pre-weighed microcentrifuge tubes. The weight of DNA and agarose was determined and distilled water was added to each tube at 1.5ml per 1g. The samples were heated at 100°C for 5 mins to melt the agarose and denature the probe DNA before storage at -20°C.

2.14.2 Blotting Agarose Gels

DNA to be probed was digested with appropriate REs, run on minigels, stained and photographed as described previously (section 2.13.4). Excess agarose was trimmed off the sides of the gel and it was then soaked in 250mM HCL for 15 mins to depurinate the DNA and thus facilitate the transfer of larger pieces of DNA from gel to nitrocellulose filter.

The DNA was denatured by soaking the gel in 1.5M NaCl, 0.5M NaOH for 2x15 mins. This was followed by neutralisation in 1M tris-HCL (pH 8.0), 1.5M NaCl for 2x15 mins. Finally the gel was soaked in 20xSSC (3M NaCl, 0.3M tri-sodium citrate, pH7.0) for 5 mins. Six pieces of Whatman 3mm paper and one piece of nitrocellulose paper (Schleicher-Schuell) all cut to the size of the gel were also soaked in 20xSSC.

The blot sandwich was assembled on the top of a glass plate. Three soaked Whatman filters were placed underneath and the gel layed on top, next the NCP was placed on the gel taking care to exclude any air-bubbles from between the two. Three more wet filter papers were layered on top followed by 6 dry papers also cut to size. A packet of paper handtowels was placed on top of these and finally a weight of approximately 500g.

In this way the buffer within the agarose gel would be drawn upwards through the NCP into the dry absorbent filters and towels and the DNA fragments would be carried simultaneously as far as the NCP where the DNA would bind to the filter. The blot sandwich was left overnight until the agarose gel was completely flattened. The NCP was then removed and sealed between 2 pieces of Whatman 3mm and baked for 2 hrs at 80^{o}C in a vacuum oven. The baked NCP was stored at room temperature until required.

2.14.3 Labelling the Probe

The probe was boiled for 5 mins to denature the DNA and then equilibrated to 37^{o}C for at least 10 mins. The probe was radioactively labelled by synthesising complementary DNA strands in the presence of deoxycytidine 5'-[α-^{32}P] triphosphate (triethylammonium salt. Amersham, U.K.). This was performed using a Random Primed DNA Labelling Kit (Boehringer Mannheim, Lewes, U.K.). 13.5μl of probe was added to 3μl of a mixture of dATP, dTTP and dGTP, 2μl of reaction mix (consisting of random hexanucleotides for priming DNA synthesis), 1μl of the Klenow fragment of DNA polymerase and 2.5μl of ^{32}P-labelled dCTP (at 10mCi/ml). The labelling mix was incubated at room temperature for at least 5 hrs. The reaction was then stopped by the addition of 5μl of 250mM EDTA.

2.14.4 Hybridisation of the Nitrocellulose Filter

The NCP to be labelled was pre-hybridised at 65°C for at least 3 hrs in 20ml of 6x SSC, 0.5% SDS, 100µg/ml denatured calf thymus DNA (Sigma) and 5 x Denhard's solution (0.1% bovine serum albumin (Sigma), 0.1% polyvinyl pyrolidone (Sigma) and 0.1% ficoll (Sigma)). The pre-hybridisation solution was then replaced by 20mls of hybridisation solution consisting of 6xSSC, 0.5% SDS, 100µg/ml denatured calf thymus DNA, 5x Denhard's solution and 50% v/v deionised formamide (BDH). The labelled probe was denatured for 5 mins at 100°C and added to the hybridisation solution.

The filter and probe were incubated overnight at 30°C ie. under low stringency conditions for DNA-DNA hybridisation.

2.14.5 Post-Hybridisation Washes

The hybridisation solution was discarded and the filter washed in 6x SSC for 4x15 mins at 4°C, and for 2x10 mins at room temperature. The filter was then dried onto a piece of Whatman 3mm paper, covered in cling film and autoradiographed overnight (section 2.12.3).

Under the above low stringency hybridisation and wash

conditions it was generally found that the probe hybridised to most of the DNA, including the negative control. The stringency was therefore increased by successive washes to the same filter at higher temperatures and with SDS in washing solutions.

The second set of washes were 0.5% SDS, 2xSSC for 5 mins at room temperature, followed by 0.1% SDS, 2xSSC for 15 mins at room temperature and finally 0.5% SDS, 0.1 xSSC for 30 mins at 37^{o}C. Stringency was increased further with the third wash; 0.5% SDS, 0.1 xSSC for 2x20 mins at 42^{o}C and finally by a fourth wash with 0.5% SDS, 0.1xSSC for 30 mins at 65^{o}C.

At each stage filters were dried down and autoradiographs taken.

CHAPTER 3: INVESTIGATION OF NEUROVIRULENCE DETERMINANTS OF αHVS THROUGH REPLICATION IN CELL CULTURE

3.1 Introduction

Neuroinvasiveness and neurovirulence in alphaherpesviruses have been the subject of many studies, particularly involving herpes simplex viruses. The regulation of neurovirulence in HSV is the result of a complex relationship between virus and host. Such factors as host age (Ben-Hur et al, 1983; Yamada et al, 1986a) and route of inoculation (Richards et al, 1981) can have a significant effect on viral pathogenesis. In addition to host factors virulence varies with virus strain, and in HSV neurovirulence has been shown to be determined multigenically (Halliburton et al, 1987; Javier et al, 1986; Sedarati and Stevens, 1987).

The viral thymidine kinase (TK) gene has been shown to be required for HSV neurovirulence in animal models (Field and Wildy, 1978; Ben-Hur et al, 1983; Chrisp et al, 1989) and for αHVS in mice (Kit et al, 1983). However, recent work has revealed an exception to this general case (Izumi and Stevens, 1988). Mutations in the DNA polymerase gene can also affect virulence following peripheral inoculation in murine models (Larder et al, 1986; Day et al, 1988).

In addition, other as yet unidentified genes have been shown to contribute to neurovirulence in various models

(RÖsen et al, 1986; Javier et al, 1988a; Thompson et al, 1989; Taha et al, 1989a). Such studies have generally identified mutants that show an increase or decrease in neuroinvasiveness on peripheral inoculation (Thompson et al, 1986; Izumi and Stevens, 1988; Goodman et al, 1989) and/or mutants exhibiting differences in neurovirulence on intracranial inoculation (Thompson and Stevens, 1983; Ben-Hur et al, 1987).

Previous studies on the two strains of alphaherpesvirus saimiri (αHVS), KM91 and KM322, studied in this project have compared a number of their molecular, pathogenic and in vitro biological characteristics (Leib, 1986; Leib et al, 1987a, 1988).

The most striking finding from these studies is the difference in neurovirulence when each virus is injected intradermally into the flank of rabbits. KM322 replicates at the periphery, enters the nerve endings and travels intraaxonally from the inoculation site to the dorsal root ganglia (DRGs) supplying nerves to the infected dermatomes. Once in the DRGs replication occurs with subsequent neuron damage, as evidenced by the development of anaesthetic areas around the inoculation site (Tosolini et al, 1982) and electron microscopic evidence of axon degeneration (Illanes, 1988; Illanes et al, 1989a and b). KM322 then

becomes established in a latent state within the DRGs and the acute infection is cleared by the host's immune response by 12 days post-inoculation. The virus has been detected in the spinal cord adjacent to infected DRGs, though it may not replicate within the cord, and the infection appears to be limited to this area (Tosolini et al, 1982; Leib, 1986).

KM91 also replicates in the rabbit skin producing erythematous lesions of equal size to those caused by KM322, ie. approximately 12mm (Tosolini et al, 1982; Leib, 1986), and it also travels centripetally along axons to the DRGs where replication occurs. At this stage the similarity between the two viruses ends as KM91 ascends the spinal cord and infects the brain stem, cerebellum and cerebral hemispheres, and animals die of encephalitis within an average of 10 days post-inoculation (Leib, 1986; Leib et al, 1988).

It would appear that KM322 has high neurovirulent potential since when inoculated directly into the brains of rabbits it replicated and killed 5 out of 6 rabbits (Tosolini et al, 1982). Also in rabbits immunosuppressed with cortisone acetate KM322 successfully invaded the central nervous system (CNS) and subsequently produced encephalitis (Leib, 1986). Thus KM322 is apparently different from KM91 in its

lack of neuroinvasiveness, since following peripheral inoculation even with very high titres (10^6 pfu) it is limited to the peripheral nervous system (PNS) while KM91 (with inocula as low as 10pfu) invades the CNS.

The experiments in which KM322 was inoculated intracerebrally into rabbits produced 5 out of 6 fatalities as compared to invariable death in rabbits injected intradermally with KM91. Indeed 3pfu of KM91 has been shown to kill a rabbit when injected into the flank (Leib, 1986). This anomaly between the two virus strains and the finding that viral titres in the brain were 40x lower in immunosuppressed rabbits infected with KM322 than in KM91-infected rabbits (Leib, 1986) warranted further study.

In order to complement the above experiments the replication of αHVS in a neuronal cell line (Neuro 2a) was investigated to further address the question of whether KM322 was restricted in its replication in nervous tissues relative to KM91. αHVS replication in Vero cells was also examined in order to determine whether any putative replication defect in KM322 might also apply to cells other than those of neuronal origin. That this might be the case is suggested by the fact that titres in the skin were lower in immunosuppressed rabbits infected intradermally with KM322 than in KM91-infected rabbits. However Leib *et al*,

(1987a) compared one step growth curves of the two αHVS strains in Veros and suggested that there was no difference in this respect between KM91 and KM322.

The interaction of HSV with neuroblastoma cell lines has been the subject of various investigations (Lancz and Zettlemoyer, 1976; Vahlne and Lycke, 1978; Vahlne et al, 1981; Ash, 1986; Kemp and Latchman, 1989). These have been concerned with the nature of the restriction of these cells on HSV replication and how it might relate to latency in neuronal tissues.

However, the murine neuroblastoma cell line Neuro 2a, derived from C1300 cells, is permissive to HSV infection and was therefore chosen for this experiment. In addition C1300 cells in vitro maintain several characteristics of undifferentiated neurons (Augusti-Tocco and Sato, 1969; Schubert et al, 1969) and therefore represent a good model for virus-neuron interaction. Day et al, (1988) found a 1000x reduction in titre between a non-neuroinvasive HSV-2 strain and a neuroinvasive intertypic recombinant when grown in Neuro 2a cells, while the two strains grew to comparable titres in Vero cells.

Comparison of KM91 and KM322 by kinetic neutralisation (McCarthy, unpublished data), restriction endonuclease

digest patterns, polypeptide immunoprecipitation and growth characteristics in tissue culture (Leib, 1986; Leib et al, 1987a) showed no significant differences between the two strains. Indeed the only potential virulence marker to arise from these studies was plaque size. KM91 showed a significantly larger mean plaque area in Vero cells than KM322, though the range of areas in the former was also larger and overlapped that of KM322 (Leib, 1986). Since this provided the only obvious virulence marker, though not necessarily virulence determinant, it was investigated further.

Plaque size has been correlated with virulence in a number of systems. Yamada et al (1986b) and Ben-Hur et al (1987) found neurovirulence of HSV-1 variants for mice to be linked to plaque size. However, in both cases increased plaque size was accompanied by a change in plaque morphology from non syncytial to syncytial. Daniel and Melendez (1970) showed that a large plaque variant (LPV) of αHVS (formerly Herpes T-virus) exhibited virulence in rabbits, mice, hamsters and embryonated eggs whereas a small plaque variant (SPV) was found to be avirulent. The LPV also showed a greater capacity to form syncytia in a number of cell lines. Other groups have shown formation of syncytia to be correlated with virulence (Kohlhage and Siegert, 1962; Benda and Cinátl, 1966; Dix et al, 1983;

Weise et al, 1987), but in the case of KM322 and KM91 both plaque morphologies are syncytial.

Ben-Hur et al (1983) showed plaque size, without change in morphology, to be related to neurovirulence of HSV-1 injected into mouse corneas. However, they attributed smaller plaque size to be due to decreased TK production in this case and KM322 and KM91 have both been shown to produce TK to high levels (Leib, 1986).

Vogt et al (1957) found plaque size reduction to be associated with attenuation in poliomyelitis viruses. Rapp (1963) found large plaque variants of HSV to be associated with virulence for rabbits and mice, but this was not the case for small plaque variants.

The parental stocks of KM91 and KM322 had no recent history of plaque-purification, therefore plaque-picking was performed to investigate whether variation in plaque size was stably inherited or whether the stocks represented mixed populations. Any stable large and small plaque variants could then be investigated for differences in pathogenicity for rabbits and in vitro growth characteristics.

To summarise, the difference in neurovirulence for rabbits

between KM91 and KM322 was investigated by experiments comparing plaque sizes in Vero cells and virus yields in neuroblastoma cells and Vero cells. The aim of these experiments was to determine whether differences in *in vitro* replication are associated with the diverse *in vivo* pathogenicity of the two strains.

3.2 Materials and Methods

3.2.1. Replication of αHVS in Neuro 2a and Vero Cells

Replication of αHVS in neuroblastoma cells and Vero cells was investigated by titration on Vero cells of virus grown in each cell line.

Neuro 2a cells and Vero cells were seeded into 24-well tissue culture plates and incubated at 37°C in 5% CO_2 to confluency (section 2.1.1 and 2.1.2). Five confluent wells of each cell type were trypsinised (section 2.1.1) and cell counts performed in a counting chamber to obtain mean numbers of cells per well.

Stocks of KM322 and KM91 were diluted to approximately 1.75×10^5 pfu/ml as starting inocula (10^0) and subsequent 10^{-4} dilutions were made from these. Both concentrations of each virus were then inoculated onto wells of Vero cells

and Neuro 2a cells at 100μl/well and 6 wells per concentration.

At the same time confluent tubes of Vero cells were inoculated with the 10^{o} inoculum of each virus in order to confirm that the two strains were at approximately the same concentration.

At day 1 and day 2 post-inoculation 3 wells per dilution of each virus were harvested and stored at -70^{o}C for subsequent titration on Vero cells in tubes (section 2.4). Results were analysed for significance using Student's t-test.

3.2.2 αHVS Plaque Size Measurements

Vero cells in petri dishes were infected with KM91 and KM322 and overlayed as described in section 2.3. After incubation for 48 hrs plaque sizes were measured using an eyepiece graticule in an inverted microscope, calibrated with a stage micrometer. Each plaque area was calculated from the mean of 3 diameters, using the equation area = $\pi d^2/4$. Thirty plaques were measured per virus strain.

The ranges of plaque diameters measured for each virus were divided into 3 groups of equal range: "large",

"intermediate" and "small". Ten plaques from the "large" and "small" groups for each strains were then picked and frozen in medium at -70°C as described in section 2.3.

Three "small" and 3 "large" plaques from each of the two αHVS strains were then plaque-purified 2 more times, picking off plaques of the appropriate size each time.

Final determinations were made by measuring 30 plaques from each of 3 "large" and 3 "small" plaque-purified stocks of KM322 and KM91 and also 30 plaques from the original parent stocks.

Results were analysed using a one-way analysis of variance.

Plaque-purified stocks were compared with parent stocks by RE digestion using HindIII, KpnI and BamHI (section 2.13.1 and 2.13.2).

Large scale stocks of each strain were then prepared from 3x plaque-purified virus; after passaging once in petri dishes of Vero cells to increase the titre before final preparation in 12oz bottles (section 2.2.2). Plaque-purified virus was used for all subsequent experiments in this thesis.

The plaque-purification technique was controlled to ensure that agarose cylinders contained only virus released from the adjacent plaque. In each of the first 10 petri dishes infected swabs were taken from the surface of the overlay and agarose cylinders, adjacent to non-infected cells but close to plaques, were assayed for infectious virus by inoculation onto Vero cells. In all cases such controls were negative.

3.3 Results

3.3.1 Replication of αHVS in Neuro 2a and Vero cells

KM91 and KM322 were inoculated into Neuro 2a and Vero cells at 2 different doses and were harvested at 24 hrs and 48 hrs. The infecting doses were determined from cell counts and back-titrations of inocula and are shown in table 3.1. The resultant yields are summarised in table 3.2.

Cell Counts

Mean no. Vero cells/well = 2.49×10^5

Mean no. Neuro 2a cells/well = 1.97×10^5

Back-Titrations of Inocula

KM91: 1.73×10^5 pfu/ml

KM322: 1.79×10^5 pfu/ml

Table 3.1. Virus dose expressed as pfu values, derived from Vero cell titrations, per cell inoculated.

Virus	Virus Dilution	Neuro 2a Cells	Vero Cells
KM322	10^{0}	$9x10^{-2}$	$7x10^{-2}$
	10^{-4}	$9x10^{-6}$	$7x10^{-6}$
KM91	10^{0}	$9x10^{-2}$	$7x10^{-2}$
	10^{-4}	$9x10^{-6}$	$7x10^{-6}$

Table 3.2. Mean virus yields (pfu/ml) from KM91 and KM322 grown in Vero cells and Neuro 2a cells. Each value represents the mean from 3 virus cultures titrated separately on Vero cells (5 tubes per dilution).

Harvest Time & Virus Dilution	Virus Strain and Cell Type: KM91 Neuro 2a	KM322 Neuro 2a	KM91 Vero	KM322 Vero
24hrs				
10^{-4}	0	0	1.01×10^{1}	3.02×10^{1}
10^{0}	2.27×10^{5}	1.71×10^{6}	4.76×10^{6}	3.07×10^{6}
48hrs				
10-4	6.27	5.73	5.13×10^{4}	3.95×10^{3}
10^{0}	4.33×10^{6}	3.17×10^{6}	3.01×10^{7}	9.84×10^{6}

Yields of each strain grown in each cell line were compared by Student's t-test. Differences in virus yields between Neuro 2a and Vero grown virus and between KM322 and KM91 grown in each cell line were significant in all cases ($P<0.001$) except between KM322 and KM91 in Neuro 2a cells with a 10^{-4} inoculum at 24hrs and 48hrs harvest time.

The results show that there is no significant difference between KM91 and KM322 titres in Neuro 2a cells at $9x10^{-6}$ dose, but results from $9x10^{-2}$ inoculum suggest that KM322 initially replicates to a greater titre, but by 48 hrs KM91 shows the greatest yield. Yields of KM91 compared to KM322 in Vero cells show the former to produce the greater titres in all cases except with the $7x10^{-6}$ inoculum harvested after 24 hrs.

KM91 virus titres in Vero cells were always greater than those from Neuro 2a cells at either dose and at either harvest time. This was also the case for KM322 grown in each cell line.

3.3.2 Plaque Size Measurements

The plaque measurements from parent stocks of αHVS and from 3x plaque-purified "large" and "small" plaque isolates in Vero cells are summarised in table 3.4.

Table 3.4: Mean plaque areas (mm^2) of KM91 and KM322 and "large" and "small" plaque isolates from each parent stock. Each value is calculated from the mean of 3 diameters from 30 plaques.

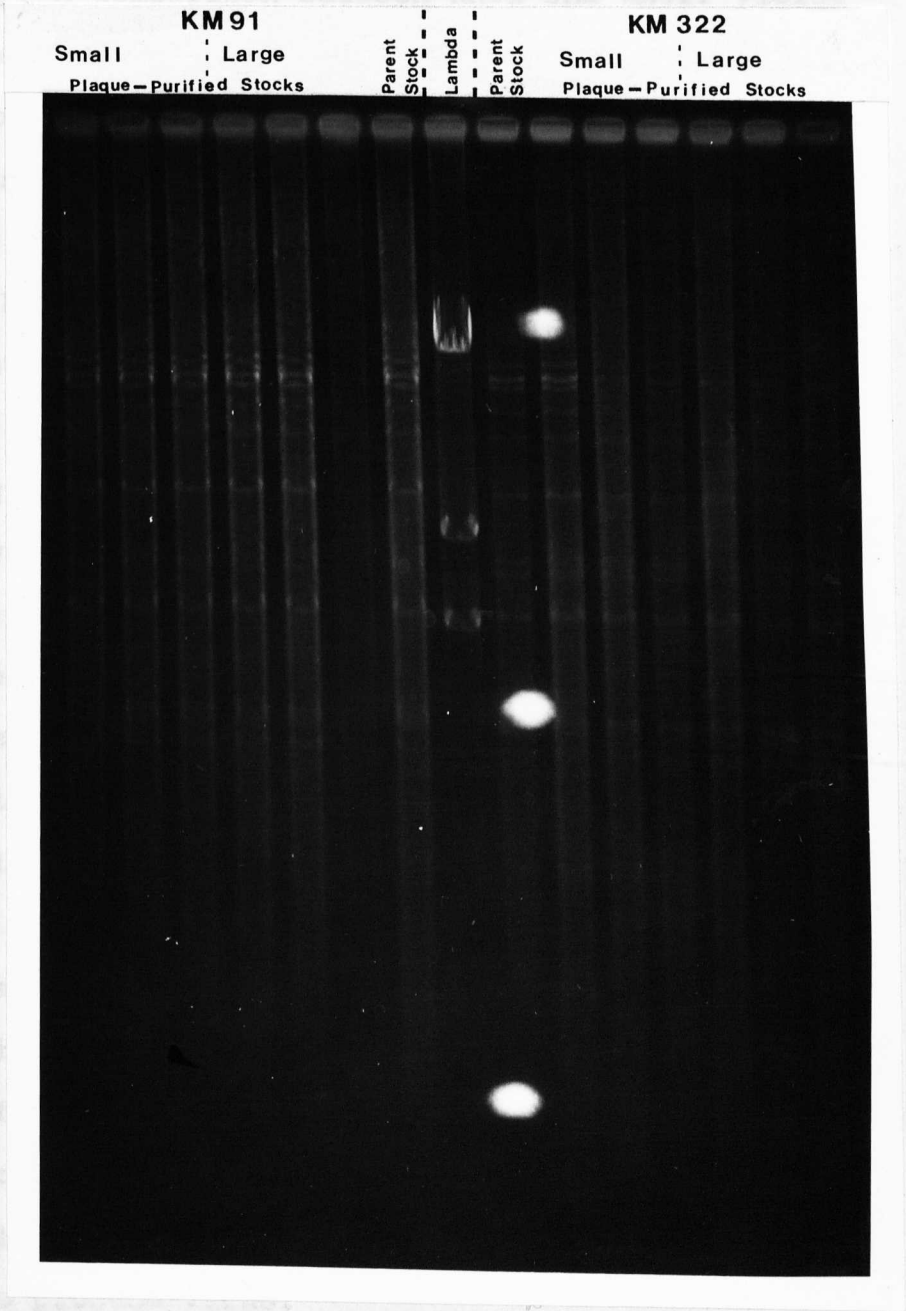
Virus Stock	Mean & Standard Deviation Plaque Areas (mm^2)			
	KM91		KM322	
	Mean	S.D.	Mean	S.D.
Parent	0.49	0.19	0.33	0.09
"Large"	0.48	0.15	0.39	0.11
"Large"	0.58	0.19	0.39	0.17
"Large"	0.55	0.17	0.32	0.15
"Small"	0.53	0.18	0.31	0.12
"Small"	0.57	0.16	0.33	0.11
"Small"	0.51	0.19	0.36	0.10

One-way analysis of variance performed on raw data revealed a significant difference between KM91 and KM322 plaque sizes ($P<<0.01$). Comparisons between "large" plaque areas and parent populations, "small" plaque areas and parent populations and "large" and "small" plaque areas within each αHVS strain revealed no significant difference in size ($P>0.05$).

The range of plaque areas in KM91 ($0.15mm^2$ to $1.02mm^2$) was larger than that in KM322 ($0.09mm^2$ to $0.68mm^2$). This observation is shown by the larger standard deviation values for KM91 mean areas in table 3.4.

RE profiles of parent virus and plaque-purified stocks showed no differences for KM91 or KM322 digested with HindIII, KpnI or BamHI (fig.3.1).

Figure 3.1: DNA extracted from plaque-purified "large" and "small" plaque isolates of KM91 and KM322 and parent virus stocks digested with HindIII.



3.4 Discussion

There is no significant difference between yields of KM91 and KM322 harvested from Neuro 2a cells inoculated with a dose of $9x10^{-6}$. A comparison between KM91 and KM322 yields in Neuro 2a cells with a dose of $9x10^{-2}$ suggests that KM322 initially replicates to a greater titre, but at 48 hrs KM91 shows a greater yield. This result may be explained by the fact that the initial KM322 inoculum was the larger, but by 48 hrs a greater replication efficiency in KM91 counteracted this early effect.

On comparing the yields of the two virus strains in Vero cells KM91 was found to replicate to greater yields except with a 10^{-4} inoculum harvested 24 hrs post-inoculation. As mentioned above, this result is thought to reflect the larger infecting dose of KM322, while the overall conclusion is that KM91 shows a greater replication efficiency in Vero and Neuro 2a cells.

Both virus strains replicate to greater titres in Vero cells than in Neuro 2a cells at each inoculum tested and at each time of harvest. This result may reflect the fact that cell counts in Vero cells were greater per well than Neuro 2a cells and therefore there were more cells available to produce virus.

All inocula were at an moi less than 1, therefore in theory cell numbers should not affect the results during the first cycle of replication, assuming that virus particles are evenly distributed within the inoculum. However by 24 hrs and 48 hrs post-inoculation secondary rounds of replication will be occurring (Leib, 1986, Leib et al, 1987a) and therefore the number of cells available for infection will influence virus yield. Such an effect would be expected to decrease with a decrease in the moi of virus inoculated. This is not the case (table 3.3) therefore it must be assumed that replication in Vero cells is more efficient for αHVS, KM322 and KM91, than in Neuro 2a cells.

Neuro 2a cells are derived from a clone of C1300 neuroblastoma cell line. Vahlne and Lycke (1977 and 1978) have shown C1300 cells to be restricted for HSV infection. The nature of this restriction is unknown, but it may be retained, to a lesser extent, by Neuro 2a cells. It is a common phenomenon for alphaherpesviruses to replicate with varying efficiencies in cells of differing phenotype.

The results presented here suggest that the lack of neurovirulence of KM322 may be due in part to a reduced replication efficiency, not necessarily specific to the CNS. This agrees with the findings mentioned in the introduction to this chapter whereby tissues sampled from

immunosuppressed KM322-infected rabbits contained lower titres of virus than tissues from KM91-infected rabbits (Leib, 1986), and fewer rabbits died from intracranial KM322 inoculation than animals inoculated intradermally with KM91 (Tosolini *et al*, 1982).

The results of one-step growth curves of KM91 and KM322 in Vero cells led Leib *et al* (1987a) to suggest that there was no sufficient difference between the replication of the two strains in Vero cells to explain the variation in neurovirulence. However, this disagreement is possibly due to the fact that no statistical significance was determined for the different virus yields.

In many cases statistical significance was found for yields of less than a 10 fold difference. It is not known whether differences of such a magnitude would be biologically significant within a host, but it is thought likely that any replication defect exhibited by KM322, relative to KM91, *in vitro* would have a greater significance *in vivo*.

It has been suggested that replication *in vivo* puts more stringent demands on viral gene functions. Thus Larder *et al* (1986) found that a mutation in the DNA polymerase of HSV-1 did not affect titres on replication in tissue culture, but it did reduce the neuroinvasiveness of the

virus in mice. In addition the viral TK gene is not essential for HSV replication in tissue culture, but has been shown to be a requirement for neurovirulence (Field and Wildy, 1978; Ben-Hur et al, 1983; Chrisp, et al, 1989). Subtle differences between KM322 and KM91, for example in the promoter of a vital gene, may exhibit a more pronounced effect on replication in vivo than in cell culture. This may apply particularly to non-replicating tissue such as neurons.

In order to clarify whether KM322 and KM91 replicate to significantly different titres in vivo, rabbit skin and DRGs from animals infected intradermally and brains from rabbits infected intracerebrally would have to be removed, homogenised and titrated on Vero monolayers to quantify virus per gramme of tissue. Such direct comparisons between immunocompetent animals infected by the same routes have never been performed.

However, such speculation as to the ability of KM322 to replicate within the rabbit may prove to be relatively insignificant as a determinant for reduced virulence. Previous experiments by Leib (1986) have suggested KM322 to be non-neuroinvasive due to its efficiency in alerting an immune response in the host. Indeed, if KM322 and KM91 are co-inoculated at the same site or at different sites the

response generated by KM322 is sufficient to neutralise KM91 (see introduction to chapter 5).

Any possible replication defect in KM322 would allow more time for an infected host to elicit an immune response, but in this system the co-inoculation experiments described above show that KM322 positively protects rabbits against KM91 inoculation. This suggests a role for the putative protective factor in the regulation of virulence on peripheral inoculation.

The plaque-purification of "small" and "large" plaques from KM322 and KM91 parent stocks and subsequent area measurements have revealed that the range of plaque area within each population is a stably inherited phenomenon and that it does not signify a mixed population of small and large plaque variants. This is further confirmed by the identical RE profiles of plaque-purified virus and parent stock for KM91 and KM322.

It was therefore considered unnecessary to perform inoculations of plaque-purified virus into rabbits to determine their neurovirulence. It was assumed that since *in vitro* characteristics were maintained *in vivo* behaviour would also resemble that of the parent stock. However, recently Taha *et al* (1988) have shown that plaque isolates

from a "pure" stock of HSV-2 exhibited varying neurovirulence in mice, but showed the same RE profiles with a range of enzymes. In the light of this information it might prove informative to perform *in vivo* experiments on αHVS plaque isolates.

KM91 mean plaque size is significantly larger than that of KM322, in agreement with the findings of Leib (1986). This situation resembles that of Daniel and Melendez (1970) whereby a large plaque variant (LPV) of αHVS exhibited greater neurovirulence in a range of host animals than a small plaque variant (SPV). However, their variants also varied in syncytial development and thermostability (Daniel and Melendez, 1968) while KM91 and KM322 do not differ in these respects (Leib, 1986).

The ranges of plaque sizes measured in these experiments differ from those determined for the same αHVS strains by Leib (1986). This is likely to be due to physiological variations in the Vero cells used in each set of experiments. Youngner (1956) showed that temperature caused variation in plaque sizes of poliomyelitis, herpes-B and vaccinia viruses and Vogt *et al* (1957) showed pH to affect the plaque size of polio viruses. For this reason all measurements for direct comparison were made on the same day. It is possible that the greater range of plaque

sizes exhibited by KM91 may be due to a greater sensitivity to local physiological variations in the Vero cell monolayer.

The results presented in this chapter showing KM91 yields in Vero cells to be significantly greater than those of KM322 suggest that the difference in plaque size may be related to differing replication efficiencies in Vero cells. The greater mean plaque size of KM91 may be due to a number of factors such as the structure or regulation of enzymes directly involved in virus replication or due to a more rapid adsorption of virus to cell or to more efficient cell to cell spread. The latter two processes are mediated by surface glycoproteins in herpesviruses (Spear, 1985). A difference in glycoprotein structure between KM91 and KM322 may provide a common link between plaque size and the diverse immune response generated in rabbits.

The biological significance of plaque size in this system is not clear. It may contribute towards virulence and it does represent a marker for neurovirulence. In order to establish whether virulence and plaque size are determined by a common genetic locus/loci in αHSV, experiments involving mutagenesis and marker-rescue of relevant fragments of DNA would need to be performed. This will be discussed further in chapter 6.

The experiments described in this chapter expand on those performed by Leib (1986) in which the biological replication of αHVS was investigated. The results presented above suggest that a reduced plaque size in Vero cells and reduced yield of KM322 in Neuro 2a and Vero cells relative to KM91 may contribute to the reduced virulence of KM322 in rabbits, particularly on intracranial inoculation. However, co-inoculation experiments (Leib, 1986) suggest that neuroinvasiveness is regulated to a greater extent by the host immune response when αHVS is inoculated by the intradermal route.

CHAPTER 4: REACTIVATION OF αHVS (KM322) FROM LATENTLY INFECTED RABBIT DORSAL ROOT GANGLIA

4.1 Introduction

The infection of rabbits with KM322 provides a useful model for alphaherpesvirus latency (Tosolini et al, 1982). After intradermal inoculation into the rabbit flank the virus replicates to produce erythematous lesions and enters the nerve endings in the skin. Following centripetal travel inside axons the virus arrives at the DRGs at about 4 days post-inoculation. Replication within the ganglion ensues producing oedema and some neuronal destruction with polymorphonuclear and mononuclear cell infiltration. Virus isolation from DRGs adjacent to those supplying the inoculated dermatomes suggests limited spread up and down the spinal cord.

Between day 6 and 8 post-inoculation areas of sensory loss develop within the dermatomes of inoculation and can be mapped by pin-prick sensitivity (Tosolini et al, 1982). A slow recovery of sensation has been observed to occur after 4-6 weeks, but anaesthetic areas have remained in some animals 3 years after infection. On day 5 or 6 severe irritation in the skin around the inoculation site has been observed, with rabbits biting and scratching at the lesions unless prevented with a protective collar.

The acute phase of infection ends by day 12 after which no

replicating virus can be recovered from homogenates of rabbit skin or DRGs. However, latent virus can be recovered from DRGs after the resolution of acute infection by cocultivation of explanted ganglion fragments with indicator Vero cells. Latent virus has reactivated from DRGs in explant cultures from rabbits up to 5 years post-inoculation. Spontaneous virus reactivation in KM322 infected rabbits has never been observed as manifest either through recrudescence of lesions or by the observation of viral cpe earlier than 5 days post-explantation to suggest the presence of active virus within the ganglion. However, reactivation in vivo has been repeatedly stimulated by immunosuppression with cortisone acetate or anti-lymphocyte serum (Tosolini et al, 1981). Induced reactivation produces recurrent cutaneous lesions in the inoculated dermatomes. This model has many characteristics in common with herpes zoster with infection producing sensory disturbances, such as segmental anaesthesia and irritation, followed by prolonged latency.

A very low proportion of ganglion explant cultures from latently infected rabbits yield reactivating virus (<20%), thus necessitating the sacrifice of a large number of rabbits to produce significant results. This may indicate that there are only small amounts of virus within any one DRG since only a small proportion of neurons in a DRG would

be likely to become infected from the 12mm lesions produced in the rabbit skin. However, it is also possible that the low isolation rate was due to a lack of sensitivity of detection. All latent virus may not reactivate on explantation or all reactivated virus may not escape from the ganglion to infect the monolayer of permissive cells.

It was decided to investigate whether reactivation of KM322 from DRGs could be increased by the incorporation of virus inducing agents into explant culture media. The agents investigated were 5-azacytidine (5-azaC); 12-o-tetradecanoylphorbol 13-acetate (TPA), sodium-n-butyrate, dimethylsulphoxide (DMSO) and hexamethylene bisacetamide (HMBA). Each of these compounds has been shown to stimulate replication or recovery from latency of viruses, including herpesviruses. The agents were chosen because of their various modes of action in the expectation that their effects on reactivation of αHVS (KM322) might provide some insight into the mechanism of reactivation from latency of αHVS and of herpesviruses in general.

Much HSV research has concentrated on the phenomenon of latency in which the viral genome resides within the host in a non-replicating state producing infectious virus only during intermittent episodes of reactivation. Herpesvirus

latency has been reviewed recently by Roizman and Sears (1987) and Stevens (1989).

It has been known since the 1930s that neurons provide the major site of latency for HSV (Roizman and Sears, 1987) suggesting that cells of this phenotype are non-permissive to HSV replication and as such are particularly competent for latency. In addition HSV DNA has been detected in extraneural tissues apparently in the absence of virus replication; for example in rabbit corneas (Cook et al, 1987; O'Brien and Taylor, 1989; Sabbaga et al, 1988), mouse footpads (Clements and Subak-Sharpe, 1988), guinea pig footpads and vaginal tissue (Scriba, 1981) and guinea pig uterine cervical tissues and genital skin (Stanberry et al, 1985). It is not known whether virus maintained at the periphery represents a low grade productive infection or true latency (Scriba, 1981). If the genome is latent at these sites it may reside in neural elements or it may be in a state different to that of virus latent within the ganglia.

The latent HSV genome within neurons seems to be circularised by ligation of the tandem repeats at the termini of the molecule (Rock and Fraser, 1983, 1985; Efstathiou et al, 1986) and is not integrated into the chromosomal DNA but exists as an episome (Mellerick and

Fraser, 1987). However this question is not completely resolved. Puga et al (1984) have suggested that latent HSV DNA is integrated into the host DNA and is not circularised and Green et al, (1987) have detected linear non-integrated and integrated molecules in latently infected rabbit trigeminal ganglia. Rziha et al (1986) have found the pseudorabies virus (an alphaherpesvirus of pigs) genome to be predominantly linear and episomal in the latent state.

Deshmane and Fraser (1989) have shown the latent HSV genome to be associated with cellular histones in a chromatin-like structure while in contrast acute viral transcription occurred on non-nucleosomal DNA. They suggested that in this way viral gene expression during latency might be controlled using cellular gene regulation factors.

The processes involved in the establishment, maintenance of and reactivation from the latent state are not yet understood. It is known that latency involves the complex interaction of host and viral gene products.

A number of processes have been shown to stimulate reactivation of HSV in vivo such as immunosuppression, neurosurgery, trauma, ultra-violet irradiation and chemical stimulation of prostaglandin synthesis (Harbour et al, 1983; Kurane et al, 1984). In addition Wilcox and Johnson

(1987, 1988) have shown HSV reactivation to be stimulated from an *in vitro* latency model using primary neuron cultures by deprivation of nerve growth factor. The effects of the above processes on reactivation are likely to be mediated by suppression of the host's immune responses within the ganglion or at the periphery or by a change in the physiological state of the cell harbouring the latent viral genome.

In addition to host factors there appear to be viral genes involved in latency and reactivation. Russell *et al* (1987) used mutants in HSV immediate early (IE) genes and concluded that ICP4 expression is not necessary for reactivation in an *in vitro* latency system, and that ICP0 expression is not needed for the establishment of latency but is required for reactivation from latency in this system. Leib *et al* (1989a) found that HSV-1 with mutations in ICP4 and ICP27 failed to replicate in the murine eye and therefore failed to establish latency in their model. ICP0 mutants showed varying abilities to establish and reactivate from the latent state, suggesting that this gene product may be involved in both processes. Sears *et al* (1985) found that an ICP22 mutant failed to establish latency when inoculated into the mouse ear, but did so after inoculation by the ocular route.

The search for "latency genes" has been focussed on the IE genes since they are expressed first during infection and as such would provide a logical source for controlling the switch from lytic to latent infection. In addition the IE genes ICP4, ICP0, ICP22 and ICP27 have been shown to have regulatory roles in the expression of other HSV genes.

It has recently been discovered that there is limited transcription from the HSV genome during latency (Stevens et al, 1987). Since this initial observation, the latency associated transcript (LAT) has been found in the neurons of mice (Croen et al, 1987; Puga and Notkins, 1987; Spivack and Fraser, 1987), rabbits (Rock et al, 1987a; Wechsler et al, 1988a) and humans (Stevens et al, 1988; Vafai et al, 1988; Gordon et al, 1988; Steiner et al, 1988; Wechsler et al, 1988b) latently infected with HSV. LAT was also found in latent infections with pseudorabies virus (Rock et al, 1988) and bovine herpesvirus-1 (Rock et al, 1986, 1987b). The LAT in HSV is transcribed from the opposite strand of DNA to that encoding the ICP0 transcript. No virus encoded protein has been reproducibly detected in latently infected cells. LAT-deficient mutants are able to establish and maintain latency, and to reactivate but with a reduced frequency (Javier et al, 1988b; Leib et al, 1989b; Steiner et al, 1989; Sedarati et al, 1989). Thus if LAT has a role in latency it appears to facilitate reactivation.

5-azaC is a DNA hypomethylating agent. The level of methylation of cytosine in CpG dinucleotides in DNA has been implicated as a means of cellular and viral gene control in a number of systems (reviewed by Doerfler, 1983). Generally a high degree of DNA methylation correlates with gene inactivation whereas low levels of methylation are associated with active parts of the genome. Inverse correlations between DNA methylation and gene expression have been observed in a number of virus replication systems, including those of herpesviruses. In an *in vitro* latency system, Youssoufian *et al* (1982) showed latent HSV-1 DNA to be extensively methylated, but this was not the case in actively replicating virus. In gammaherpesvirus saimiri DNA is methylated in non-producing lymphoid cell lines but not in producing lines (Desrosiers *et al*, 1979). Szyf *et al* (1985) showed that hypomethylation of cellular and viral DNA preceded Epstein-Barr virus (EBV) amplification following induction. Sutter and Doerfler (1980) found an inverse correlation between methylation of integrated adenovirus type 12 DNA sequences in transformed cells and viral gene expression. Also, the experimental induction of a previously inactive TK gene in HSV-1 is associated with concurrent demethylation of the gene promoter (Clough *et al*, 1982; Ben-Hattar and Jiricny, 1988). Furthermore, Tasseron-de Jong *et al* (1989) showed that inactivation of a transfected

TK gene in human cells was mediated by de novo methylation.

5-azaC is a cytosine analogue, with the carbon-5 atom replaced by nitrogen thus inhibiting methylation at this position when it is incorporated into DNA. An additional and more significant mode of action is by irreversibly binding to and inhibiting cellular methylases (Taylor and Jones, 1982). Creusot et al (1982) showed that a 0.3% incorporation of 5-azaC inactivated more that 95% of the cell's methyltransferase.

The mechanism by which methylation regulates gene expression is unknown. The presence of methylated bases may affect protein binding directly or by eliciting or stabilising structural changes in DNA (Doerfler, 1983). Buschhausen et al (1987) and Graessmann and Graessmann (1988) found the formation of chromatin to be essential in meditating the regulatory effect of DNA methylation on gene expression in the HSV TK gene that had been microinjected into cells. It has been proposed that methylation of DNA regulates gene expression by changing or stabilising chromatin structures which in turn affects the binding of sequence-specific proteins such as transcription factors. Eukaryotic DNA in nucleosomes contains 4 times more methyl groups that DNA in regions between them (Kolata, 1985; Ben-Hattar and Jiricny, 1988). It is noteworthy that

Deshmane and Fraser (1989) have suggested that latent HSV is associated with nucleosomes in a chromatin structure. Ben-Hattar et al (1989) have shown that cytosine methylation inhibits transcription of the HSV TK gene by a mechanism other then direct blocking of transcription factor binding.

Whitby et al (1987) and Stephanopoulos et al (1988) have used 5-azaC to increase reactivation from explanted neural tissues latently infected with HSV. The model used for the experiments reported here extends the investigation to a different alphaherpesvirus (αHVS) which produces latency in the rabbit, and unlike other models does not exhibit spontaneous reactivation.

The phorbol ester, TPA, exerts a variety of effects on cells. In particular, it is a potent tumour promoter. In certain cells TPA may inhibit or stimulate differentiation, stimulate DNA synthesis, induce the production of various growth factors, hormones and other cellular secretions and induce proto-oncogenes (Yamamoto, 1984). TPA has been used in virus systems to enhance human immunodeficiency virus (HIV) replication in MT-4 cells (Harada et al, 1986), to stimulate transcription from the simian virus 40 enhancer (Chiu et al, 1987) and to induce EBV replication in producer lymphoblastoid cell lines (Zur Hausen et al, 1978;

Laux et al, 1988).

The pleiotropic effects of TPA on cells are thought to be mediated through stimulation of protein kinase C, a plasma membrane signal transducer (Nishizuka, 1984, 1986). Protein kinase C relays information in the form of extracellular signals, such as hormones, neurotransmitters and growth factors, across the membrane to regulate a number of Ca^{2+} dependent processes.

Sodium-n-butyrate induces a wide variety of effects on cells. Generally it modifies gene expression without reducing protein synthesis but in specific cells it may prevent division and induce the production of certain proteins (Kruh, 1982). Butyrate has been used as a differentiation inducer in a number of cell lines in culture (Leder and Leder, 1975; Schneider, 1976). The agent was selected for this experiment because it has been shown to enhance virus expression despite inhibiting cellular DNA replication in a number of systems. It has been used to stimulate EBV replication in producer cell lines (Luka et al, 1979; Saemundsen et al, 1980) and to increase reactivation of HSV from latently infected trigeminal ganglia (Hino and Sekizawa, 1986). Butyrate has also been shown to activate HIV long terminal repeat-directed gene expression (Bohan et al, 1987) and to

permit HSV replication in otherwise restricted neuroblastoma cells in culture (Ash, 1986). However, the effect of butyrate does vary according to cell type and virus tested and it has been shown to inhibit adenovirus type 2 replication in 3T3 cells (Iseki and Baserga, 1983) and polyoma virus replication in mouse kidney cells (Wawra et al, 1981).

It has been proposed that n-butyrate modifies gene expression by inhibition of histone deacetylase and thus hyperacetylation of histones (Kruh, 1982). Histone acetylation is correlated with gene activation, possibly by decreasing histone-DNA binding. As stated previously gene expression in the latent HSV genome may be mediated by histones (Deshmane and Fraser,1989).

5-azaC, TPA and butyrate were the agents studied most extensively in these experiments. In addition a limited investigation was made using DMSO and HMBA. Both of these drugs act as differentiation inducers and promote demethylation (Reuben et al, 1976; Christman et al, 1977; Razin et al, 1986).

DMSO has been shown to enhance the maturation of an influenza A virus, Newcastle disease and Semliki Forest viruses in culture, presumably by facilitating particle

assembly (Scholtissek and Müller, 1988). It has also been used to induce reactivation of latent HSV in ganglia *in vivo*, possibly by stimulating prostaglandin synthesis (Harbour *et al*, 1983) and to increase the rate of reactivation of HSV from mouse ganglion explants (Whitby *et al*, 1987; Leib *et al*, 1989a).

HMBA appears to actively induce DNA demethylation in Friend erythroleukaemia cells by the replacement of 5-methylcytosine with cytosine (Razin *et al*, 1986). It has also been shown to enhance reactivation of HSV from latently infected tissues *in vitro* (Bernstein and Kappes, 1988).

The agents described above were tested in explant cultures of rabbit DRGs latently infected with KM322 in order to investigate whether they had any effect on virus reactivation. In addition the effects of 5-azaC, TPA and butyrate on productive KM322 infection in Vero cells were determined.

Tosolini *et al* (1982) cocultivated rabbit skin from the inoculation site in an attempt to detect the presence of latent virus within these tissues. No reactivation was observed. In an attempt to increase the sensitivity of the assay this experiment was repeated with the inclusion of

5-azaC in culture media.

4.2 Materials and Methods

Rabbits were infected with KM322 as described in section 2.6. After a period of at least 1 month post-infection rabbits were dissected and their ganglia removed and explanted onto Vero monolayers as described in section 2.7. Reagents used in explant cultures are described in section 2.7.

Areas of rabbit skin ($1cm^2$) were removed from anaesthetic areas in the infected dermatomes of 3 rabbits, minced and explanted into 20 tubes per section of tissue as for DRGs (section 2.7).

All isolates were confirmed as input virus by RE analysis of viral DNA (section 2.13).

The effects of 5-azaC, butyrate and TPA on plaque formation efficiencies of KM322 were determined by infection of Vero monolayers in tubes with $2x10^2$ to $5x10^2$ pfu KM322 in maintenance medium containing increasing concentrations of each drug. After 40 hrs plaque counts were made and compared to those of drug-free controls.

The supernatants from these cultures were then harvested, centrifuged to remove cellular debris and titrated on Vero cells to determine virus yields (section 2.4).

4.3 Results

Cultures treated with 5-azaC (50μM) reactivated more rapidly (fig. 4.1) and to a greater extent (table 4.1) than controls. By 8 weeks post-explantation 42% of treated cultures had reactivated as compared to 16% of controls ($P<0.00001$).

Neither TPA (5ng/ml) nor butyrate (2mM) showed any significant difference in frequency (table 4.1) or speed (Figs. 4.2 and 4.3) of reactivation compared to controls.

Within the limited number of cultures tested there was no significant difference in reactivation of cultures treated with DMSO or HMBA and controls (table 4.1).

In all control cultures the timing of the appearance of cpe around explanted ganglion fragments was consistent with reactivation of latent virus. Isolation of virus was more rapid in 5-azaC treated tubes, but as stated previously this is unlikely to represent the detection of non-latent virus.

All isolations were found to be identical to input virus by RE analysis.

Table 4.1. Number of explant cultures yielding reactivated virus. In each case fragments from the same ganglion were cultivated without drugs as controls. N.S. denotes no significant difference.

	Drug		Control		P-value
50μM 5-azaC	57/132	43%	21/129	16%	<0.00001
5ng/ml TPA	8/45	18%	6/46	13%	N.S
2mM butyrate	2/44	5%	3/44	7%	N.S
200mM DMSO	0/12	0%	1/12	8%	N.S
5mM HMBA	0/8	0%	1/8	13%	N.S

Values represent number reactivated/ number cultivated.

Figure 4.1 Reactivation of αHVS (KM322) from explant cultures containing 5-azaC (50μM) and drug-free controls.

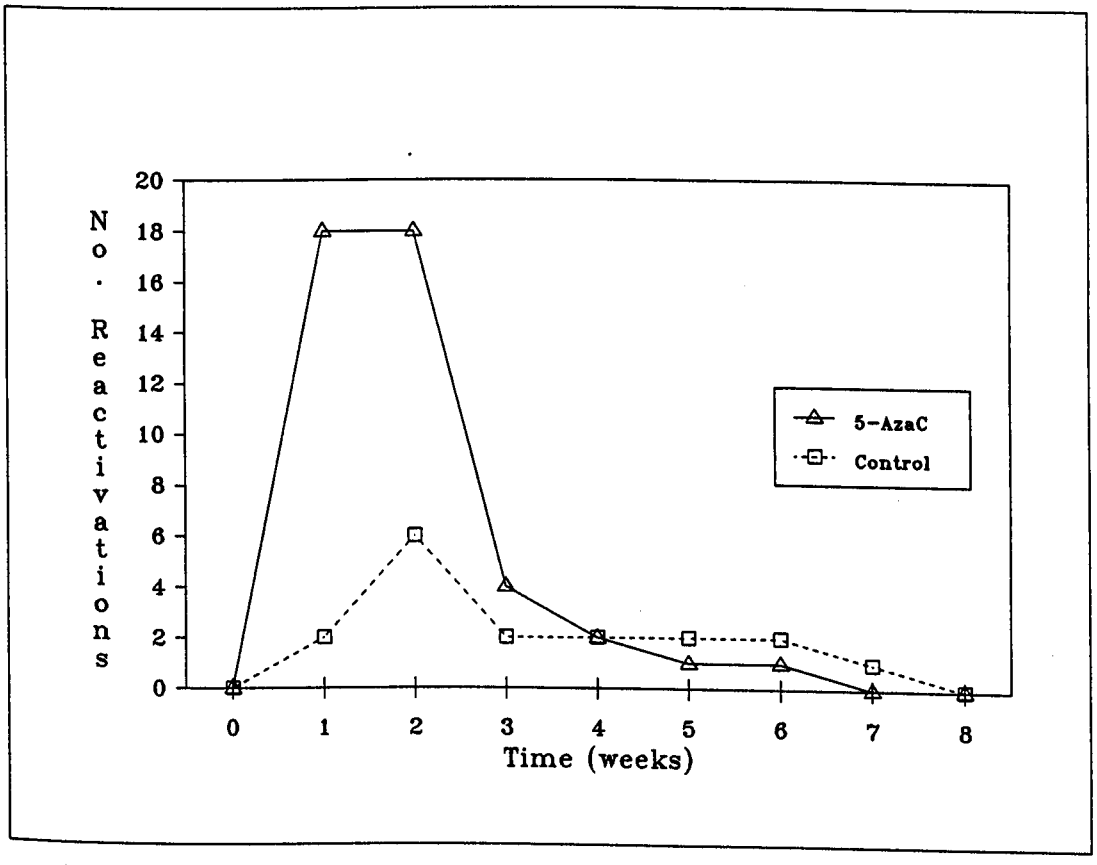


Figure 4.2 Reactivation of αHVS(KM322) from explant cultures containing TPA (5ng/ml), 5-azaC (50μM) and drug-free controls.

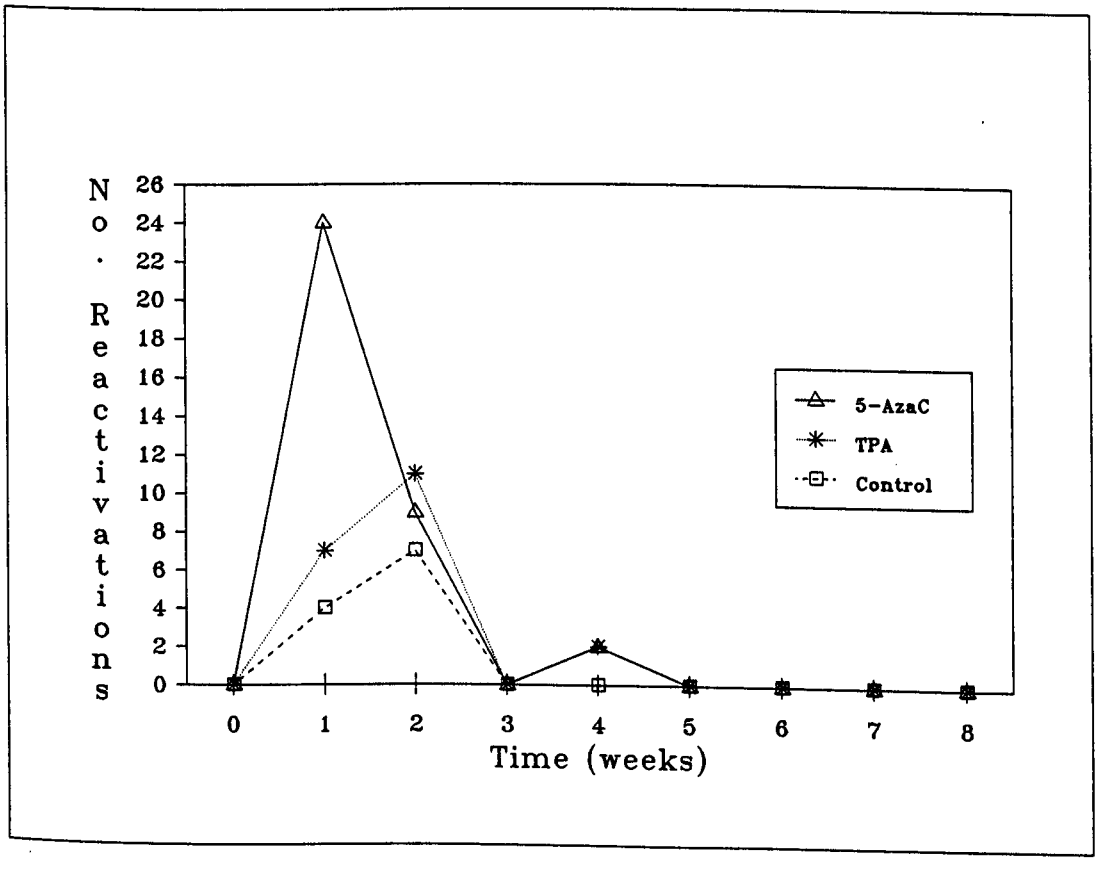


Figure 4.3 Reactivation of αHVS(KM322) from explant cultures containing butyrate (2mM), 5-azaC (50μM) and drug-free controls.

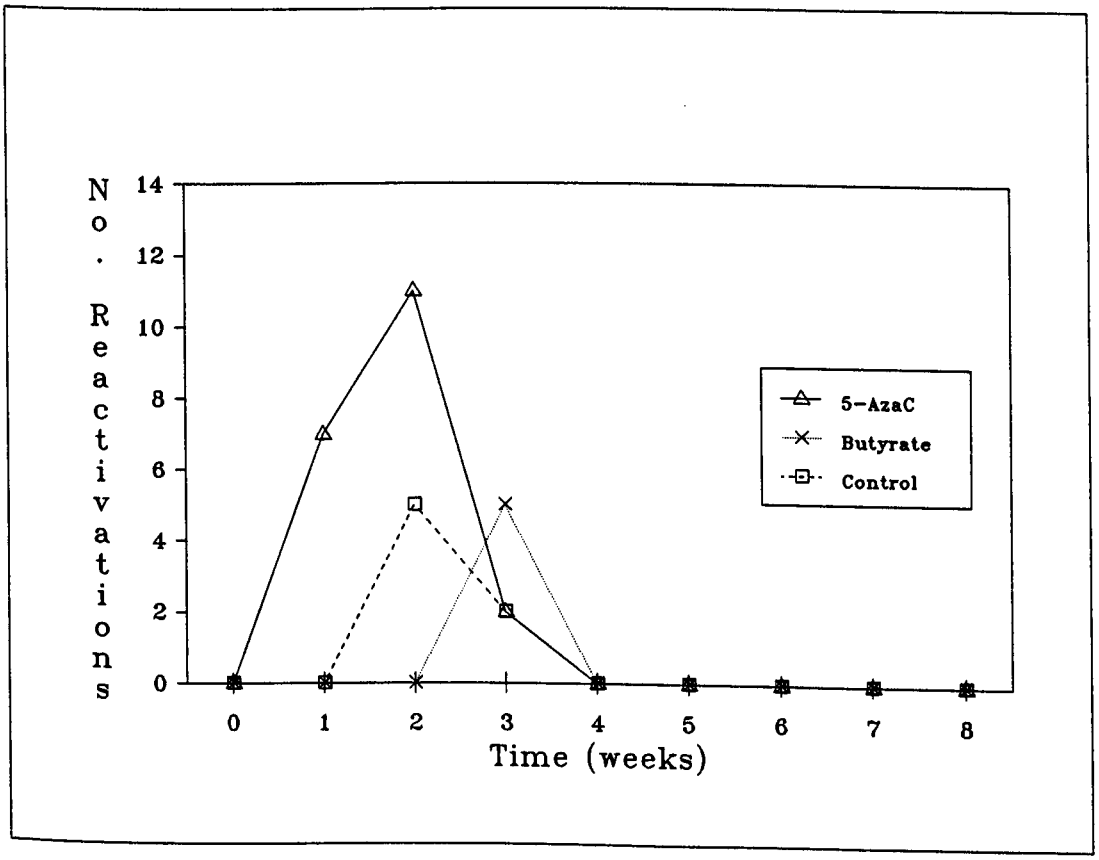


Figure 4.4 Effect of 5-azaC on KM322 plaque formation efficiency in Vero cells.

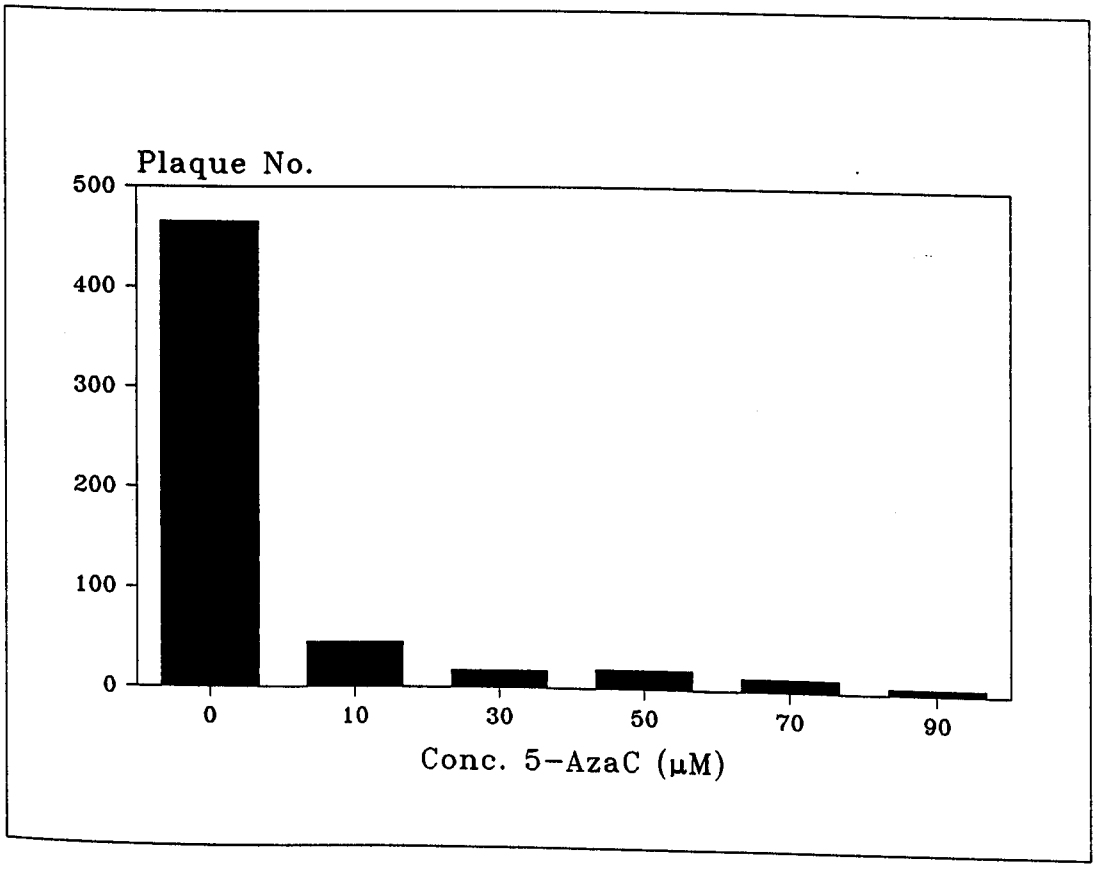


Figure 4.5 Effect of 5-azaC on KM322 yield in Vero cells.

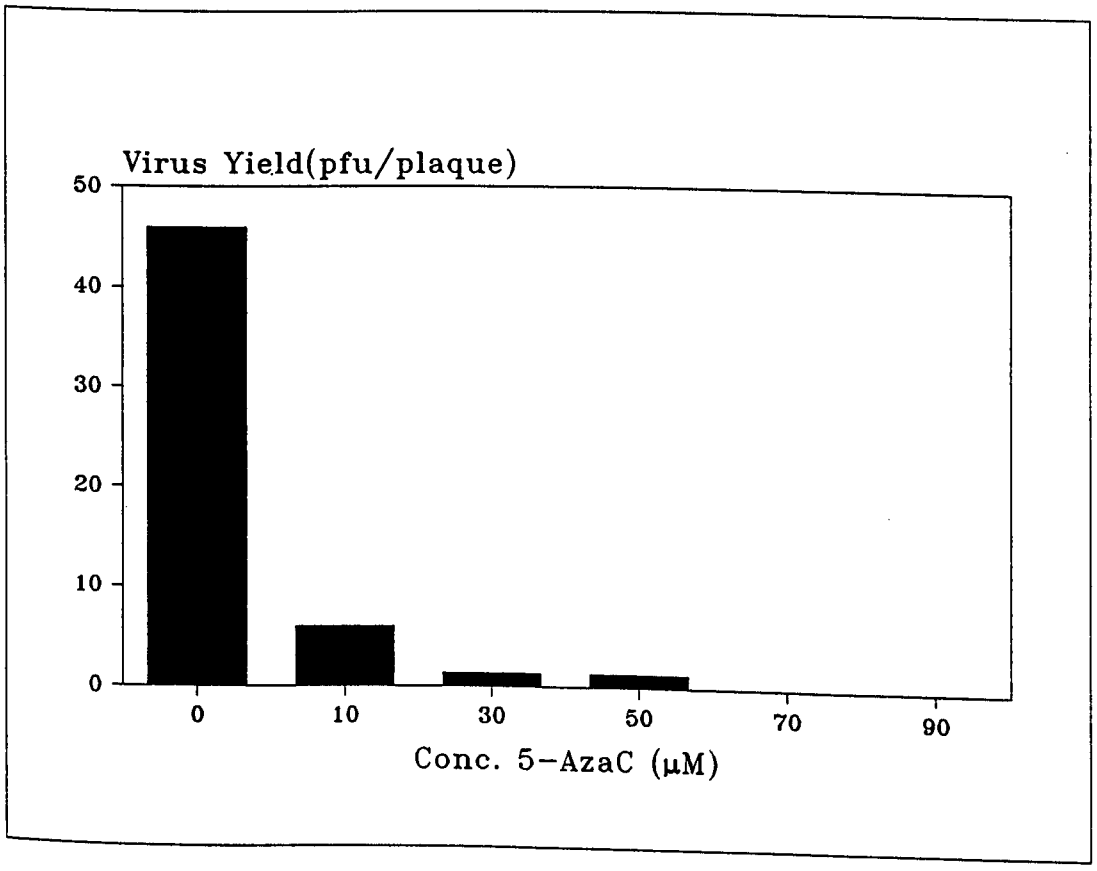


Figure 4.6 Effect of TPA on KM322 plaque formation efficiency in Vero cells.

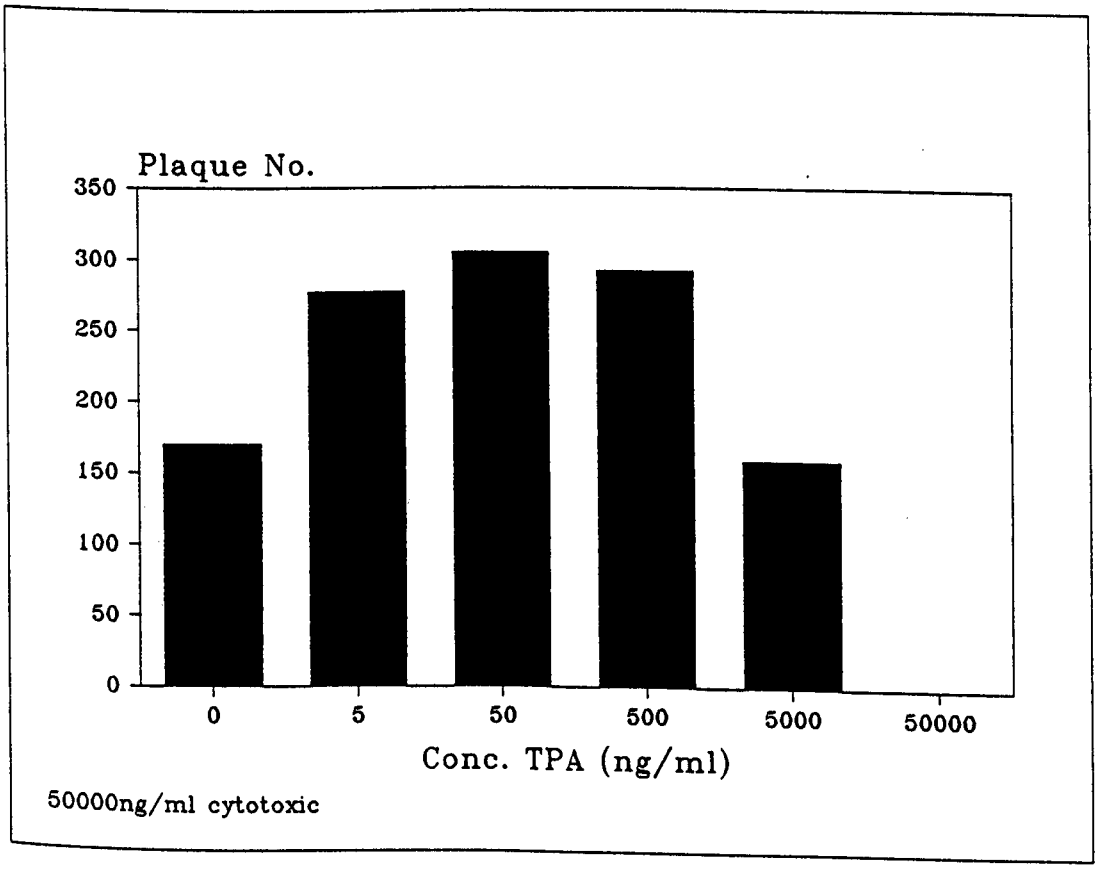


Figure 4.7 Effect of TPA on KM322 yield in Vero cells.

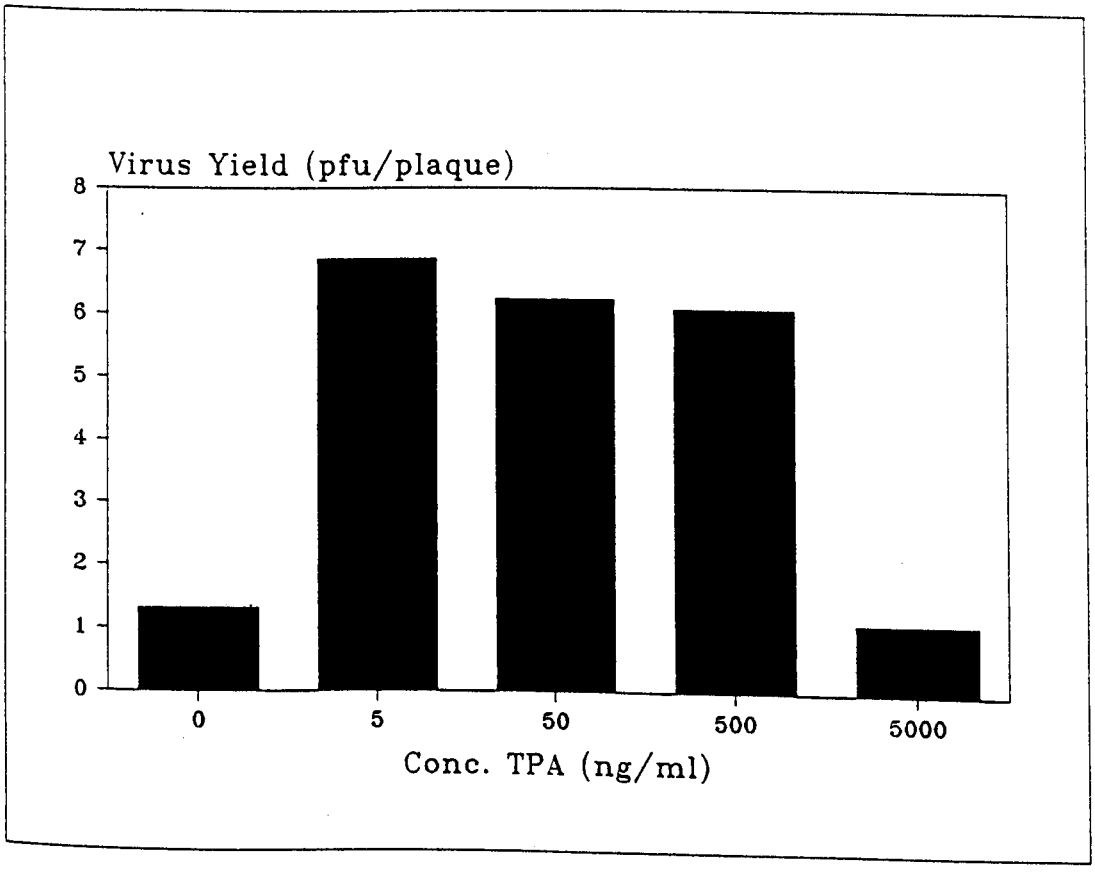


Figure 4.8 Effect of butyrate on KM322 plaque formation efficiency in Vero cells.

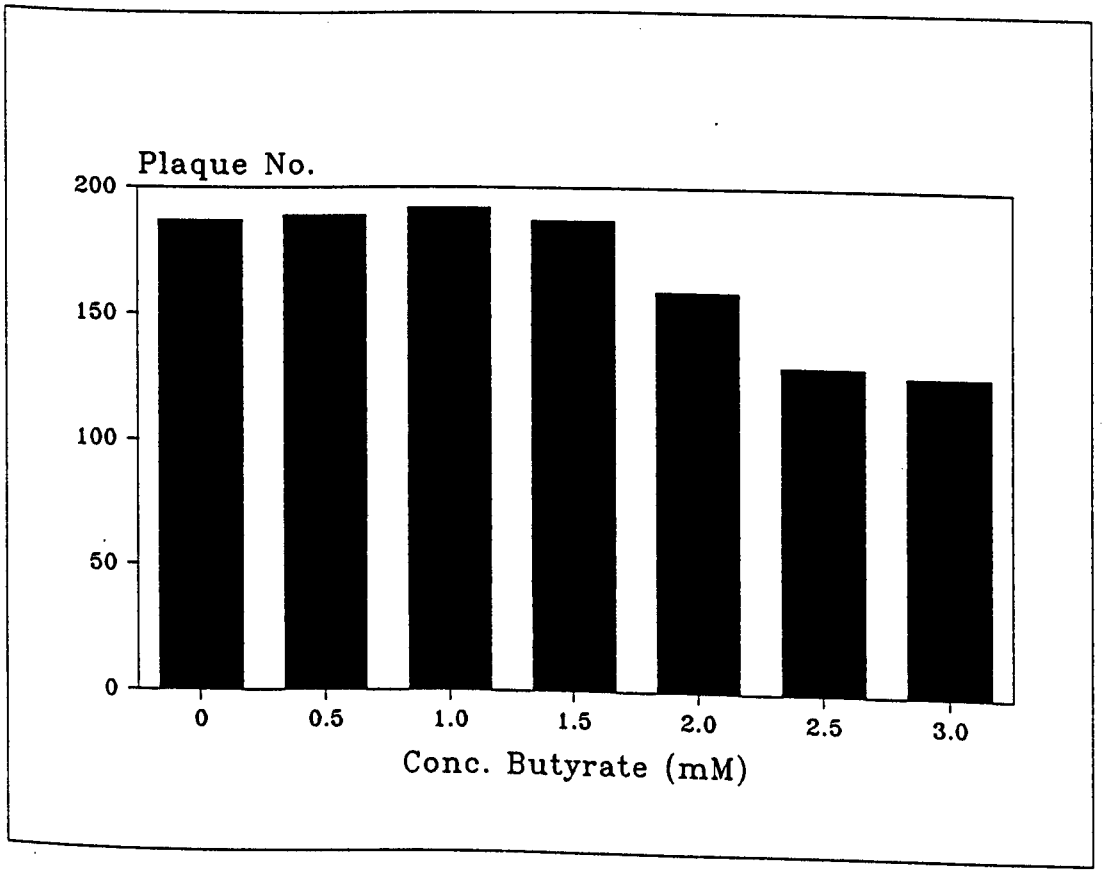
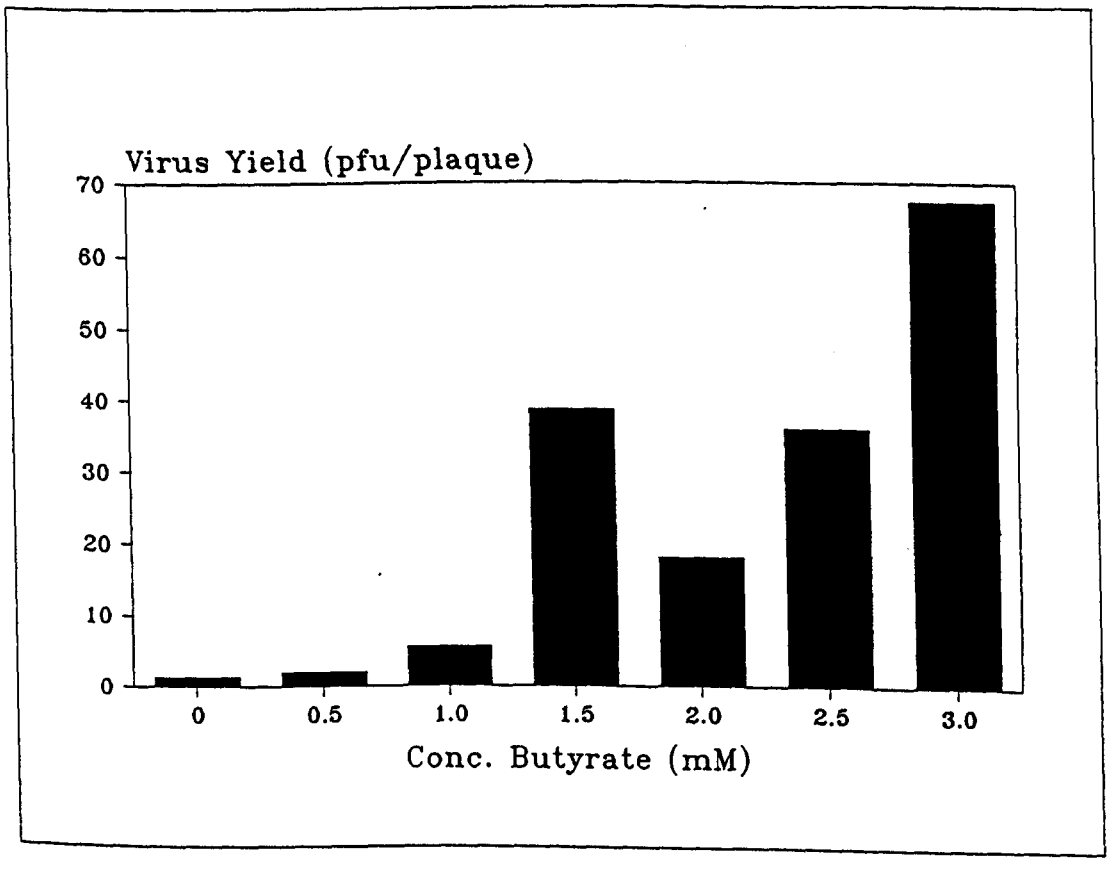


Figure 4.9 Effect of butyrate on KM322 yield in Vero cells.



Increasing concentrations of 5-azaC decreased both plaquing efficiency and virus yield (fig.s 4.4 and 4.5). TPA increased plaque formation efficiency and virus yield at low concentrations but inhibited viral replication at higher concentrations (5000ng/ml) (fig.s 4.6 and 4.7). The drug was cytotoxic at this concentration. Butyrate had no effect on plaque formation at low concentrations but was inhibitory at concentrations >2mM. Conversely an increase in virus yield was seen with increasing concentrations of butyrate (fig.s 4.8 and 4.9).

Explant cultures of rabbit skin fragments yielded no reactivations with 5-azaC or without drug.

4.4 Discussion

The fact that no virus was isolated from rabbit skin cultures even in the presence of 5-azaC suggests that this tissue may not be a site for latent virus in this model. However, it is possible that the observation resulted from a very low concentration of latent virus within the peripheral tissue. In such a case a very large area of skin would need to be sampled to increase the probability of isolating latent virus.

HMBA and DMSO had no effect on reactivation of αHVS from

latently infected DRGs *in vitro*. This result contrasts with findings for HSV reactivation in other models (Bernstein and Kappes, 1988; Whitby *et al*, 1987; Leib *et al* 1989a). It is not immediately clear why this should be the case since similar concentrations were used. It is highly probable that mechanisms controlling latency are conserved within the alphaherpesviruses since this phenomenon is so unique and within the group characteristics of latency are very similar, such as type of reactivating stimulus and site of latency. Therefore any differences between the two viruses to explain the differing results are likely to be in the stringency of control of latency between αHVS in rabbits and HSV in mice rather than gross differences in mechanisms of latency or reactivation. Indeed there are precedents within strains of HSV that show differing abilities to reactivate (Gerdes and Smith, 1983; Centifanto-Fitzgerald *et al*, 1987).

It may be the case that a virus less prone to reactivate might require a higher concentration of inducing agent than another strain. Concentrations of drug used in cultures are limited by cytotoxicity to ganglion fragments and Vero cells.

It cannot be discounted that the differing results seen with HSV and αHVS (KM322) reactivation with DMSO and HMBA

are due to differences in culture methods.

TPA and butyrate had no effect on αHVS reactivation from explant cultures either in frequency or speed. In contrast TPA increased plaque formation efficiency and virus yield at the concentration used in explant cultures (5ng/ml). Butyrate (2mM) decreased plaque formation by only 13%, but virus yield showed an increase from control cultures at this concentration. Butyrate has been shown to increase virus replication in other systems (Luka et al, 1979; Saemundsen et al, 1980).

Hino and Sekizawa (1986) found that butyrate (2mM) in explant cultures induced reactivation of HSV from murine trigeminal ganglia. This is not in agreement with the results presented here, and this may be explained as were the results with DMSO and HMBA above.

The failure of TPA to affect reactivation rates may be due to a lack of the appropriate receptors on the neurons harbouring the latent virus, to the inability of protein kinase C activation or other TPA-induced effect to stimulate reactivation, or due to limits on concentration used imposed by cytotoxic effects in explant cultures.

Clearly neither DMSO, HMBA, TPA nor butyrate are of use in

increasing the sensitivity of the cocultivation system used for detection of latent KM322 in rabbit dorsal root ganglia.

5-azaC treated cultures reactivated more rapidly and to a greater extent than control cultures ($P<0.00001$). At the concentration of 5-azaC used in explant cultures (50μM) both plaque formation and virus yield per plaque were decreased. This may be caused by cytotoxicity or possibly by inhibition of a transient methylation of the herpesvirus genome during replication, as has been shown to occur in HSV-1 (Sharma and Biswal, 1977). The fact that 5-azaC inhibits αHVS replication in Vero cells suggests that it is reactivation not subsequent replication that is being stimulated in the explant cultures.

That 5-azaC stimulates αHVS reactivation does not directly prove a role for methylation of DNA in the control of herpesvirus latency. Methods of action other that its more widely reported role as a hypomethylating agent have been described for this agent, such as inhibition of protein synthesis (Reichman and Penman, 1973). Also, Robert et al (1988) have suggested that activation of the major late promoter in adenovirus transformed cells by 5-azaC was not accompanied by extensive DNA demethylation.

The other agents used in these experiments; TPA, butyrate, HMBA and DMSO have all been shown to produce demethylation of DNA coincident with induction of differentiation in erythroleukaemia cell lines (Szyf et al, 1985; Reuben et al, 1976). However, induction by these agents produces active demethylation of DNA as opposed to the passive hypomethylation produced by 5-azaC which is dependent on DNA replication. These differences in mechanism may explain why 5-azaC may reactivate αHVS by hypomethylation while the other agents do not in this system. Whitby et al (1987) argued that stimulation of HSV reactivation with 5-azaC as well as other hypomethylating agents suggested that this was the mechanism responsible for activating the virus.

Rziha et al (1986) have found the pseudorabies virus genome not to be extensively methylated in the latent state. Dressler et al (1987) have produced evidence which suggests that the latent HSV-1 genome is not extensively methylated in vivo. Their analysis covered only 3% of CpG dinucleotides in the genome. However, Ben-Hattar and Jiricny (1988) showed that a single CpG methylation in the TK promoter segment resulted in a 20-fold reduction in transcription.

Honess et al (1989a) argued against methylation of latent

alphaherpesvirus genomes because of the fact that their DNA is G+C-rich. This differs from the case in gammaherpesviruses in which the genomes are A+T-rich with local clusters of CpG dinucleotides in putative regulatory regions. Honess et al stated that methylation of CpG dinucleotides gives rise to increased mutations to produce TpG dinucleotides and therefore the G+C-rich genome of alphaherpesviruses is evidence that their latent genomes are not methylated.

Thus there is evidence for and against methylation of DNA playing a role in control of herpesviral gene expression during latency. In order to clarify this controversy regions of DNA important in regulating reactivation need to be analysed specifically for methylation in latency, since widespread methylation is not necessary for the control of gene transcription (Ben-Hattar and Jiricny, 1988). Alternatively it may be the case that hypomethylation of neuronal DNA initiates transcription from otherwise silent genes which may in turn induce reactivation of latent herpesvirus DNA.

If replication of the αHVS genome is almost totally suppressed during latency, except for limited transcription as seems to be the case for HSV, and since it resides in non-dividing nervous tissue, it would appear that any

hypomethylation will be effected through DNA repair processes. Such unscheduled repair synthesis has been shown to occur in cultured neurons (Sanes and Okun, 1972).

The results presented here confirm those of Stephanopoulos et al (1988) and Whitby et al (1987) and extend them to a different alphaherpesvirus, αHVS (KM322). They suggest a role for methylation of host and/or viral DNA in the control of αHVS latency. In addition the use of 5-azaC in explant cultures provides a useful tool for increasing the sensitivity of detection of latent virus.

CHAPTER 5: ANALYSIS OF αHVS POLYPEPTIDES BY SDS PAGE AND WESTERN BLOTTING

5.1 Introduction

Previous work on αHVS, KM91 and KM322, in rabbits (Leib, 1986; Leib et al, 1988) has suggested that the most significant factor determining the difference in neurovirulence between these two strains is related to their ability to elicit a differing immune response in the host. Thus KM322 promotes an immune response in the inoculated rabbit which confines the virus to the DRGs without allowing extensive replication within the CNS and subsequent encephalitis. Conversely KM91 produces a lethal encephalitis in all rabbits within 12 days of inoculation. The neurovirulence of KM91 could be explained in two ways in terms of host response; either the virus is less sensitive than KM322 to the various forms of immune control or it fails to produce the protective response that KM322 inoculation promotes. Co-inoculation and superinfection experiments in rabbits with the two virus strains have suggested the latter explanation to be the case (Leib et al, 1988).

Rabbits inoculated with 75 pfu KM91 survived this lethal challenge if co-inoculated with 1500 pfu live or inactivated KM322 at the same site or with 1500 pfu KM322 on the opposite flank. In addition, rabbits survived KM91 infection if co-inoculated at the same site with BPL-inactivated KM91 virus particles. Animals previously infected with KM322 or

with live and inactivated KM91 were immune to superinfection with live KM91 (Leib et al, 1988).

These results imply that an immune response stimulated in the host by KM322 protects the animal from both KM322 and KM91 neurovirulence. This explanation is strengthened by the fact that KM322 protects against KM91 even when inoculated at a distant site. The fact that inactivated KM91 also protects against simultaneous inoculation of live KM91 suggests that this virus might produce the protective effect less efficiently than KM322 or in an altered form. In this way the balance between virus replication and host defense might be in the favour of KM91 on live virus inoculation, but the addition of non-infective particles could produce greater stimulation of the protective effect and therefore allow the host to overcome virus spread.

A similar effect has been noted with inoculation of pathogenic and apathogenic strains of HSV into mice. A series of experiments with the apathogenic strain HSV-1 ANG have shown this strain capable of protecting mice against a lethal challenge with a pathogenic strain on simultaneous intraperitoneal inoculation into mice (Schröder et al, 1981). The apathogenicity of HSV-1 ANG in mice is not a function of interferon (IFN) induction or sensitivity or of natural killer (NK) cell activation on intraperitoneal (Engler et al,

1981) or peripheral inoculation (Kümel et al, 1982). Kümel et al, (1982) also suggested that virus restriction was independent of T-cell and macrophage functions, but that an age-dependent defence mechanism was involved.

Schröder et al, (1983) further examined the nature of the protective effect generated in HSV-1 ANG-inoculated mice. They found mice to be protected from intraperitoneal inoculation of pathogenic virus if inoculated with HSV-1 ANG 4 hours later. They also found that 1-3 days after inoculation with the protective strain, mice could survive intracranial inoculation of a pathogenic HSV-1 strain. These results led them to suggest the presence of an early antigen-induced immune response possibly restricted to the CNS.

Yamada et al, (1986a) protected mice from lethal intraperitoneal infection with a virulent HSV-1 variant by preinoculation with an attentuated one. Resistance to the attenuated variant was shown to be age-related since newborn mice were much more susceptible to infection than young adult mice. They suggested the protective effect elicited by the apathogenic variant was due to the induction of natural resistance factors such as macrophages, IFN and NK cells. They discounted the involvement of T-cells and antibodies because of the short interval between protective inoculation

and challenge (0 to 24 hrs).

The study of immunity to HSV is complicated by virus latency and recurrence since recurrences represent subsequent exposures of virus to the immune system and the host immune status does mature after primary infection. Immune mechanisms are thought to play an integral role in control of viral latency and recrudescence. Most of the knowledge on host immune responses to alphaherpesvirus infections has been derived from studies with HSV inoculation into mice. Mice represent useful animal models since so much information on their immune system exists. However, results must be applied to human or other animal systems with caution since the virus-host interaction is extremely complex and varies with virus strain and the host involved. Nevertheless, with this in mind the results of such experiments can provide much useful information on the immunobiology of herpesvirus infections.

The following review of immune responses to herpesviruses will deal only with primary acute infections since this is of greatest relevance to the study of the pathogenicity of αHVS in rabbits.

Langerhans cells represent the most peripheral immune cellular elements in the body. Their presence in the

epidermis allows them to interact with invading viruses and they are believed to be important in HSV antigen presentation to T-cells. Sprecher and Becker (1986) showed that depletion of Langerhans cells in the skin produced a very severe infection even with a non-pathogenic strain of HSV.

Natural resistance mechanisms are thought to be significant in limiting virus dissemination during the initial stages after infection since they do not require prior exposure to antigen and a response is seen 2-4 hours post-inoculation. Such responses are selective but are not antigen-specific. Factors involved in natural defense against HSV infections include macrophages, NK cells and IFN (Mogensen, 1984). HSV replication in macrophages is abortive and thus prevents virus spread in this way. In addition, these cells secrete arginase which depletes local arginine concentrations required for HSV replication. Macrophages have been shown to kill HSV infected cells directly (Seid et al, 1986; Best, 1986; Mogensen, 1984; Visser and Verhoef, 1986). The lysis of HSV infected cells by NK cells is thought to play an important role against early HSV infection (Weinberg et al, 1986; Visser and Verhoef, 1986). IFN may be of particular importance in natural resistance to HSV because of its general antiviral effect and because it augments macrophage and NK cell function. Oberman and Panet (1988) have suggested that human IFN-α blocks HSV-1 replication during

immediate early transcription.

Both natural and adaptive immune responses are thought to be important in recovery from primary infection and in limitation of HSV spread. Cell mediated immunity (CMI) seems to play a more important role than humoral immunity in protection against HSV infections. Observations in humans and animal models have shown that selective impairment of CMI is often accompanied by severe herpesvirus infections, while this is not so with selective humoral immunity impairment. In addition recrudescence of HSV occurs in the presence of high titres of circulating antibodies. The spread of herpesviruses from cell to cell without exposure to the surrounding environment also suggests that CMI mechanisms will have greater significance in host defence.

High titres of neutralising antibody against HSV can limit viral spread from the inoculation site to sensory ganglia if administered at the appropriate time (Erlich and Mills, 1986; McKendall *et al*, 1979). However, in the mouse model this appropriate time occurs before the appearance of neutralising antibody *in vivo* (Rouse, 1984; Wildy and Gell, 1985). Thus antibodies *in vivo* may be produced too late and in too low a titre to be important for protection in acute primary infection.

In addition to neutralisation, antibodies have been shown to act against HSV-infected cells *in vitro* by antibody-dependent cellular cytotoxicity (ADCC). This effect is mediated by IgG and non-immune leukocytes possessing Fc receptors. Kohl and Loo (1986) have provided evidence to suggest that ADCC may be important in the defense against HSV *in vivo*. Cytolysis of HSV-infected cells mediated by complement occurs *in vivo*, but the significance of this to the protective response is unclear (Wildy and Gell, 1985).

T-cells are likely to play a more significant role than antibodies in anti-HSV immune responses since they occur earlier in infection and also recruit non-adaptive components of the immune system to participate. Support for the role of T-cells in the immune response against HSV has been obtained from adoptive transfer experiments in mice (Rouse, 1984; Mogensen, 1984).

The relative roles played by cytotoxic, delayed-type hypersensitivity-mediating or lymphokine-producing T-cells is a matter of some controversy. Nash *et al* (1987) used monoclonal antibodies (McAbs) to selectively deplete cytotoxic T lymphocytes (CTLs) (Lyt 2) and T-helper (T_H) cells (L3T4) in mice which they infected with HSV. They found differential anti-herpes activity of T-cells in different target tissues. In the skin T_H cells were more

significant while CTLs were more significant in the nervous system. Thus it seems that more than one subset of T-cells are likely to be important and that they interact with each other and with natural resistance and antibody responses to form the host immune response against infecting herpesviruses.

Many of the immunological responses of an HSV-infected host are directed against the viral glycoproteins. These are found on the surface of infected cells and on the surface of the virion where they can be seen as projecting spikes by electron microscopy (Stannard et al, 1987). The presence of these molecules on the surface of infected cells is of significance in immune cytolysis. The HSV glycoproteins are involved in virus-cell and infected cell-cell interactions and as such are important in determination of tissue and species tropisms.

Seven HSV glycoproteins have been described; gB, gC, gD, gE, gG, gH, and gI (reviewed by Spear, 1985; Courtney, 1984; Maingay et al, 1988; Norrild, 1980; for gI see Longnecker et al, 1987). Glycoproteins D, B and H are essential for replication in tissue culture but this is not the case for the other glycoproteins. Glycoprotein B and gD are involved in fusion of infected cells to form syncytia and gB is possibly involved in virus-cell adsorption with gD mediating

penetration into the cell. Glycoprotein D is one of the major virus-specific glycoproteins present on the surface of the infected cell. Glycoprotein C is a receptor for the C3b constituent of complement and gE and possibly gI are receptors for the Fc region of immunoglobulin. Glycoprotein E may play a role in in vivo pathogenesis since gE-negative mutants show reduced neurovirulence in mice. Glycoprotein H is thought to mediate egress of virions from infected cells. All the glycoproteins have HSV type-specific and type-common epitopes. The dominance of such epitopes may vary according to the host, route of inoculation, immune status etc.

The roles of different glycoproteins in generating the various arms of the immune response are not clear since conflicting results have been obtained. However, a general consensus is that gC dominates in eliciting a CTL response, gB in T_H induction and gD and gC in delayed-type hypersensitivity generation. All glycoproteins seem to induce neutralising antibodies, with gD and gH being the most potent. Actual antibody titres in humans are highest against gB (Maingay et al, 1988). The glycoproteins of HSV are being used to develop subunit vaccines (reviewed by Hall and Katrack, 1986; Dix, 1987).

A number of studies have suggested that an earlier and more significant immune response against HSV primary infection is

directed against internal structural proteins, in particular within the molecular weight range 34-49K, that are antigenically related to a nucleocapsid protein, p40 (Eberle et al, 1985; Bernstein et al, 1986; McKendall et al, 1988a and b). Martin et al (1988) suggest that non-structural immediate early polypeptides are significant in anti-HSV immunity in vivo.

Leib (1986) examined the polypeptides of αHVS infected cells by immunoprecipitation and SDS PAGE. The presence of about 14 virus-specific polypeptides of αHVS was noted and some cross-reaction between αHVS and HSV-1. However, no differences between KM91 and KM322 polypeptides were detected. It was therefore decided to examine the antigenic nature of αHVS polypeptides by the more sensitive technique of immunoblotting or Western blotting of polypeptides separated by SDS PAGE (Towbin et al, 1979).

It has been shown previously that immunoblotting allows the identification of a greater number of polypeptides than immunoprecipitation. It has also been used to demonstrate a greater diversity of McAbs to viral proteins (McKendall et al, 1988a; Braun et al, 1983). Braun et al (1983) suggested that immunoprecipitation is less sensitive because of the low concentration of minor polypeptides in lysates, different rates of incorporation of isotope in lysates and the

unavailability of poorly soluble proteins in lysates. Immunoblotting reduces these problems and also provides signal amplification through the use of indirect labelling procedures.

SDS PAGE separates proteins according to molecular weight. The effect of charge on protein migration is negated by the incorporation of SDS which complexes with the polypeptides to produce a uniform negative charge (Shapiro et al, 1967). However, proteins modified by processes such as glycosylation migrate with less predictability since the SDS complexes only with the polypeptide part of the molecule (Pitt-Rivers and Impiombato, 1968).

SDS PAGE of αHVS, KM91 and KM322, and HSV-1 virion polypeptides was performed followed by Western blotting using hyperimmune rabbit sera. The main objective of these experiments was to compare the antigenicity and patterns of KM91 and KM322 structural polypeptides with a view to explaining the diversity of immune response to each virus seen in rabbits. Herpes virions rather than infected cells were analysed since it was felt that the latter would produce gels far too complex to distinguish virus-specific polypeptides, and structural proteins are generally considered most significant in immune responses.

A number of groups have analysed the polypeptides of various herpesviruses and a common finding with each virus is the large number of polypeptides and a broad molecular size range. The results of some of these studies are summarised in table 5.1.

Table 5.1 SDS PAGE analysis of herpesvirus polypeptides

Virus	Virion/ IPCs	No. of poly-peptides	Molecular weight range	No. of glyco-proteins	Reference
HVS-1 (αHVS)	ICPs	>30	>250-30K	6	Mou et al (1986)
HT (αHVS)	ICPs	23			Desrosiers& Falk (1981)
BHV-1	Virion ICPs	25-33 + 15	330K-12K	11	Misra et al (1981)
PRV	Virion	>20	230K-20K		Stevely (1975)
EHV-1	Virion	28	270K-16K	9	Perdue et al (1974)
B-virus	ICPs	50	239K-26K	9	Hilliard et al (1987)
HSV	Virion	33	250K-25K		Lonsdale et al (1979)

ICPs are infected cell polypeptides. ICPs include regulatory and DNA replicative polypeptides not present in the purified virion.

Virus abbreviations are αHVS, alphaherpesvirus saimiri; BHV-1, bovine herpesvirus type 1; PRV, pseudorabies virus; EHV-1, equine herpesvirus type 1 and HSV, herpes simplex virus.

Analysis of herpes polypeptides has been used to distinguish between strains. Lonsdale et al (1979) compared strains of HSV and found polypeptides to vary in presence, mobilities and intensities. They suggested mobility differences to be due to differences in amount or type of post-translational modification or loss/gain of amino acid residues, and different intensities to be due to differential rates of processing or glycosylation. Metzler et al (1985) used SDS PAGE to compare bovine herpesvirus-1 (BHV-1) isolates and Westenbrink et al (1985) compared Marek's disease herpesvirus strains by the same method.

The technique of Western blotting has been widely used to analyse immune responses to herpesvirus polypeptides (Eberle et al, 1985; Cohen et al, 1986; Bernstein et al, 1986; McKendall et al, 1988a and b).

In addition to comparisons between strains of αHVS the polypeptides of these viruses were also compared to those of HSV-1. There is known to be a certain amount of conservation of polypeptides between herpesviruses. Norrild et al (1978) identified a common antigen of HSV, B-virus and bovine herpes mammillitis virus in a 125K molecular weight glycoprotein. Petrovskis et al (1986) showed homology of two glycoproteins between pseudorabies virus (PRV), HSV and varicella-zoster virus (VZV). Glycoprotein B of HSV has been found to have

homologues in Epstein-Barr virus (EBV) (Pellet et al, 1985), cytomegalovirus (CMV) (Cranage et al, 1986), equine herpesvirus-1 (Whalley et al, 1989) and VZV (Davison and Scott, 1986). Other groups have shown antigenic cross-reactivity between different herpesviruses (Hilliard et al, 1987) including between αHVS and HSV (Tischendorf, 1969; Blue and Plummer, 1973; Desrosiers and Falk, 1981; Leib, 1986).

Monoclonal antibodies were raised against purified KM322 virions for use in characterisation of αHVS polypeptides and antigenicity. These McAbs were analysed against blots of αHVS and HSV-1 polypeptides for further comparisons between the three viruses.

5.2 Material and methods

Hyperimmune rabbit sera were prepared against αHVS, KM91 and KM322, a standard αHVS strain, termed αHVS Boston, and HSV-1 as described in sections 2.8 and 2.9. Monoclonal antibodies were prepared by J.Atkinson, Department of Immunology, The Liverpool School of Tropical Medicine according to the method of Lane (1985).

KM322 antigen was prepared for McAb production as described in section 2.8 and supernatants screened by

immunofluorescence as described in section 2.10.

Virus antigen was prepared, SDS PAGE gels run and Western blotting performed as described in sections 2.11 and 2.12. Where McAbs were not reactive against protein blots gels were run under non-reducing conditions (section 2.11.2).

Blots were incubated with normal rabbit serum and NS-1 cell culture supernatant as negative controls for rabbit sera and McAb supernatants respectively.

5.3 Results

From reducing SDS PAGE gels greater than 26 virion polypeptides were identified for KM91 and KM322 and greater than 23 for HSV-1. The molecular weights of these range from 155K to less than 20K. The molecular weights of polypeptides for each virus are listed in table 5.2 and are shown in fig. 5.1.

The polypeptide patterns are very similar for KM322, KM91 and HSV-1. A region of variable polypeptides between the viruses exists between 47K and 37K molecular weights. Band differences are marked on fig. 5.1 and include differences in intensity (i), mobility (m) and absence of a band (a).

Immunoblots labelled after incubation with hyperimmune rabbit sera are shown in fig. 5.2. The results show a high degree of cross-reactivity between HSV-1, αHVS; KM91, KM322 and Boston strains.

Bands with molecular weights 48K and 45K on KM322 and HSV-1 samples are replaced on KM91 by a band with a molecular weight of 47K. The bands at 54K are less intense on the KM91 samples

Table 5.2: Molecular weights of polypeptides of αHVS, KM322 and KM91, and HSV-1 identified from reducing SDS PAGE gels.

Molecular Weight x10^3		
KM91	KM322	HSV-1
155	155	155
111	111	111
107	107	-
100	100	100
98	98	-
97	97	97
90	90	-
87	87	-
-	84	84
76	76	76
71	-	71
69	69	69
64	64	-
62	62	62
54	54	54
51	51	51
-	48	48
47	-	-
-	45	45
42	42	42
39	-	39
-	38	-
37	-	37
33	33	33
30	30	30
28	28	28
26	26	26
24	24	24
23	23	23
20	20	20

than on KM322 and HSV-1. The 37K protein of KM91 has greater intensity than the 38K protein of KM322. These two bands are only significantly labelled on αHVS strains labelled with anti-KM91 and anti-KM322 immune sera.

The bands mentioned above constitute the most immuno-reactive proteins on Western blots but are minor proteins on the SDS PAGE gel (fig. 5.1). The molecular weights of these polypeptides are not altered under non-reducing conditions (fig. 5.3).

Other prominent bands common to all immunoblots include those of molecular weights 90K, 69K and high molecular weight bands ranging between 110K and 130K in size.

The blot incubated with anti-αHVS, Boston, serum shows an overall similarity between this serum and the other αHVS sera. However there is a difference to all other blots with the presence of a doublet at 69K and 71K with KM91 and HSV-1 samples where a single 69K molecular weight band is present with other sera.

The blot with anti-KM322 serum shows KM322 and HSV-1 to have the same band patterns. The same is not true of the anti-HSV-1 serum blot since HSV-1 has extra bands at 97K and 76K. Additional bands at 112K are seen with KM91 and KM322

and a further band of 87K on KM322 polypeptides.

Figure 5.1.

SDS PAGE of αHVS, KM91 and KM322, and HSV-1 (HFEM) polypeptides under reducing conditions on a 10% acrylamide gel. Uninfected Vero lysates are included as a control. Differences between intensity (i), mobility (m) and absence (a) of a band are marked to the left of each lane.

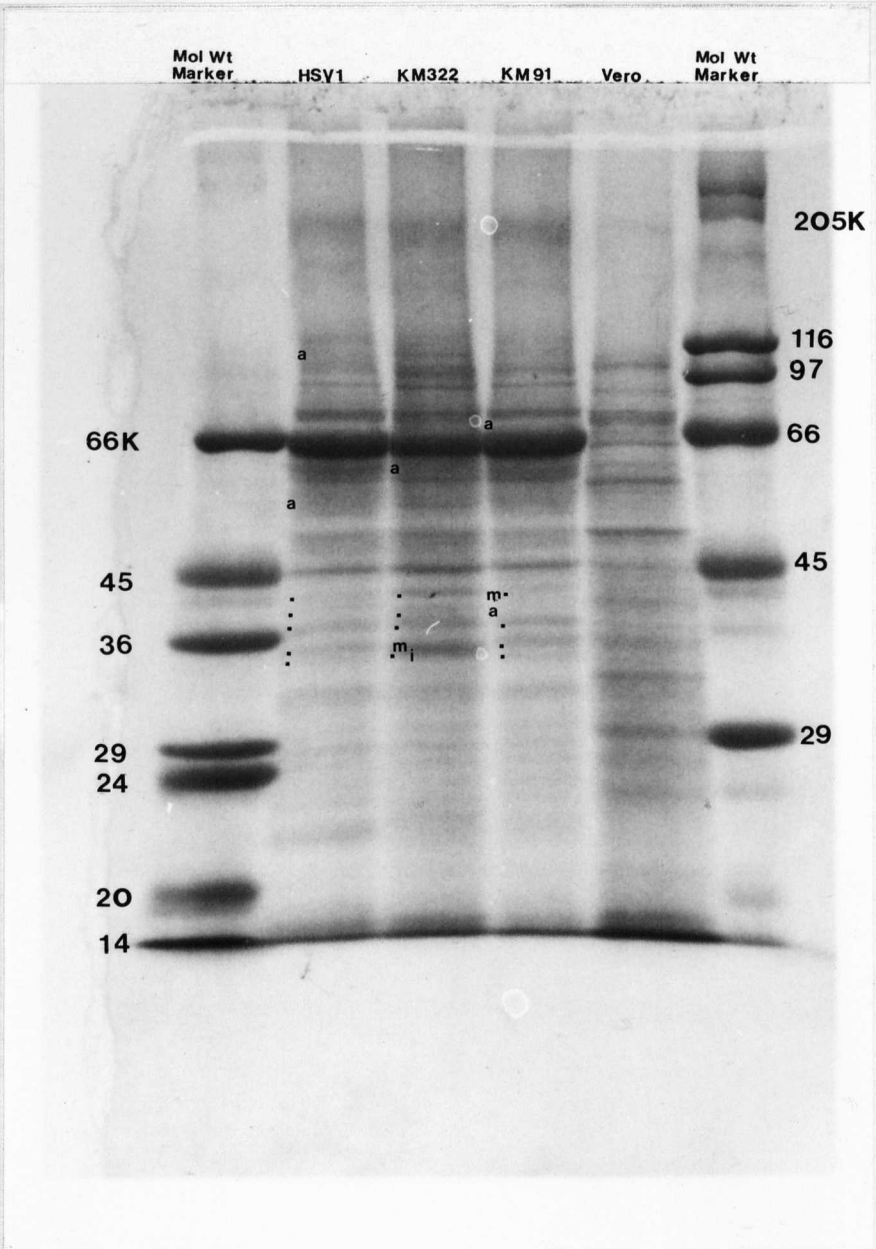
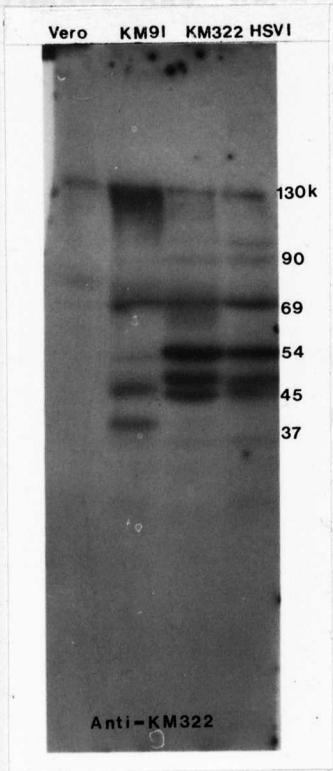
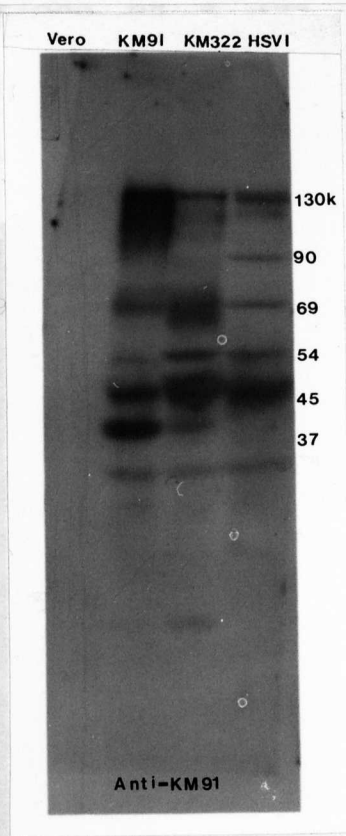
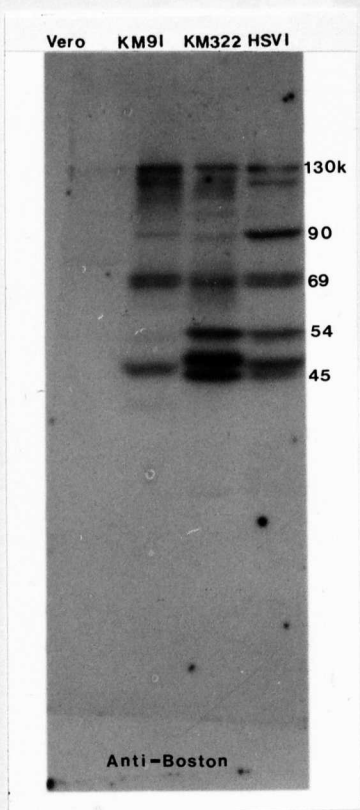


Figure 5.2. Immunoblots from αHVS and HSV-1 (HFEM) polypeptides, separated on 10% acrylamide gels, reacted with rabbit hyperimmune sera diluted 1/30. αHVS (Boston) is a standard strain of αHVS. Molecular weight markers on each blot were stained with Ponceau S prior to incubation with sera.







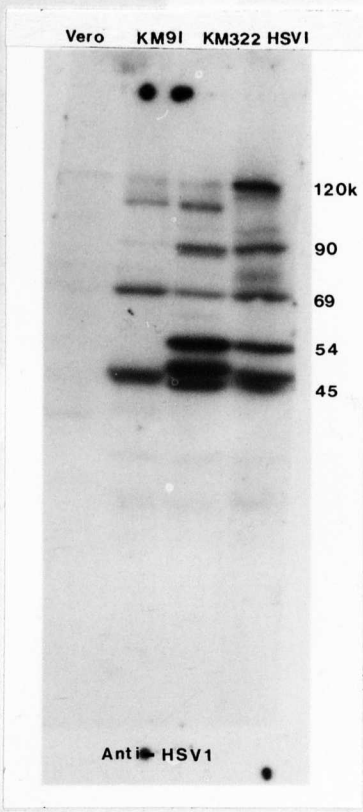
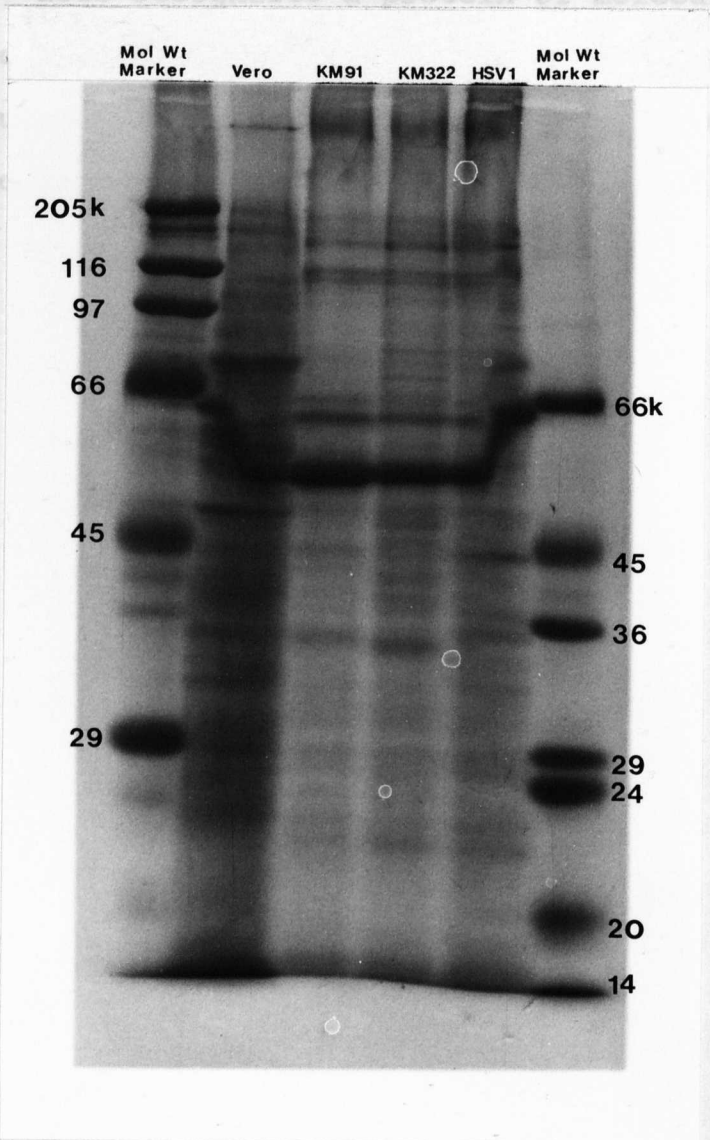


Figure 5.3.

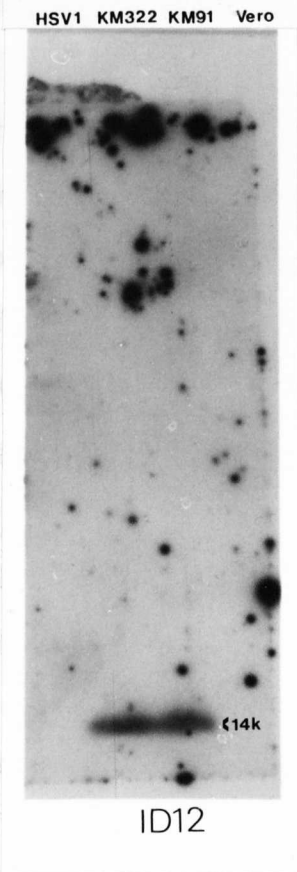
SDS PAGE of αHVS, KM91 and KM322, and HSV-1 (HFEM) polypeptides under non-reducing conditions on a 10% acrylamide gel.

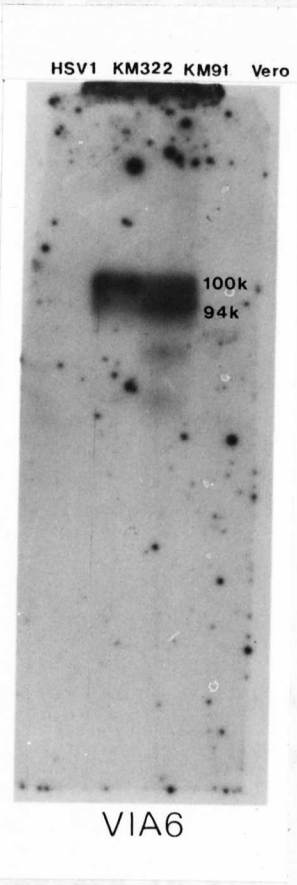


Blots of virion proteins labelled with McAbs reveal that the antibodies are all specific to αHVS (fig. 5.4). Both IIIC5 and VIA6 antibodies recognise 2 protein bands each of high molecular weights; 133K and 122K and 100K and 94K respectively. ID12 recognises a protein of molecular weight below the resolution of this PAGE system, i.e. less than 14K. All McAbs required non-reducing conditions to recognise the specific epitopes. A non-reducing gel is shown in fig. 5.3.

Figure 5.4.

Immunoblots from αHVS and HSV-1 (HFEM) polypeptides reated with McAb supernatants. Polypeptides were separated on 10% acrylamide gels. Molecular weight markers on each blot were stained with Ponceau S prior to incubation with supernatants.





5.4 Discussion

It can be seen from the SDS PAGE gel shown in fig. 5.1 that the polypeptide patterns of αHVS and HSV-1 are very similar. At least 26 virion-specific polypeptides have been identified for KM322 and KM91 and at least 23 for HSV-1. This compares well with previous work on alphaherpesvirus proteins as described earlier (table 5.1). The molecular weights of the polypeptides range from 155K to less than 20K in this study. This shows a lower maximum than previous work and suggests that the conditions used here do not allow for the detection of higher molecular weight proteins. As mentioned below it may be that Coomassie Brilliant Blue staining is not detecting all polypeptides.

It is noteworthy that the 155K protein found in these gels is in agreement with the findings of Desrosiers and Falk (1981) who proposed this protein to be the major nucleocapsid structural protein of αHVS. This protein exists in all herpesviruses and has a molecular weight of approximately 150K (Killington et al, 1977).

From the results of Western blots labelled with hyperimmune rabbit sera the prominently labelled polypeptides of αHVS and HSV-1 can be divided into regions of molecular weight 110-130K, 90K, 69-71K and 37-47K. The first and last of

these regions were also noted to be particularly immunogenically reactive for HSV-1 in mice (McKendall et al, 1988a).

The immunoblots illustrate a striking difference between KM322 and KM91 band patterns. Bands at 48K and 45K on KM322 and HSV-1 samples are not present on KM91 but are replaced by a band of 47K molecular weight. The 54K band of KM91 is labelled less intensely than those of KM322 and HSV-1. On blots labelled with anti-KM91 and anti-KM322 sera a band at 37K molecular weight is more intense on KM91 than a 38K protein of KM322.

These bands fall within the area of variable polypeptides, noted in the SDS PAGE gel (fig. 5.1), between 37K and 47K molecular weights. These bands do not represent major proteins on gels despite being immunodominant. These results are in close agreement with those of Eberle et al (1985), Bernstein et al (1986) and McKendall et al (1988a and b) who found proteins in the range 34-49K to be prominent in humoral responses against HSV-1. The molecular weight of the bands within this variable region are not changed on non-reducing gels suggesting that they do not have a subunit structure linked by disulphide bridges.

These differences between immunogenic polypeptides of KM91

and KM322 may be responsible for the varying immune response shown by rabbits against these two strains and therefore for their differing pathogenicity.

The presence of bands between 110K and 130K on immunoblots is surprising since these bands are not present on gels (fig. 5.1). It is difficult to explain this reproducible difference except by suggesting that the Coomassie Brilliant Blue stain is less sensitive than the labelling used for Western blots and therefore does not detect these proteins.

It is interesting to note that the size of two of these proteins at 130K and 120K is the same as the sizes of HSV-1 gC and gB respectively. This suggests the possibility that αHVS may have antigenically cross-reactive equivalent molecules. However, this needs to be investigated further, possibly by the use of McAbs against gB and gC of HSV-1.

Differences between immunoblots with different hyperimmune sera are restricted to a few bands only as detailed in the results section. In general there is a high degree of cross-reactivity between αHVS; KM91, KM322 and Boston and HSV-1 immune sera.

Thus the SDS PAGE gel (fig. 5.1) and immunoblots (fig. 5.2) show a high degree of similarity between the proteins of αHVS

and HSV-1. Indeed KM322 appears to resemble HSV-1 more closely than KM91 in antigenic cross-reactivity However, the isolation of 3 McAbs specific to αHVS, with no prior selection against HSV-1 reactivity, suggests that the difference between HSV-1 and αHVS is more extensive than is apparent from the above experiments with polyclonal sera.

The degree of homology between HSV-1 and αHVS polypeptides could be analysed further by the use of existing McAbs directed against known HSV-1 proteins. It would be interesting to ascertain whether HSV-1 inoculation into rabbits can protect against subsequent KM91 challenge.

The McAbs used in these experiments were only reactive against non-reduced proteins. This suggests that the epitopes recognised were dependent on disulphide bonds for their structure. McAb IIIC5 recognised proteins at 133K and 122K. McAb VIA6 recognised proteins at 100K and 94K and McAb ID12 recognised a protein(s) at molecular weight less than 14K and therefore not resolved by the gel. The fact that IIIC5 and VIA6 both recognised two proteins suggests that they might be binding to modified molecules such as glycoproteins and their non-modified, lower molecular weight precursors.

Two other McAbs were tested against virus polypeptides but

were negative despite being positive by immunofluorescence. This may be due to SDS denaturation of the specific antigenic sites involved. Such a possibility could be investigated further by the use of non-denaturing gels (Cohen et al, 1986).

It was hoped that the many hundreds of McAb supernatants screened initially would yield at least one to distinguish between KM91 and KM322. This was not achieved, but the αHVS-specific McAbs obtained are potentially very useful in further characterising the proteins of αHVS. Such uses include examination of cross-reactivity with other alphaherpesviruses, location of genes specifying polypeptides by the use of mutants and identification of the site of accumulation of proteins in the infected cell by pulse-labelling experiments.

The use of Western blotting to analyse the immune response of rabbits to αHVS, KM322 and KM91, is limited by the fact that it only detects humoral responses and that hyperimmune sera are likely to reflect a different immune status to that seen in rabbits with a primary acute infection. However, the results presented here have shown there to be a significant difference between KM91 and KM322 immunoreactive proteins.

The nature of these proteins needs to be investigated further

in order to establish the degree of difference between the molecules of each strain and the functions of the proteins. It is possible that they are internal structural proteins as has been found for a 40K and other related immunoreactive polypeptides of HSV-1 (Eberle et al, 1985; Bernstein et al, 1986; McKendall et al, 1988a and b). If this proves to be the case these antigens would not be present on the cell membrane or virion envelope which rules out a direct antiviral action for antibodies generated against them. However, McKendall et al (1988b) have postulated that such antibodies might have an indirect effect on antiviral defence.

This system has potential for future research into the development of a subunit vaccine against KM91. The use of a low molecular weight αHVS polypeptide would complement the work on HSV-1 and 2 vaccines currently being developed using the major glycoproteins (reviewed by Hall and Katrack, 1986; Dix, 1987).

Further work on the immune response of rabbits to KM91 and KM322 could investigate the nature of the restriction of KM322. For example, at what point is KM322 restricted? It may prove to be within the ganglion itself; Gebhart and Hill (1988) have shown that T-cells can infiltrate the ganglia of rabbits. The timing of the immune response could be

investigated by inoculating a protective dose of KM322 at various times after KM91 infection. This would provide information on the nature of the immune response involved. Furthermore, selective depletion of various arms of the immune response, for example by silica treatment against macrophages, in KM322 infected animals would also provide valuable information. In addition passive transfer of immune elements such as antibodies and/or T-cells from KM322 immunised animals into rabbits before KM91 infection might indicate the immune constituent(s) responsible for the KM322 protective effect.

To summarise, the results presented here show a high degree of similarity between the patterns of polypeptides of KM91, KM322 and HSV-1 by SDS PAGE. There is also a high degree of antigenic cross-reactivity which extends to a standard strain of αHVS, Boston, as seen by Western blotting with hyperimmune rabbit sera. However, there are differences between KM91 and KM322 polypeptides, and Western blots show these to be highly immunoreactive. These protein differences suggest a possible explanation for differences in KM91 and KM322 pathogenicity. Finally, three McAbs raised against KM322 have been shown to be specific to αHVS polypeptides of varying molecular weights.

CHAPTER 6: RESTRICTION ENDONUCLEASE ANALYSIS OF αHVS DNA

6.1 Introduction

The genome of herpesviruses consists of a double stranded DNA molecule which in the case of HSV is complexed with polyamines and wrapped around a protein spool within the core of the virion (Furlong et al, 1972). The variability of herpesvirus genomes is greater than that within any other virus family. The genomes vary in size (129-230 kilobases, kb), base composition (32-75% G + C) and in the arrangement of large scale repeated sequences. Of almost 100 herpesviruses identified, relatively few genomes have been characterised. HSV-1 is particularly well studied, indeed the entire genome has now been sequenced (McGeoch et al, 1985; McGeoch et al, 1986; McGeoch et al, 1988; Perry and McGeoch, 1988). The structure of the HSV genome has been reviewed by Roizman (1979, 1980), Honess (1984), Roizman and Batterson (1986) and McGeoch (1989).

The HSV genome consists of two covalently linked segments of DNA, the long (L) and short (S) regions. Each segment contains unique sequences (U_L and U_S) flanked by repeated sequences in opposite orientation (R_L and R_S) (Grafstrom et al, 1974; Sheldrick and Berthelot, 1974). The internal and terminal repeats are referred to as IR_L, IR_S and TR_L, TR_S respectively. The sequences of R_L and R_S are distinct

(Clements et al, 1976; Wilkie et al, 1977) except for a 220-500 base pair direct repeat termed the 'a' sequence at each terminus and at least one copy at the L-S junction in the opposite orientation (Wilkie and Cortini, 1976; Davison and Wilkie, 1981).

Within a population of HSV virions there are four isomeric arrangements of DNA in equal quantities. These isomers are produced by inversion of the L and S regions relative to the joint region. A single virion can give rise to progeny of all isomeric forms and each isomer seems to be functionally identical. The isomers have been designated prototype (P), inversion of S (I_S), inversion of L (I_L) and inversion of S and L (I_{SL}).

Genome isomerism produces fragments sub-molar relative to intact DNA when HSV DNA is digested with certain restriction endonucleases (REs). Enzymes cutting outside the inverted repeats produce four terminal fragments in 0.5M amounts and four 0.25M junction fragments. All other fragments are produced in 1M amounts. Thus the theoretical model for HSV DNA structure described above, first proposed by Sheldrick and Berthelot (1974), was confirmed by the detection of sub-molar fragments in HSV-1 and HSV-2 DNA digested by REs (Hayward et al, 1975a and b; Skare et al, 1975; Wilkie, 1976; Wilkie and Cortini, 1976; Clements et al, 1976; Skare

and Summers, 1977; Cortini and Wilkie, 1978).

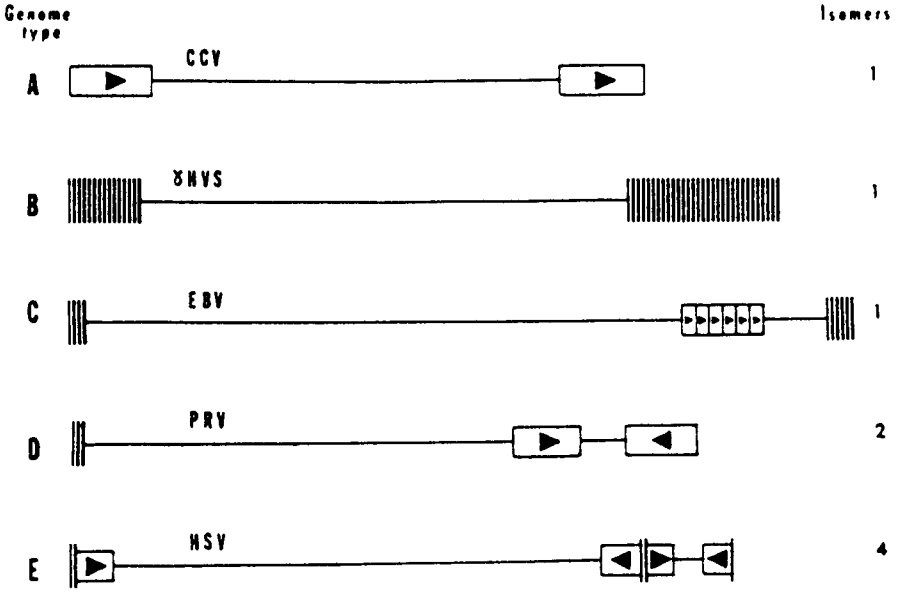
A similar isomeric structure is present in the DNA of other alphaherpesviruses, for example bovine herpesvirus-2 (BHV-2) and the simian virus SA8. The DNA of the betaherpesvirus human cytomegalovirus (HCMV) is also present as four isomers, although recently an isolate has been found with eight isomers due to an invertible segment within the L region (Takekoshi et al, 1987). Varicella-zoster virus (VZV) DNA also exists in four isomers but two are twenty times more abundant (Davison and Scott, 1986).

A second structural arrangement of DNA existing within the herpesvirus family produces two isomers due to inversion within the S segment only. Examples of viruses with this structure include the alphaherpesviruses pseudorabies (PRV), equine herpesvirus-1 (EHV-1) and bovine herpesvirus-1 (BHV-1). Other herpesvirus DNA structures produce only one isomer. The genomes of channel catfish herpesvirus (CCV) and possibly human herpesvirus-6 (HHV-6) consist of a single unique sequence flanked by a pair of large direct repeats. The genome of gammaherpesvirus saimiri (γHVS) consists of a unique sequence bracketed by multiple reiterations of one set of sequences in the same orientation at both termini. The remaining identifiable genome structure is exhibited by Epstein-Barr virus (EBV) in which multiple reiterations of a

set of sequences exists at each terminus in the same orientation, with an internal repeat. Examples of each genome arrangement are shown in figure 6.1.

Figure 6.1

Sequence arrangements in the five types of genomes of herpesviruses. Horizontal lines represent unique regions. Large scale reiterated sequences are shown as rectangles, arrows represent orientation. Long vertical lines represent terminal direct repeat sequences (designated 'a' sequences in type E genomes). From Roizman and Batterson (1986).



There are conflicting reports on the structure of αHVS DNA. Kit et al (1980) and Desrosiers and Falk (1981) have suggested that the genome had a D arrangement with two isomers and only 0.5M fragments present in RE digests (See figure 6.1). However, Leib et al (1987b) detected 0.25M RE fragments and therefore proposed an E structure with four isomers of αHVS DNA.

The inversion of genome segments is thought to occur by site-specific recombination between the 'a' sequences. Evidence for the role of 'a' sequences comes from studies by Mocarski et al (1980) in which the addition of extra 'a' sequences into the HSV genome produced additional genome inversions. However, Weber et al (1988) have suggested that segment inversion is not site- specific since they found that a bacterial transposon inserted into the HSV genome inverted at high frequency with recombination directed by the DNA replication machinery. The 'a' sequences are believed to be involved in genome maturation by signalling the cleavage of DNA concatamers produced in DNA replication (Davison and Wilkie, 1981; Nasseri and Mocarski, 1988).

The biological significance of genome isomerism is not clear. Hayward et al (1975a) and Roizman (1979) have suggested that the L and S segments of HSV may have been derived from separate genetic sources during the evolution of these

viruses. Honess (1984) has disputed both this theory and earlier suggestions that inversion of DNA segments might be involved in the regulation of latency and reactivation.

Table 6.1

Genome diversity in the herpesviruses. The data has been derived from Roizman (1982) with additional information on the human herpesviruses, MHV-2, BHV-1, αHVS, CTHV and AHV-1 from McGeoch (1989), Johnson and Whalley (1987), Mayfield *et al* (1983), Leib (1987 a and b), Foulon and Cebrian (1989) and Bridgen *et al* (1989).

Virus	Sub-family	Genome Model (No. Isomers)	Size DNA(kb)	%G+C
Herpes simplex-1 (HSV-1)	α	E (4)	152	68
Herpes simplex-2 (HSV-2)	α	E (4)	152	69
Varicella-zoster virus (VZV)	α	E (4)	125	46
SA8	α	E (4)	150	67
Macropodid herpesvirus-2 (MHV-2)	α	E (4)	135	50
Bovine herpesvirus-1 (BHV-1)	α	D (2)	138	72
Equine herpesvirus-1 (EHV-1)	α	D (2)	141	57
Pseudorabies virus (PRV)	α	D (2)	137	74

Alphaherpesvirus saimiri (αHVS)	α	D/E (2/4)	148/153	67
Channel catfish herpesvirus (CCV)	α	A (1)	129	56
Human Herpesvirus-6 (HHV-6)	β	A (1)	-	42
Human cytomegalovirus (HCMV)	β	E (4)	230	56
Murine cytomegalovirus (MCMV)	β	-	198	59
Herpesvirus aotus type 1	β	E (4)	218	56
Epstein-Barr virus (EBV)	ϑ	C (1)	172	60
Gammaherpesvirus saimiri (γHVS)	ϑ	B (1)	155	46
Cottontail herpesvirus (CTHV)	ϑ	E (4)	150	45
Marek's disease herpesvirus	ϑ	E (4)	165	46
Herpesvirus ateles (HVA)	ϑ	B (1)	135	48
Alcelaphine Herpesvirus type 1 (AHV-1)	ϑ	B (1)	160	50

It now seems likely that isomerism of herpesvirus genomes is the result of the evolution of a site-specific recombination-cleavage system necessary for maturation of the genome (Honess, 1984) and in itself has no specific biological function. This latter conclusion is supported by the fact that no transcript crosses the L-S junction and by the finding that a strain of PRV with four isomers as opposed to the two isomeric forms found in all other isolates shows no significant biological differences (Lomniczi et al, 1984). In addition the isomeric structure varies throughout the herpesvirus family and no temperature-sensitive non-inverting HSV mutants have yet been isolated.

As mentioned earlier herpesvirus genomes also vary in G+C content and genome size. Table 6.1 illustrates this diversity. Clustering of high G+C regions has been observed in several herpesvirus genomes. In the case of HSV and HCMV IR_S and TR_S are particularly rich in G+C as is the internal repeat of EBV. This clustering is particularly marked in the genomes of ϒHVS and HVA where the unique sequences are very low in G+C content but the reiterated sequences are G+C rich (Honess et al, 1989a).

There is some non-exclusive correlation between the size of

unique sequences, G+C content and arrangement of reiterated sequences with the biological properties of herpesviruses (Roizman, 1982; Honess, 1984). These factors have been analysed in considering the evolution of the Herpesviridae (Roizman, 1980; Honess, 1984).

The HSV genome has been studied extensively by RE analysis. This technique has been used to study genome stability after reactivation from latent infection and after serial passage in cell culture. The epidemiology of herpes simplex viruses has also been studied by analysis of RE fragment variability in related and unrelated incidents of HSV infection. Such studies have led to the conclusion that RE analysis provides a sensitive and reliable technique for strain differentiation (Buchman et al, 1980; Roizman and Tognon, 1983).

Generally the HSV genome is very stable with mutations being acquired over a long time span. Blondeau et al (1988) and Darville et al (1987) analysed the genomes of HSV isolates after reactivation from latently infected mice and found them to be stable. Studies of isolates from humans have also illustrated the stability of HSV genomes (Lonsdale et al, 1979; Lonsdale et al, 1980; Whitley et al, 1982; Maitland et al, 1982). Indeed isolates from recrudescent lip lesions taken from an individual 12 years apart were shown to be identical (Buchman et al, 1980).

However, although the HSV genome shows an overall stability there are regions of the DNA that do show a high degree of variability both between strains and within strains. Hayward et al (1975b), Skare and Summers (1977), Wilkie et al (1977), Lonsdale et al (1979), Maitland et al (1982) and Darville et al (1987) found variation in the mobilities of certain terminal and subterminal fragments. Analysis of the nucleotide sequence of the L-S joint region of HSV-1 and HSV-2 led Davison and Wilkie (1981) to propose that size heterogeneity of terminal and internal fragments corresponded to the size of 'a' sequences and of smaller insertions / deletions within junction sequences. They suggested that the tandem reiterations at the joint region could produce size variability by unequal cross-over events. Thus regions of HSV-1 and HSV-2 vary in size as a result of amplification and reduction of sequences at the termini and in other sites within the genomes shown to be hypervariable such as regions within U_L and U_S close to the termini (Roizman and Tognon, 1983). Roizman and Tognon (1982) proposed that such fragment mobility variations might vary on serial propagation and as such should not be used for strain identification purposes.

In addition to changes in the mobilities of RE fragments other variations are produced by the loss or gain of RE cleavage sites due to spontaneous insertions, deletions or

substitutions at non-lethal sites (Roizman and Tognon, 1983). Such restriction fragment length polymorphisms (RFLPs) could be produced by single base pair changes. These variations are relatively stable and are therefore reliable as strain identity markers (Roizman and Tognon, 1982). However, Alam et al (1989) found RFLPs within isolates from different organs of an individual and suggested that all isolates were derived from a single infecting strain. Thus RFLPs within the unique regions were not stable in this case. Umene et al (1984) have suggested that RFLPs are scattered over the length of the HSV genome while mobility variations are mainly restricted to the inverted repeats and the S segment of the genome. Furthermore, they suggested that specific regions of U_S are more variable than others with regards to mobility variations (Umene and Yoshida, 1989). Chaney et al (1983) analysed 84 HSV-1 isolates and suggested that certain viral DNA sequences are more prone to RFLPs than others.

Other herpesviruses have also been analysed by RE digestion of viral DNAs. Strains of BHV-1 have been differentiated by RE studies (Engels et al, 1981; Mayfield et al, 1983; Engels et al, 1986/7). Straus et al (1984) used RE analysis to compare varicella and zoster viruses and to show their identity within single individuals. Leib et al (1986) analysed strains of avian infectious laryngotracheitis virus by RE digestion of viral DNA and Wall et al (1989) have

recently used this technique to compare strains of B-virus.

The genomes of other herpesviruses also show good conservation with the exception of variable regions. Dambaugh et al (1980) found variation in the EBV genome within the internal and terminal repeats or in the number of copies of the repeat sequences. Hayakawa et al (1986) examined the DNA of clinical isolates of VZV and investigated genome stability after serial passage in vitro. They found the genome to be stable with the exception of variable regions within U_L and in TR_S. EHV-4 isolates contain variable regions within U_S, within the repeat sequences and in the terminus of U_L. Such variations were also found in the same regions of EHV-1 after passage in heterologous cell cultures (Studdert et al, 1986). Rota et al (1986) found variation within the feline herpesvirus-1 genome to occur mainly at the termini and in the internal repeat sequences. The PRV genome has hypervariable regions at the junction between U_S and repeat sequences, at the terminus of U_L and within an internal region of U_L (Todd and McFerran, 1985).

The ordering of restriction fragments within a piece of DNA can be determined by the use of double digests, using two REs simultaneously. The sizes of fragments produced from each single digest and from double digests are then used to determine where RE sites are distributed along the length of

DNA in relation to each other and to sites of the second enzyme. In this way restriction site maps can be determined for herpesviral DNA for characterisation of the genome and for reference purposes in genetic manipulations. The construction of RE maps for herpesvirus genomes is aided by the presence of sub-molar terminal and internal fragments. However, the genomes are relatively large and often produce a complex array of fragments in RE digests such that additional techniques may be required to produce RE maps. Many of the herpesvirus genome maps determined so far have been assisted by hybridisation studies and/or isolation of specific fragments before digestion with a second enzyme. Herpesvirus genomes mapped to date include HSV-1 (Wilkie, 1976; Wilkie et al, 1977; Skare and Summers, 1977), HSV-2 (Cortini and Wilkie, 1978), BHV-1 (Engels et al, 1986/7; Mayfield et al, 1983), MHV-1 (Johnson and Whalley, 1987) and CTHV (Foulon and Cebrian, 1989).

Leib et al (1987a) analysed the genome of αHVS with REs and compared the restriction profiles of KM91 and KM322. They found mobility differences of certain fragments with the enzymes used (BamHI,XhoI,KpnI and HindIII). These differences were found to be reproducible (Leib, 1986; Mossman, unpublished observation) but following the guidelines of Roizman and Tognon (1982) they do not represent reliable strain markers.

The genomes of KM91 and KM322 were analysed with further REs in the hope of identifying a stable difference in the genomes of the two strains in the form of RFLPs. Such genomic differences would provide a firm basis for future attempts to identify the genetic determinant(s) responsible for the differences in pathogenicity between KM91 and KM322. Such "virulence genes" have been identified for HSV in which neurovirulent phenotypes have been correlated with specific regions of DNA (Javier et al, 1988a; Thompson et al, 1989; Taha et al 1989b; Chaney et al, 1983). Double digests were performed on αHVS (KM322) DNA in order to investigate the genome structure.

6.2 Materials and Methods

DNA was extracted from αHVS, KM91 and KM322, by the large-scale technique described in section 2.13.3.

DNA was digested with restriction endonucleases and electrophoresed on full-size gels as described in section 2.13.4. Only fragments greater than about 1.5kb were measured. Double digests were performed in a universal incubation buffer (New England Biolabs, Beverley, USA)

Photographs of EtBr stained gels were analysed by scanning densitometry using a Joyce-Loebl Chromoscan 3 densitometer.

Fragment molarities were calculated from densitometer scans by the following equation:

$$\text{Molarity} = \frac{\text{fragment density / total density}}{\text{fragment size (kb) / Genome size (kb)}}$$

Genome lengths were calculated as follows:

Length = Σ (1M fragment sizes) + Σ ½ (0.5 M fragment sizes).

6.3 Results

The restriction endonuclease profiles for KM322 and KM91 digested with HindIII, EcoRI, KpnI, BamHI and HpaI are shown in fig.s 6.2 and 6.3. KM322 digested with pairs of enzymes are shown in fig.s 6.3 and 6.4.

Sizes, molarities and band designations are tabulated for each digest in tables 6.2-11.

KM91 and KM322 DNA digested with BamHI and KpnI show identical profiles between the two strains (Fig. 6.2). Molarities calculated for each set of fragments indicate that BamHI digests contain four 0.5M fragments while KpnI fragments are all 1M (tables 6.3 and 6.5). Profiles from DNA

digested with EcoRI and HindIII show band mobility differences between KM91 and KM322 in HindIII B,C and K and EcoRI H and I (fig. 6.2 and tables 6.2 and 6.4). HindIII digests contain 0.5M fragments and EcoRI fragments are all 1M.

KM322 and KM91 digested with HpaI exhibit a RFLP; KM322 contains an extra HpaI cleavage site relative to KM91. Fragments E plus P of KM322 equal 14.50kb, the approximate size of KM91 fragment D. All HpaI fragments are 1M with respect to whole viral DNA (fig. 6.3, table 6.6).

KM91 and KM322 digested with SmaI, AvaI, SalI, BglII, XbaI, MluI and BstEII exhibit mobility differences between the two strains (fig. 6.5). SalI and BglII show RFLPs between KM91 and KM322. The SalI fragments of KM322 (5.52kb and 3.90kb) summate to produce the approximate size of the extra KM91 fragment (10.17kb). The BglII fragments unique to KM91 (26.98kb and 2.14kb) are together equivalent to the approximate value of KM322 27.42kb (fig. 6.5).

Table 6.2 DNA fragments generated by digestion of αHVS with HindIII. Fragment sizes and molarities apply to KM322 and KM91 except where differing KM91 values are included in brackets.

HindIII

Band	Size (kb)	Calculated Molarity	Postulated Molarity
A	24.66	0.43	0.5
B	22.41	1.02 (0.59)	1 (0.5)
C	21.05	0.41 (1.07)	0.5 (1)
D	18.51	0.38	0.5
E	16.00	0.45	0.5
F	12.11	1.11	1
G	9.09	0.90	1
H	8.63	1.03	1
I,J	7.27	1.79	1 + 1
K	6.01 (5.77)	1.28	1
L,M	4.82	2.42	1 + 1
N	4.76	1.03	1
O	4.32	1.35	1
P	2.94	1.07	1
Q	2.69	0.79	1
R	2.39	0.99	1
S	2.19	1.30	1

Table 6.2 continued

T	1.95	1.07	1
U	1.61	0.95	1
V	1.44	1.08	1

Genome size = 146.83kb (145.91kb)

Table 6.3 DNA fragments generated by digestion of αHVS (KM322 and KM91) with BamHI.

BamHI

Band	Size (kb)	Calculated Molarity	Postulated Molarity
A	23.54	0.49	1
B	21.56	0.32	0.5
C	18.97	0.31	0.5
D	17.34	0.31	0.5
E,F	15.00	1.25	1 + 0.5
G	14.03	0.67	1
H	13.21	0.77	1
I	10.05	0.97	1
J	8.02	1.10	1
K	7.23	1.18	1
L	6.91	1.10	1
M	5.43	1.36	1
N	4.79	1.15	1
O	4.49	1.49	1
P	3.15	1.07	1

Table 6.3 continued

Q,R	3.00	1.76	1 + 1
S	2.51	1.23	1
T	2.21	0.98	1
U	2.11	0.99	1

Genome size = 165.12kb

Table 6.4 DNA fragments generated by digestion of αHVS with EcoRI. Fragment sizes apply to KM322 and KM91 except where differing KM91 values are included in brackets.

EcoRI

Band	Size (kb)	Calculated Molarity	Postulated Molarity
A	35.49	0.95	1
B	32.35	1.03	1
C	24.58	0.81	1
D	20.66	0.65	1
E	9.44	1.13	1
F,G	4.95	1.63	1+1
H	4.51 (4.31)	1.25	1
I	3.10 (2.68)	1.04	1
J,K	2.39	1.61	1 + 1

Genome size = 144.81 kb (144.19kb)

Table 6.5 DNA fragments generated by digestion of αHVS (KM322 and KM91) with KpnI.

KnpI

Band	Size (kb)	Calculated Molarity	Postulated Molarity
A	21.38	0.75	1
B	19.20	0.59	1
C	17.28	0.70	1
D	14.09	0.88	1
E	13.42	0.86	1
F	12.27	1.13	1
G	7.80	0.96	1
H	7.49	0.98	1
I,J	6.38	2.22	1 + 1
K	6.04	1.23	1
L	3.95	0.62	1
M,N	3.07	2.32	1 + 1
O	2.73	0.89	1
P	2.48	1.23	1
Q	1.90	0.75	1
R	1.57	1.14	1

Genome size = 150.50kb

Table 6.6 DNA fragments generated by digestion of αHVS with HpaI. Fragment sizes apply to KM322 and KM91 except where differing KM91 values are included in brackets.

HpaI

Band	Size (kb)	Calculated Molarity	Postulated Molarity
A	32.17	0.92	1
B	28.34	0.66	1
C	22.36	1.17	1
D (91)	14.60	1.34	1
E (322)	11.87	1.02	1
F,G	6.08	1.85	1+1
H,I,J,K,L,	4.75	4.70	1+1+1+1+1
M,N,O,	3.84	2.70	1+1+1
P (322)	2.63	1.31	1

Genome size = 144.80kb (144.90kb)

Table 6.7 DNA fragments generated by double digestion of KM322 with EcoRI, HindIII.

EcoRI, HindIII

Size (kb)	Calculated Molarity	Postulated Molarity
24.02	0.46	0.5
22.39	1.27	1+ 0.5
17.81	0.50	0.5
16.20	0.47	0.5
13.71	0.92	1
12.25	0.98	1
7.28	2.17	1 + 1
6.00	1.42	1
5.78	0.85	1
4.84	1.75	1 + 1
4.68	1.10	1
4.29	0.69	1
2.92	1.82	1 + 1
2.76	0.81	1
2.60	0.69	1
2.50	0.73	1
2.32	1.59	1 + 1
2.12	1.12	1

Genome size = 154.01kb

Table 6.8 DNA fragments generated by double digestion of KM322 with BamHI, HindIII.

BamHI, HindIII

Size (kb)	Calculated Molarity	Postulated Molarity
22.38	0.65	1
19.44	0.31	0.5
18.18	0.33	0.5
16.85	0.40	0.5
15.26	0.47	0.5
8.36	0.96	1
7.63	1.46	1 + 1
7.25	0.65	1
6.84	0.68	1
5.16	2.10	1 + 1
4.94	0.90	1
4.62	1.85	1 + 1
4.16	1.19	1
3.82	1.12	1
3.13	2.90	1 + 1 + 1
2.66	0.71	1
2.51	0.70	1
2.19	2.66	1 + 1 + 1
2.00	0.74	1
1.51	2.16	1 + 1

Genome size = 153.59kb

Table 6.9 DNA fragments generated by double digestion of KM322 with HindIII, KpnI.

HindIII, KpnI

Size (kb)	Calculated Molarity	Postulated Molarity
22.76	0.85	1
16.37	0.78	1
15.15	0.73	1
13.33	1.15	1
8.10	1.74	1+1
7.65	0.99	1
6.53	0.85	1
6.15	0.90	1
5.23	1.71	1+1
4.47	1.09	1
4.26	1.11	1
3.96	0.62	1
3.64	1.17	1
3.10	0.94	1
2.89	1.71	1+1
2.56	2.34	1+1
2.29	1.25	1
2.09	1.71	1+1

Table 6.9 continued

1.82	2.46	1+1+1
1.49	1.99	1+1
1.36	0.75	1
1.21	0.58	1
1.14	0.71	1

Genome size = 163.55kb

Table 6.10 DNA fragments generated by double digestion of KM322 with EcoRI, KpnI.

EcoRI, KpnI

Size (kb)	Calculated Molarity	Postulated Molarity
23.21	0.82	1
18.59	0.76	1
15.56	0.81	1
14.61	0.75	1
13.51	0.80	1
9.00	1.13	1
8.04	1.69	1 + 1
6.86	1.92	1 + 1
6.46	0.59	1
5.34	1.11	1
4.84	1.28	1
4.27	1.10	1
3.70	1.17	1
3.25	2.30	1 + 1
2.58	0.63	1
2.34	0.74	1
2.11	0.96	1

Genome size = 162.42kb

Table 6.11 DNA fragments generated by double digests of KM322 and KM91 DNA with HindIII, HpaI. Data apply to KM322 and KM91 except where specified.

HindIII, HpaI

Size (kb)	Calculated Molarity	Postulated Molarity
24.03	0.37	0.5
22.03	0.40	0.5
19.57	0.84	1
18.36	1.39	1 + 0.5
15.98	0.44	0.5
12.13	1.15	1
8.62	1.20	1
7.38	0.79/1.83	KM322 : 1/KM91: 1+1
KM322: 5.22	1.08	KM322 : 1
4.88	0.93	1
4.74	1.12	1
4.53	0.99	1
3.56	3.75	1+1+1+1
2.93	1.17	1
2.59	1.97	1+1
2.33	1.37	1+1
2.21	0.84	1

Table 6.11 continued

2.10	0.65	1
1.89	3.86 / 2.90	KM322 : 1+1+1+1 / KM91: 1+1+1
1.74	0.85	1

Genome size = KM322: 166.25kb / KM91: 166.52kb

Figure 6.2 Gels showing digestion of αHVS (KM322 and KM91) with HindIII, EcoRI, BamHI and KpnI. Bands showing differences between the two strains are marked. Samples were run in duplicate.

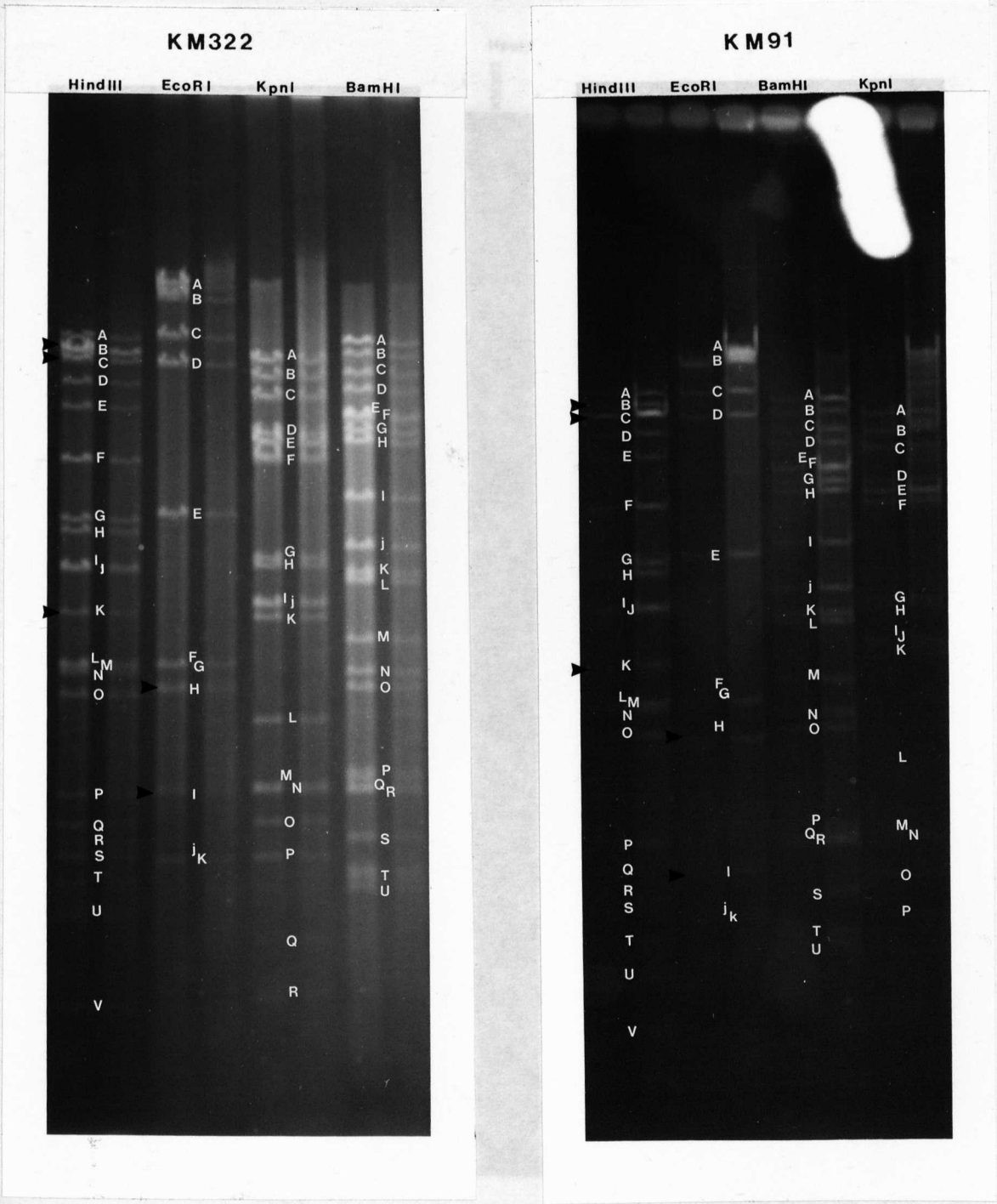


Figure 6.3 Gel showing digestion of αHVS (KM322 and KM91) with HpaI and HindIII, HpaI. Bands showing differences between the two strains are marked.
Samples were run in duplicate.

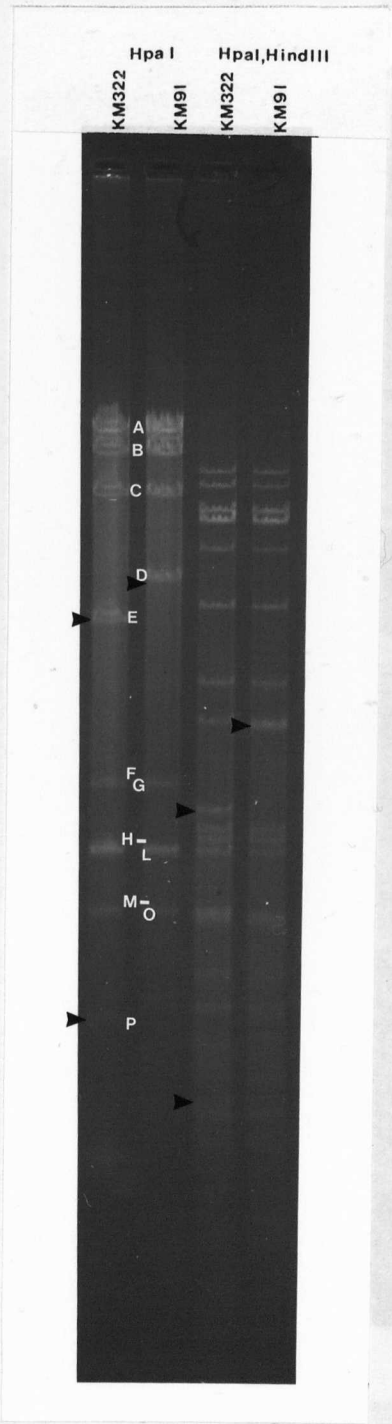


Figure 6.4 Gels showing digestion of KM322 with EcoRI, HindIII; BamHI, HindIII; HindIII, KpnI and EcoRI, KpnI. Samples were run in duplicate.

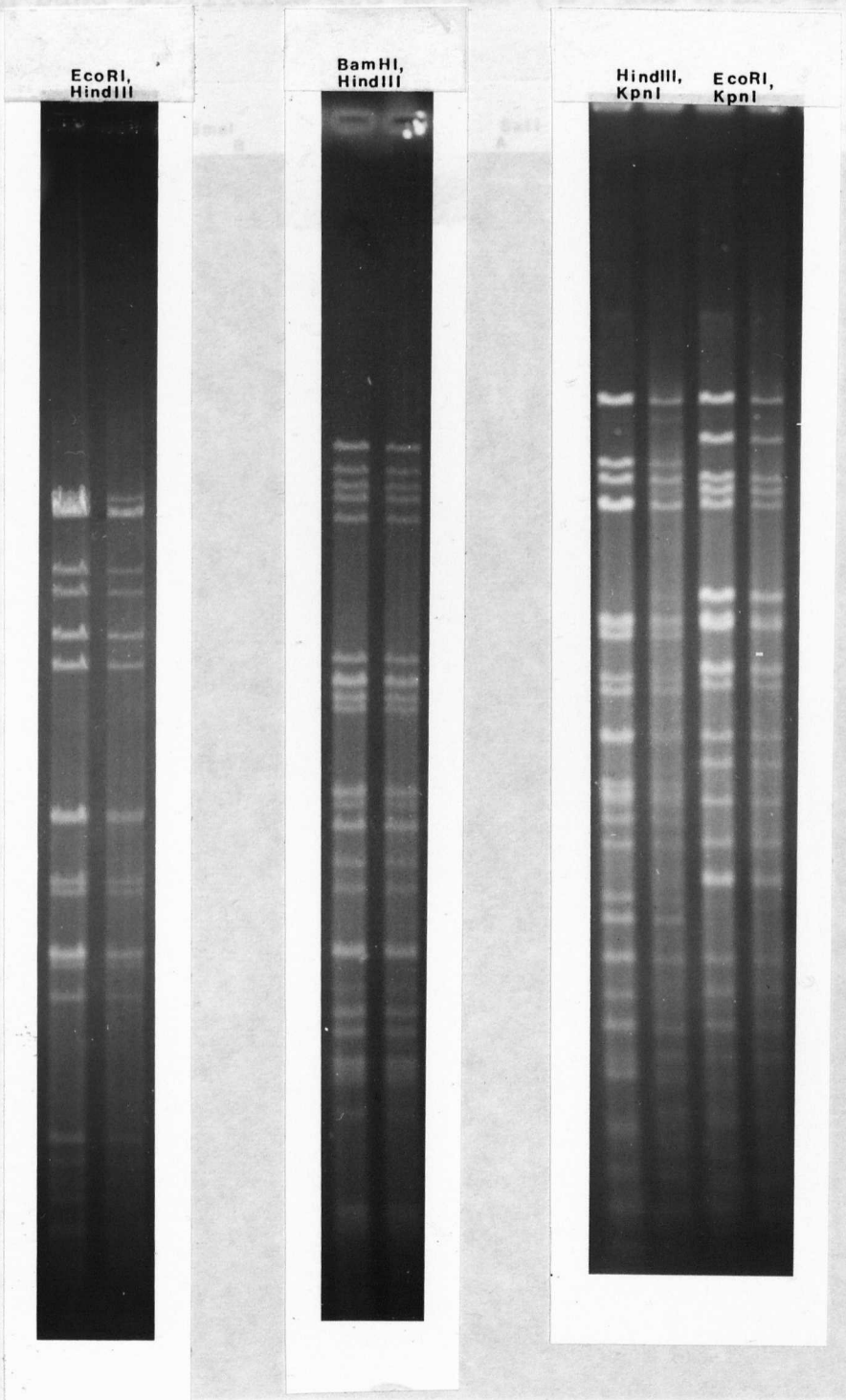
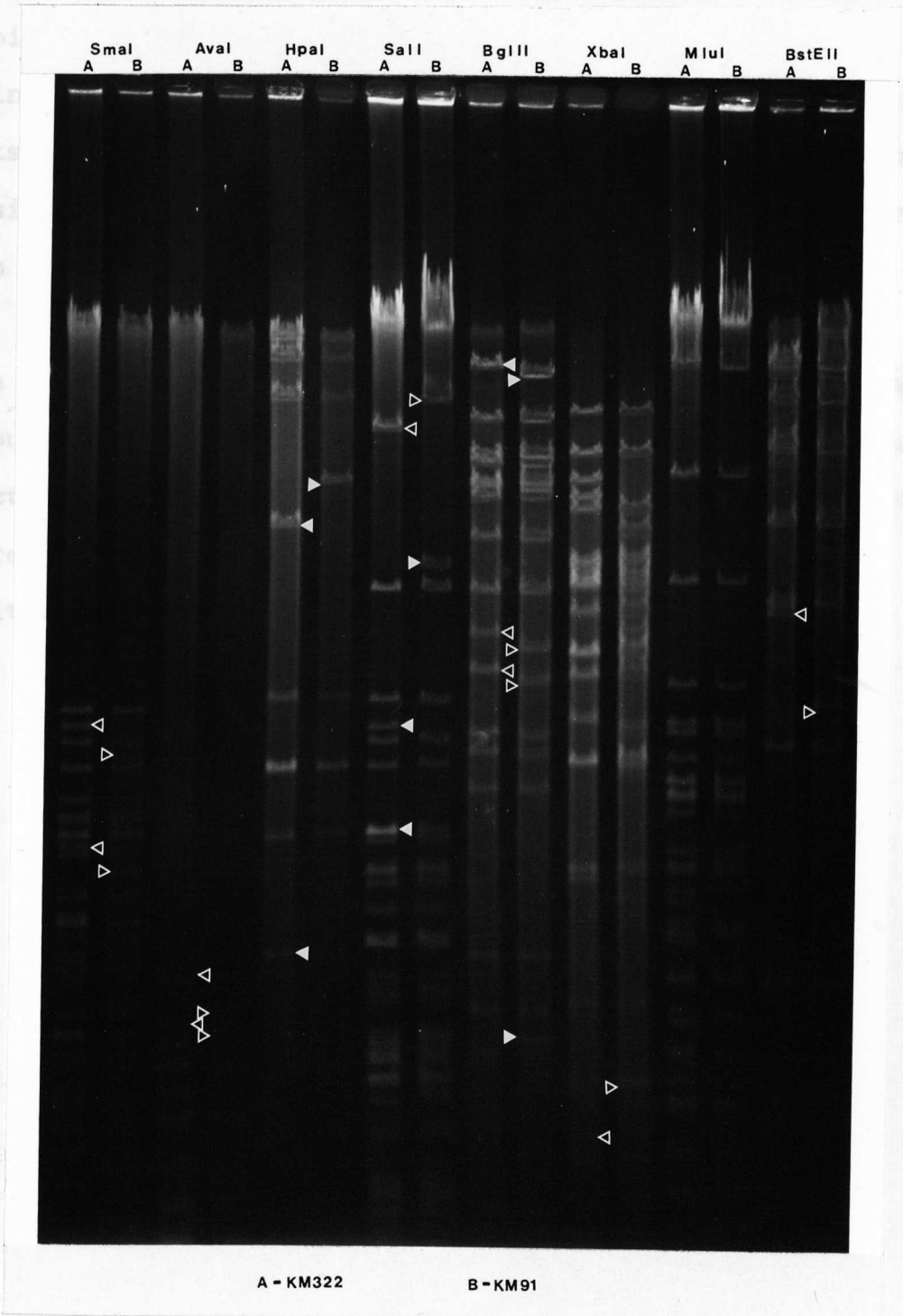


Figure 6.5

Gel showing digestion of αHVS (KM322 and KM91) with SmaI, AvaI, HpaI, SalI, BglII, XbaI, MluI and BstEII. Differences in band mobilities are marked (△) and RFLPs are marked (▲).

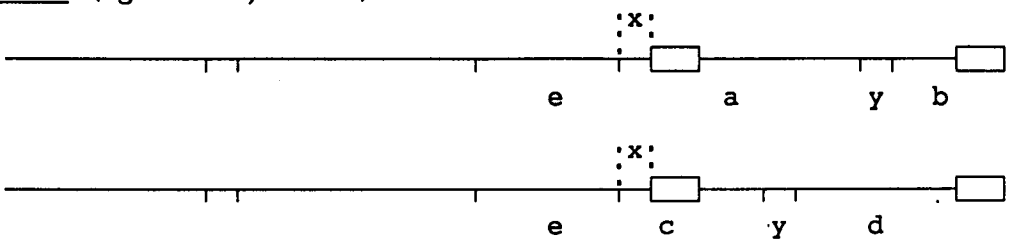


None of the above enzymes or PstI or XhoI (data not shown) revealed 0.25M fragments in αHVS DNA. In order to rule out the possibility of 0.25M fragments being obscured due to co-migration with other fragments double digests were performed. The analysis of fragment molarities and relationships from these results distinguish between the possibility of αHVS being a D or E structure (see fig. 6.1). Assuming the presence of 0.25M fragments in HindIII and BamHI digests in a number of permutations it was not possible to make single and double digests agree with respect to cleavage models for 4 isomeric arrangements (Wilkie et al, 1977).

Once a D model for αHVS DNA was applied to the data single and double digest cleavage models were found to agree with respect to submolar fragments. The cleavage models for αHVS DNA are illustrated in fig. 6.6 with the corresponding molarities and fragment relationships.

Figure 6.6 Cleavage models for the two αHVS genome arrangements. Molar ratios and size relationships of DNA fragments are noted.

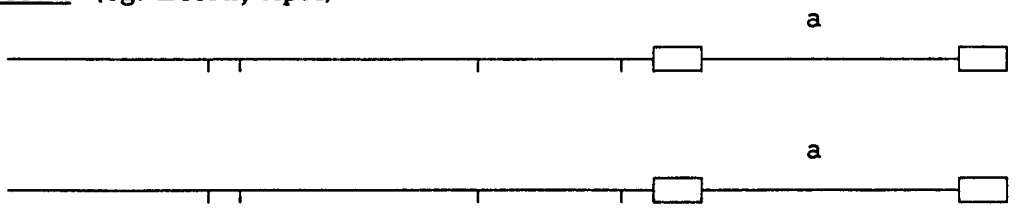
Model 1 (eg. BamHI, HindIII)



0.5 M fragments: a, b, c, d. All other fragments 1M

$a + b = c + d$, $a - d = x$, $c - b = x$
$S = a + b - x + y$, $S = c + d - x + y$

Model 2 (eg. EcoRI, HpaI)



All fragments 1M, $a > S$

Model 3 (eg. KpnI)

All fragments 1M

In double digests:

Model 1 + 1 = 1	Model 2 + 2 = 2
Model 1 + 2 = 1	Model 2 + 3 = 3
Model 1 + 3 = 3	Model 3 + 3 = 3

The αHVS fragments from double and single digests of KM322 were analysed according to these models.

HindIII

KM322:

Model 1

a = 24.66 a + b = 40.66

b = 16.00 c + d = 39.56

c = 21.05 x = a - d = 6.15

d = 18.51 x = c - b = 5.05

$\bar{x}$ = 5.60

e = 22.41

S = 35.06 + y

S = 33.96 + y

$\bar{S}$ = 34.51 + y

HindIII

KM91:

Model 1

a = 24.66 a + b = 40.66

b = 16.00 c + d = 40.92

c = 22.41 x = a - d = 6.15

d = 18.51 x = c - b = 6.41

$\bar{x}$ = 6.28

e = 21.05

S = 34.38 + y

S = 34.64 + y

$\bar{S}$ = 34.51 + y

BamHI

KM322 and KM91: a = 21.56 a + b = 36.56

b = 15.00 c + d = 36.31

Model 1 c = 18.97 x = a - d = 4.22

d = 17.34 x = c - b = 3.97

$\bar{x}$ = 4.10

S = 32.46 + y

S = 32.21 + y

$\bar{S}$ = 32.34 + y

EcoRI

Km322 and KM91: a = 35.49 > S

Model 2

HpaI

KM322 and KM91: a = 32.17 > S

Model 2

KpnI

KM322 and KM91: KpnI cuts within IR_S/TR_S

Model 3

EcoRI, HindIII

KM322: a = 24.02 a + b = 40.22

b = 16.20 c + d = 40.20

Model 1	c = 22.39	x = a - d = 6.21
Model 2 + 1 = 1	d = 17.81	x = d - b = 6.19
0.5M fragments determined		$\bar{x}$ = 6.20
by HindIII sites		

S = 34.02 + y

S = 34.00 + y

$\bar{S}$ = 34.01 + y

HpaI, HindIII

KM322:	a = 24.03	a + b = 40.01
	b = 15.98	c + d = 40.39
Model 1	c = 22.03	x = a - d = 5.67
Model 2 + 1 = 1	d = 18.36	x = c - b = 6.05
0.5M fragments determined		$\bar{x}$ = 5.86
by HindIII sites		

S = 34.15 + y

S = 34.53 + y

$\bar{S}$ = 34.34 + y

BamHI, HindIII

KM322:	a = 19.44	a + b = 34.70
	b = 15.26	c + d = 35.03
Model 1	c = 18.18	x = a - d = 2.59
Model 1 + 1 = 1	d = 16.85	x = c - b = 2.92
0.5M fragments determined		$\bar{x}$ = 2.76
by BamHI.		

S = 31.94 + y

S = 32.27 + y

$\bar{S}$ = 32.11 + y

KpnI, HindIII

KM322:

Model 3

Model 3 + 1 = 3

All fragments 1M.

EcoRI, KpnI

KM322:

Model 3

Model 2 + 3 = 3

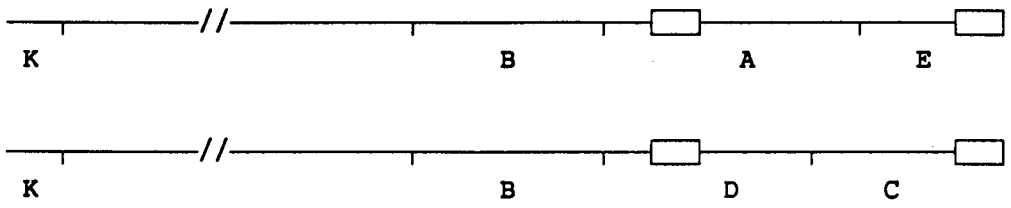
All fragments 1M.

The digests producing model 1 cleavage patterns were used to calculate a mean minimum length for S. A mean maximum length for S was provided from model 2 digest patterns. BamHI terminal fragments are smaller than those of HindIII as seen from double digests with HindIII, BamHI. Therefore the theoretical fragment(s), y, have a value of at least 34.29-32.23 = 2.06kb. Only 0.5M fragments determined by HindIII sites were used to calculate a minimum S length.

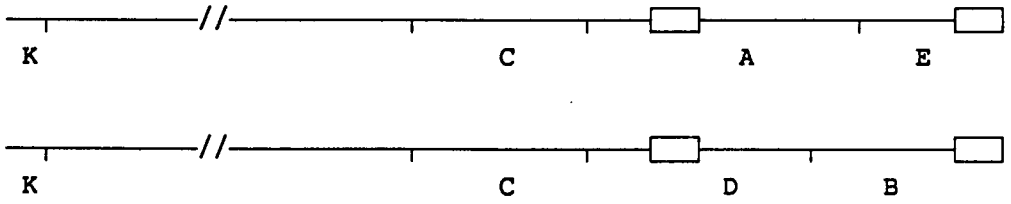
Minimum length S (kb)	Maximum length S (kb)
HindIII: 34.51	EcoRI: 35.49
EcoRI, HindIII: 34.01	HpaI: 32.17
HpaI, HindIII: 34.34	
mean = 34.29	mean = 33.83

Figure 6.7 Postulated genome positions of specific αHVS (KM91 and KM322) HindIII, BamHI, EcoRI and HpaI fragments.

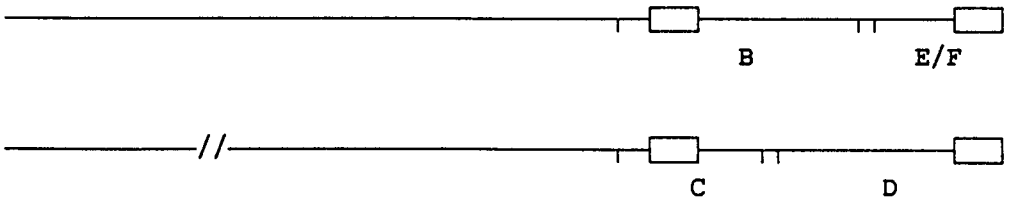
HindIII KM322



HindIII KM91



BamHI, KM322 and KM91



EcoRI, KM322 and KM91

HpaI, KM322 and KM91

The minimum length for S is close to the maximum length. These two values were therefore used to calculate an approximate length for S.

$$S = 34.06kb$$

These calculations suggest that in HindIII digests the fragment(s) y are very small or do not exist.

The mean genome length was calculated from the totals of all KM322 digests as 155.19kb.

S represents $\frac{34.06}{155.19} \times 100 = 22\%$ of the genome

L is therefore 121kb long i.e. 78% of the genome

The postulated locations of specific RE fragments within the αHVS genome are shown in figure 6.7.

6.4 Discussion

The analysis of single and double digests of αHVS DNA with HindIII, EcoRI, BamHI, KpnI and HpaI suggest that the genome has two isomeric arrangements. This is in agreement with the findings of Kit et al (1980) and Desrosiers and Falk (1981), but contrary to the finding of Leib et al (1987b). No 0.25M

fragments were detected with any of the above enzymes or with SmaI, AvaI, SalI, BglII, XbaI, MluI, BstEII, PstI or XhoI (data not shown). Cleavage models for a two isomer structure only would fit the data produced by single and double RE digests.

HindIII cuts within U_S and the long segment but not within the repeat sequences producing 0.5M internal and terminal fragments within S. Calculations for the length of S suggest that the 0.5M fragments of HindIII comprise the complete short segment and that y is very small or does not exist. KM91 and KM322 differ with respect to a 0.5M fragment in HindIII digests (table 6.2). KM322 has a 22.41kb 1M fragment and a 21.05kb 0.5M fragment while the reverse is true of KM91. The difference in 0.5M fragment sizes is probably due to a different value for x between the two viruses (see fig. 6.6) such that the fragment termed e, which defines the size of x, would also differ for each strain. Therefore it is postulated that fragment e is 21.05kb in KM91 and 22.41kb in KM322. This model also predicts that the other internal 0.5M fragment, HindIII A, will also differ between the two viruses. It is likely that such a difference is not being detected in the gels. Differences in magnitude between larger fragments are less well resolved than between lower molecular weight fragments.

BamHI also cuts within L and U_S but not IR_S/TR_S, producing 0.5M fragments. The HindIII, BamHI double digest suggests that in BamHI digests the short segment is composed of the 0.5M internal and terminal fragments and one or more fragments, y, totalling approximately 2kb.

EcoRI RE sites are situated only within the long segment of αHVS and therefore all digest fragments are 1M. It is postulated that the 35.49kb fragment encompasses the entire short segment and this is confirmed by comparison with the mean estimates for the length of S. Double digests of αHVS DNA with EcoRI and HindIII (table 6.7) and also EcoRI, BamHI (data not shown) are in agreement with the postulate that EcoRI does not cleave within the short segment.

KpnI cleavage of αHVS DNA produces only 1M fragments. The recognition sites are situated within L,U_S and IR_S/TR_S. This is confirmed by the fact that double digests with any enzyme and KpnI produces only 1M fragments.

HpaI digestion of αHVS DNA produces a type 2 cleavage model (fig. 6.6). As with EcoRI this enzyme does not cut within S. The 32.17kb fragment of HpaI cleaved αHVS DNA represents the entire short region. This model is confirmed by the results of double RE digests with a model 1 enzyme, HindIII (table 6.11).

Further experiments involving hybridisation studies of αHVS fragments with other αHVS fragments and isolation of RE fragments before second digestion should allow the completion of restriction endonuclease maps for αHVS. In addition electron microscopy studies such as those described by Ruyechan et al (1982) will allow the size of the inverted repeats bracketing U_S to be determined.

RE analysis of KM91 and KM322 shows minor mobility differences between the two in HindIII fragments B, C and K (Table 6.2, fig. 6.2). The variation in B and C, described above, suggests a local rearrangement of sequences within L near the junction of L and IR_S, a region of high variability in herpesvirus genomes. The mobility difference of HindIII K is in agreement with the findings of Desrosiers and Falk (1981) who noted the variability in the size of this fragment between isolates of αHVS. The difference of approximately 240bp is probably produced by a deletion / insertion of tandem repeat sequences at the terminus of the long segment (Roizman and Tognon, 1983). Thus, it is postulated that HindIII K is the terminal or a subterminal fragment of the long segment.

Mobility differences were also observed within EcoRI H and I between KM322 and KM91 (table 6.4, fig. 6.2). These

fragments are likely to be terminal or sub-terminal L fragments or fragments close to the L-S junction.

HpaI cleavage of αHVS DNA produces a RFLP with KM91 having one less HpaI site than KM322 (table 6.6, fig. 6.3). KM322 HpaI fragments E and P approximate to the size of KM91 HpaI D. This variation provides a stable strain marker to differentiate KM91 and KM322 (Roizman and Tognon, 1982). This difference in nucleotide sequence is located within the L segment of αHVS.

Further studies are required to establish whether the locus / loci determining the variation in neurovirulence of KM91 and KM322 is related to the region of the genome containing the variable HpaI site. Completion of restriction site maps for αHVS would be most useful for such studies and would permit the precise localisation of the HpaI RFLP to be established. Possible approaches for future work include site-directed mutagenesis of αHVS and analysis of subsequent phenotypes, marker-rescue of neurovirulence of KM322 or protective immunogenicity of KM91 by construction of intra-strain recombinants using fragments of DNA known to include differences between the two strains. A further area to be investigated by molecular biological techniques is the question of whether varying plaque size and varying immunogenicity between KM91 and KM322 are specified by the

same genetic locus/loci.

The gel illustrated in fig. 6.5 shows two further RFLPs between KM322 and KM91 with the enzymes SalI and BglII. The two extra SalI fragments of KM322 summate to the approximate value of the extra KM91 fragment. Two extra KM91 BglII fragments approximate to the value of the extra KM322 fragment. A large number of mobility differences between the two αHVS strains are also evident with enzymes SmaI, AvaI, SalI, BglII, XbaI, MluI and BstEII (fig. 6.5). These enzymes were not used in double digests due to the large number of RE sites. The identification of three enzymes (HpaI, SalI and BglII) exhibiting RFLPs between KM91 and KM322 provide a focus for further investigations to identify the genetic basis for phenotypic differences between the two strains. It would be interesting to establish whether the three RFLPs occur within the same region of DNA.

To summarise, the αHVS genome has been partially characterised. The evidence presented here strongly suggests a structure with two isomeric forms with the S segment only inverting. The length of S has been estimated as 34kb and the total genome as 155kb. L is therefore 121kb and S and L represent 22% and 78% of the total genome respectively. Three enzymes have been identified producing stable RE profile differences between KM322 and KM91. These should be

useful for determining genetic differences between the αHVS strains.

CHAPTER 7: ANALYSIS OF αHVS AND HSV-1 DNA SEQUENCE HOMOLOGY BY SOUTHERN BLOTTING

7.1 Introduction

The study of the evolution of the Herpesviridae has included analysis of nucleotide sequence homology between individual members of the family. Thus the studies on gross genome structure discussed in Chapter 6 have been complemented by more detailed examination of DNA sequences.

Early experiments comparing HSV-1 and HSV-2 by RE analysis showed considerable differences between the profiles of the two strains (Skare et al, 1975; Cortini and Wilkie, 1978). These were more significant than intrastrain variations as would be expected from the biological differences between the two viruses.

Comparative studies were also performed using the technique of Southern hybridisation or Southern blotting (Southern, 1975). This involves transferring DNA fragments from gels onto filters and thus immobilising them for use in hybridisation studies using labelled DNA probes of either complete viral DNA or specific fragments. In this way Ludwig et al (1972) found 70% homology between the DNAs of HSV-1 and HSV-2 and 8 to 10% sequence homology between pseudorabies virus (PRV) and the herpes simplex viruses. Kieff et al (1972) performed Southern hybridisation experiments with whole HSV-1 and HSV-2 DNA and detected approximately 50%

homology. However, they noted that homology varied across the genome suggesting the presence of variable and invariable regions of DNA. Wilkie et al (1979) used Southern blotting to demonstrate that the inverted repeat sequences of HSV-1 and HSV-2 share considerably less sequence homology than the unique sequences. This finding supports the results of RE studies described in Chapter 6 in which the repeat sequences are less stable than the unique regions in HSV (Roizman and Tognon, 1983).

Davison and Wilkie (1983) performed hybridisation experiments to investigate homologous regions in the genome of HSV-2, HSV-1, equine herpesvirus-1 (EHV-1), PRV and varicella-zoster virus (VZV). They found highly conserved regions of DNA between HSV and the other three alphaherpesviruses and suggested colinearity of genes within these regions. The genes lying within these HSV regions, and therefore possible candidates for the highly conserved genes, included the major DNA binding protein, the major capsid protein, the DNA polymerase and the immediate early protein ICP4. These results were in good agreement with the fact that antigenic cross-reactivity and amino-acid sequence homology have been detected between the major DNA binding proteins of HSV-1, HSV-2, PRV, bovine herpesvirus-1 (BHV-1) and EHV-1 (Yeo et al, 1981; Littler et al, 1981). Antigenic cross-reactions were also observed between the major capsid proteins of these

viruses and between glycoproteins of HSV-1, HSV-2 and BHV-1 (Yeo et al, 1981).

The location of specific genes on the HSV genomes has been determined by the analysis of gene products produced by mutants and HSV-1/HSV-2 intertypic recombinants (Wilkie et al, 1979; Morse et al, 1978).

The genomes of the human herpesviruses; HSV-1, VZV and EBV, have now been entirely sequenced (McGeoch et al, 1985; McGeoch et al, 1986; McGeoch et al, 1988; Perry and McGeoch, 1988; Davison and Scott, 1986; Baer et al, 1984). Thus the arrangement of many of the viral genes has been elucidated and comparisons of the positions of genes and their nucleotide sequences have been made between the herpesviruses.

The HSV-1 genome is now known to encode 56 genes within U_L, 12 in U_S and 1 in R_L and R_S. There are 72 genes coding for 70 distinct proteins recognised so far (the genes encoding ICPO and ICP4 are within the repeat sequences and are therefore diploid). There is the possibility that other genes exist overlapping or between recognised coding sequences. Functions have been identified for 27 of the HSV-1 genes (McGeoch, 1989). Eleven of the genes within U_S are dispensable for growth of HSV-1 in tissue culture (Longnecker and Roizman, 1987; Longnecker et al, 1987; Weber

et al, 1987).

The information derived by sequencing the HSV-1 genome has allowed confirmation of the fact that

the repeat sequences show greater divergence between HSV-1 and HSV-2 than unique sequences. Coding sequences of HSV-1 and corresponding HSV-2 genes show 70 to 80% identity (McGeoch, 1989).

The VZV genome encodes at least 70 genes coding for 67 unique proteins (Davison and Scott, 1986). Most HSV-1 genes have VZV counterparts, some with a high degree of homology (up to 59%) and others with only slight similarities, such as HSV-1 ICPO and VZV61 (McGeoch, 1989). The two genomes show a similar functional organisation but with regions of divergence (Davison and Scott, 1986). These differences are most pronounced within the S segments.

EBV codes for approximately 80 proteins. There are EBV counterparts to 29 of the 67 VZV genes and all are in the U_L component of the genomes (Davison and Taylor, 1987). These conserved genes do show related layouts within the two genomes, but there are large scale rearrangements of these colinear regions. There are no EBV counterparts of VZV U_S genes.

Human cytomegalovirus (HCMV) shows some gene homology with the other human herpesviruses including the DNA polymerase and major DNA binding protein. These genes show good conservation and colinearity with EBV. The U_S region of HCMV has been sequenced and shows no homology with HSV-1, VZV or EBV (Mach et al, 1989). The HCMV terminal 'a' sequence is related to that of HSV. As with HSV and PRV the immediate early genes of HCMV map close to and/or within the reiterated sequences (Roizman, 1980).

Analysis of the DNA of the recently isolated human herpesvirus-6 (HHV-6) suggests that this virus is more closely related to HCMV than to EBV or the alphaherpesviruses (Efstathiou et al, 1988).

The genes conserved within the herpesvirus family from DNA sequencing studies include gB and gH homologues, the major DNA binding protein and the DNA polymerase (McGeoch, 1989).

Comparative studies of herpesvirus genomes reveal valuable information on the evolution of this diverse family of viruses. The studies reviewed here and in the introduction to Chapter 6 imply a common ancestor, with divergent evolution producing a wide variation in the genomes and extensive phenotypic change (Honess, 1984; Roizman, 1980; McGeoch, 1989). Phylogenetic trees have been constructed to

illustrate the divergence of herpesviruses from comparative analysis of viral genes (Gentry et al, 1988; Honess et al, 1989b).

Previous workers have performed experiments comparing αHVS DNA with that of HSV-1. Desrosiers and Falk (1981) used whole HSV-1 DNA to probe fragments of αHVS DNA. They found 16% cross-hybridisation across the length of the αHVS genome under stringent conditions. Otsuka et al (1981) performed hybridisation studies on the TK genes of αHVS and HSV-1. They found no homology between the two, and their gene products have been shown to be antigenically distinct. Otsuka and Kit (1984) have sequenced the TK gene of αHVS and found no significant sequence homology with the TK genes of HSV-1 and HSV-2. Honess et al (1989b) found a 40% amino-acid sequence homology between the TK genes of αHVS and HSV-1 from the predicted polypeptides of these two viruses.

The experiments presented in this chapter describe the use of Southern hybridisation to investigate sequence homology between HSV-1 (KOS) and αHVS, KM91 and KM322. It was hoped that such experiments would provide information on the genetic relatedness between HSV-1 and αHVS and the organisation of certain genes within the αHVS genome. A number of selected fragments of HSV-1 DNA, representing 40%

of the genome, were used as probes against separated αHVS DNA fragments.

7.2 Materials and Methods

Table 7.1 lists the genes present in each HSV-1 (KOS) DNA fragment used as a probe. Also included are gene functions where known and % identity to VZV genes.

Fragments EcoRI n and f lie adjacent within U_L. EcoRI ek crosses the end of U_L, IR_L and ends within IR_S in the prototype isomer of HSV-1 DNA. EcoRI h lies within U_S and the terminus of the short repeat. pLPB1 is a PstI/BamHI subfragment of BamHI b and lies within the long repeat and therefore within EcoRI ek.

The HSV-1 EcoRI restriction map is illustrated in figure 7.1

Viral DNA was separated from vector DNA as described in section 2.14.1. EcoRI cloned fragments were digested with EcoRI and pLPB1 was digested with PstI and BamHI.

Southern hybridisations were performed as described in section 2.14.2-5. DNA from αHVS, KM91 and KM322, was extracted as described in section 2.13.3 and digested with HindIII and with EcoRI as described in section 2.13.4. HSV-1

(KOS) DNA digested with EcoRI and pBR325 linearised with EcoRI were included on each gel as positive and negative controls respectively.

Hybridisation was performed at Tm-32^{o}C for homologous hybrids and post-hybridisation washes were at Tm-60^{o}C for the second washes, Tm-55^{o}C for the third wash and Tm-32^{o}C for the fourth wash (Maniatis et al, 1982). Washing solutions are described in section 2.14.5.

Figure 7.1 The EcoRI restriction site map for prototype HSV-1 to illustrate the distribution of HSV-1 probes. (Skare and Summers,1977).

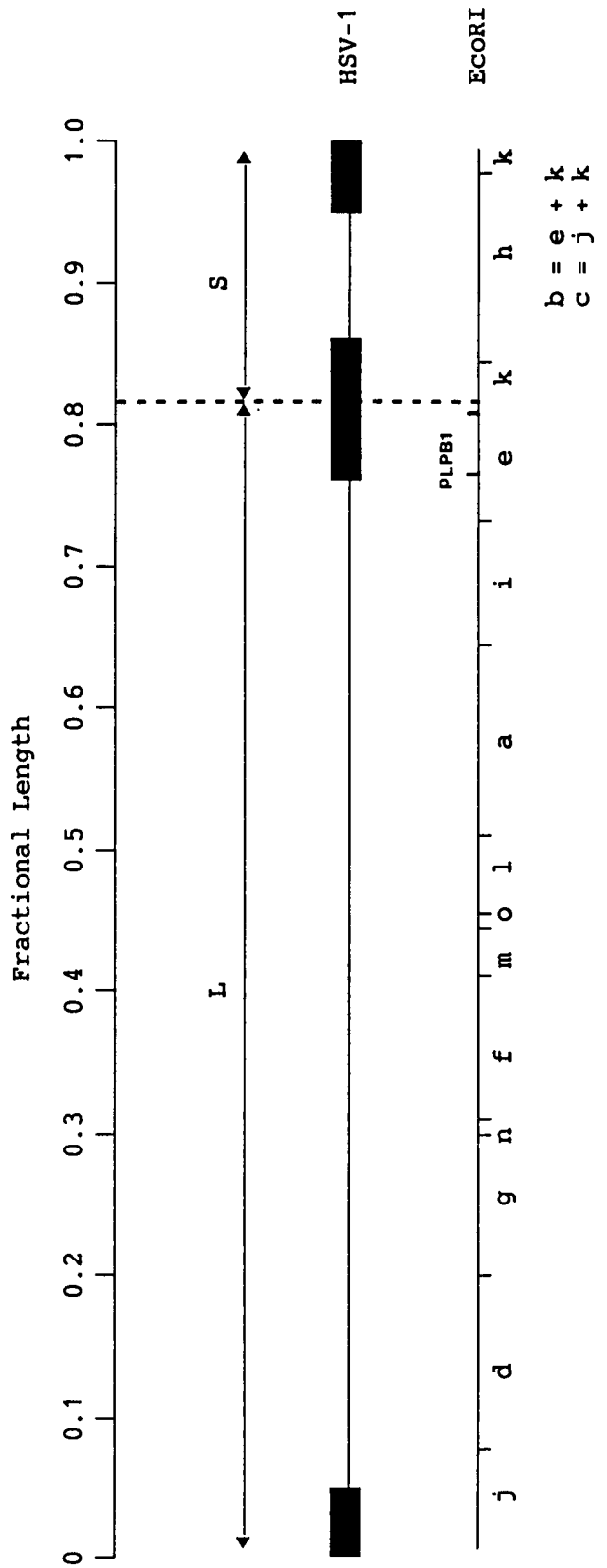


Table 7.1 Genes contained within HSV-1 (KOS) DNA fragments used to probe αHVS DNA, U_L and U_S denote genes within the unique long and unique short regions respectively. Functions where known are included in brackets. * indicates where only part of a gene is encoded (McGeoch, 1989).

DNA Fragment (Plasmid Vector)	Size (kb)	Genes Contained	% Identity to VZV
EcoRI n (pBR325)	2.416	UL22* (gH)	25
		UL23 (TK)	28
		UL24*	32
EcoRI f (pBR325)	16.135	UL24*	32
		UL25 (Virion protein)	44
		UL26 (DNA packaging)	34
		UL27 (gB)	45
		UL28 (structural)	46
		UL29 (DNA binding protein)	50
		UL30* (DNA polymerase)	52
EcoRI ek (pBR325)	21.439	UL52* (DNA replication)	37
		UL53 (Membrane protein)	29
		UL54 (IE protein)	20
		UL55	27
		UL56	--

Table 7.1 continued

		IE 110 (ICPO)	?
		IE 175 (ICP4)	?
EcoRI h	15.159	USI (IE 68)	?
(pBR325)		US2	--
		US3 (protein kinase)	33
		US4 (gG)	--
		US5 (glycoprotein)	--
		US6 (gD)	--
		US7 (gI)	23
		US8 (gE)	22
		US9 (virion protein)	24
		US10 (virion protein)	?
		US11	--
		US12	--
pLBP1	4.592	IE110* (LAT)	--
(PUC8)			

7.3 Results

A representative gel used for Southern blotting is shown in figure 7.2.

In all cases where positive results were detected, KM91 and KM322 showed hybridisation to equivalent fragments.

Autoradiographs were interpreted as being positive when hybridisation was observed with αHVS but not with the negative control. Conversely results were considered negative where the αHVS hybridisation disappeared at a lower or equal stringency wash to negative control DNA. αHVS HindIII and EcoRI band designations are listed in Chapter 6.

EcoRI n fragment of HSV-1 DNA hybridised under conditions of low stringency to αHVS HindIII fragments B or C (22.41kb KM322 / 21.05kb KM91), G (9.09kb) and I and/or J (7.27kb) and EcoRI fragments B (32.35kb) and E (9.44kb). These results were obtained after the 2nd washes (fig. 7.3). All hybridisation except the positive control was lost after the 3rd wash.

EcoRI f produced the strongest hybridisation signals of all fragments tested. Hybridisation was seen even after the 3rd wash involving αHVS HindIII fragments A (24.66kb) and H

(8.63kb) and EcoRI fragments A (35.49kb), E (9.44kb) and F and/or G (4.95kb) (Figure 7.4). The only hybridisation observed after a 4th wash was to the positive control DNA.

EcoRI ek was hybridised to a number of fragments of αHVS DNA after the 2nd wash. The negative control was also positive at this stage and the results are therefore not so reliable (figure 7.5). However, the results are noted since not all αHVS fragments hybridised to the probe suggesting some degree of specificity. Hybridising fragments were HindIII D (18.51kb), E (16.00kb), F (12.11kb) and an αHVS variable fragment K (KM91 5.77kb and KM322 6.01kb). EcoRI fragments were C (24.58kb) and a variable fragment H (KM91 4.31kb and KM322 4.51kb). All hybridisation except that to the positive control was lost after stringency was increased with a 3rd wash.

EcoRI h DNA produced no specific hybridisation with αHVS DNA fragments even at low stringency conditions (fig. 7.6). Neither αHVS fragments nor the negative control hybridised to probe DNA after the 2nd set of washes. However, there was no hybridisation to the positive control.
Similarly, pLPB1 did not hybridise with αHVS DNA at conditions of low stringency (Fig. 7.7). αHVS DNA did not bind to probe DNA after the 2nd set of washes while the negative control DNA remained hybridised.

These results are summarised in table 7.2 and postulated positions of specific αHVS fragments in the genome are shown in Figure 7.8 (see also Chapter 6).

Figure 7.2 A representative gel used for Southern blots. αHVS, KM322 and KM91, DNA cleaved with EcoRI and HindIII and HSV-1 (KOS) DNA and pBR325 DNA cleaved with EcoRI.

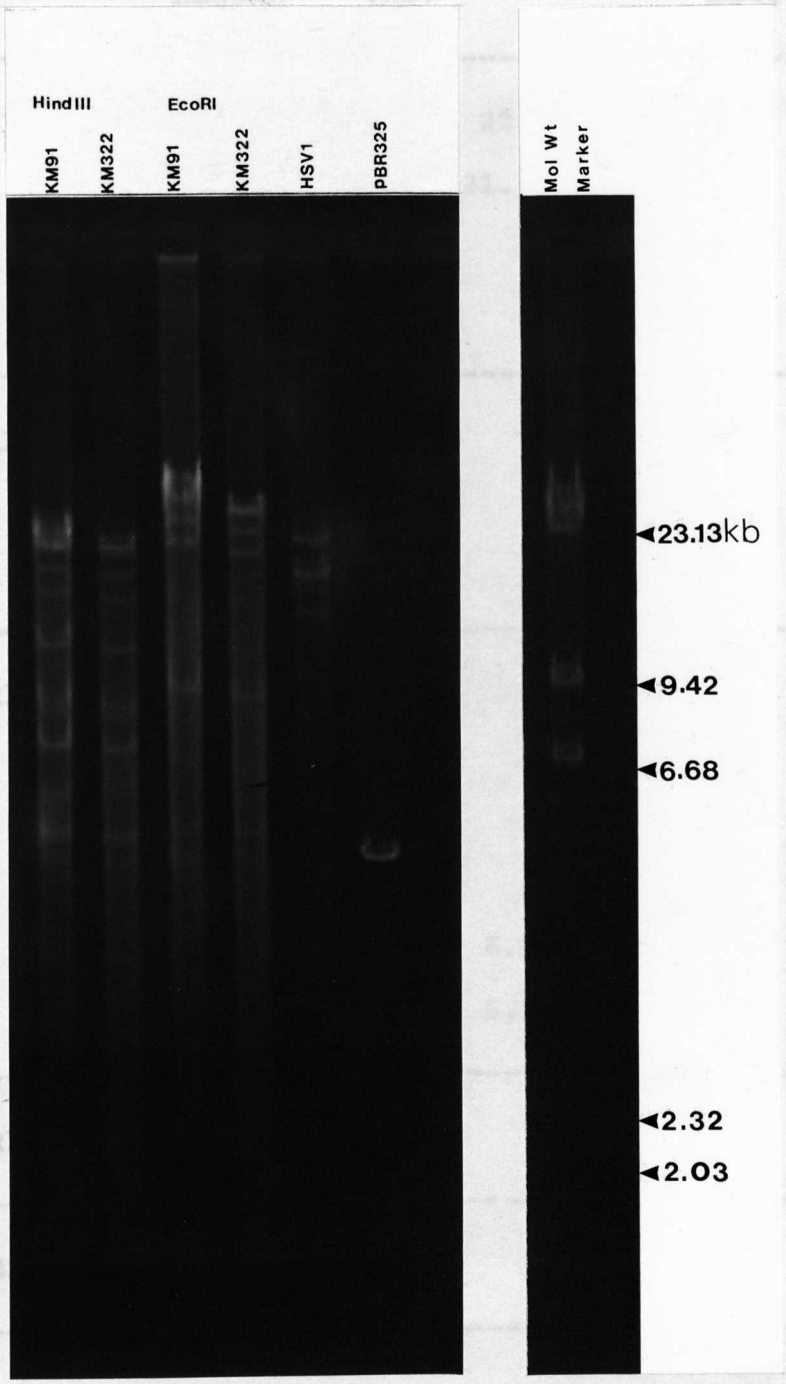


Table 7.2 Fragments of αHVS DNA hybridised to HSV-1 (KOS) probes at low stringencies.

HSV-1 Probe	αHVS HindIII Fragments		αHVS EcoRI Fragments	
	Genome Region	Length (kb)	Genome Region	Length (kb)
EcoRI n	L	KM322 22.41 KM91 21.05	L	32.35
	L	9.09	L	9.44
	L	7.27		
EcoRI f	S	24.66	S	35.49
	L	8.63	L	9.44
			L	4.95
EcoRI ek	S	18.51	L	24.58
	S	16.00	L	KM322 4.51 KM91 4.31
	L	12.11		
	L	KM322 6.01 KM91 5.77		
EcoRI h		--		--
pLPB1		--		--

Figure 7.3 Hybridisation of HSV-1 EcoRI n to αHVS DNA digested with HindIII and EcoRI.

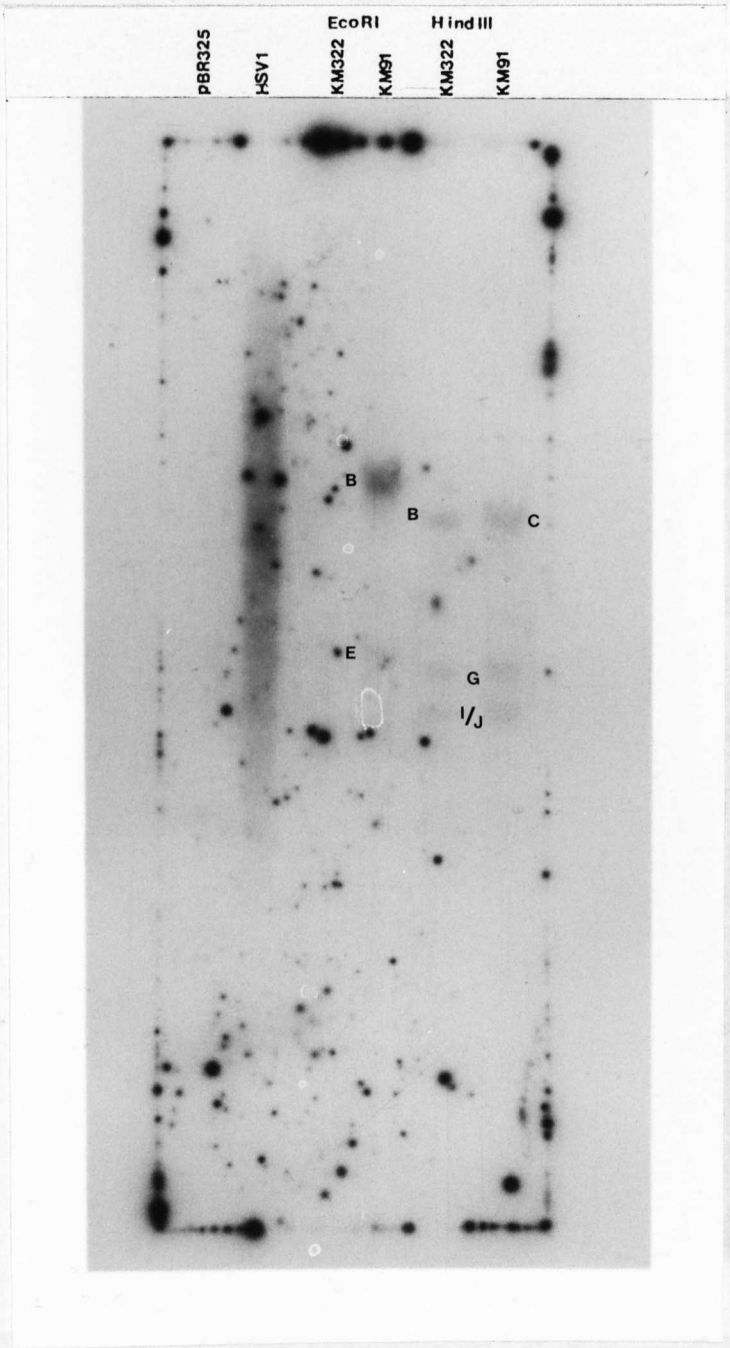


Figure 7.4 Hybridisation of HSV-1 EcoRI f to αHVS DNA digested with HindIII and EcoRI.

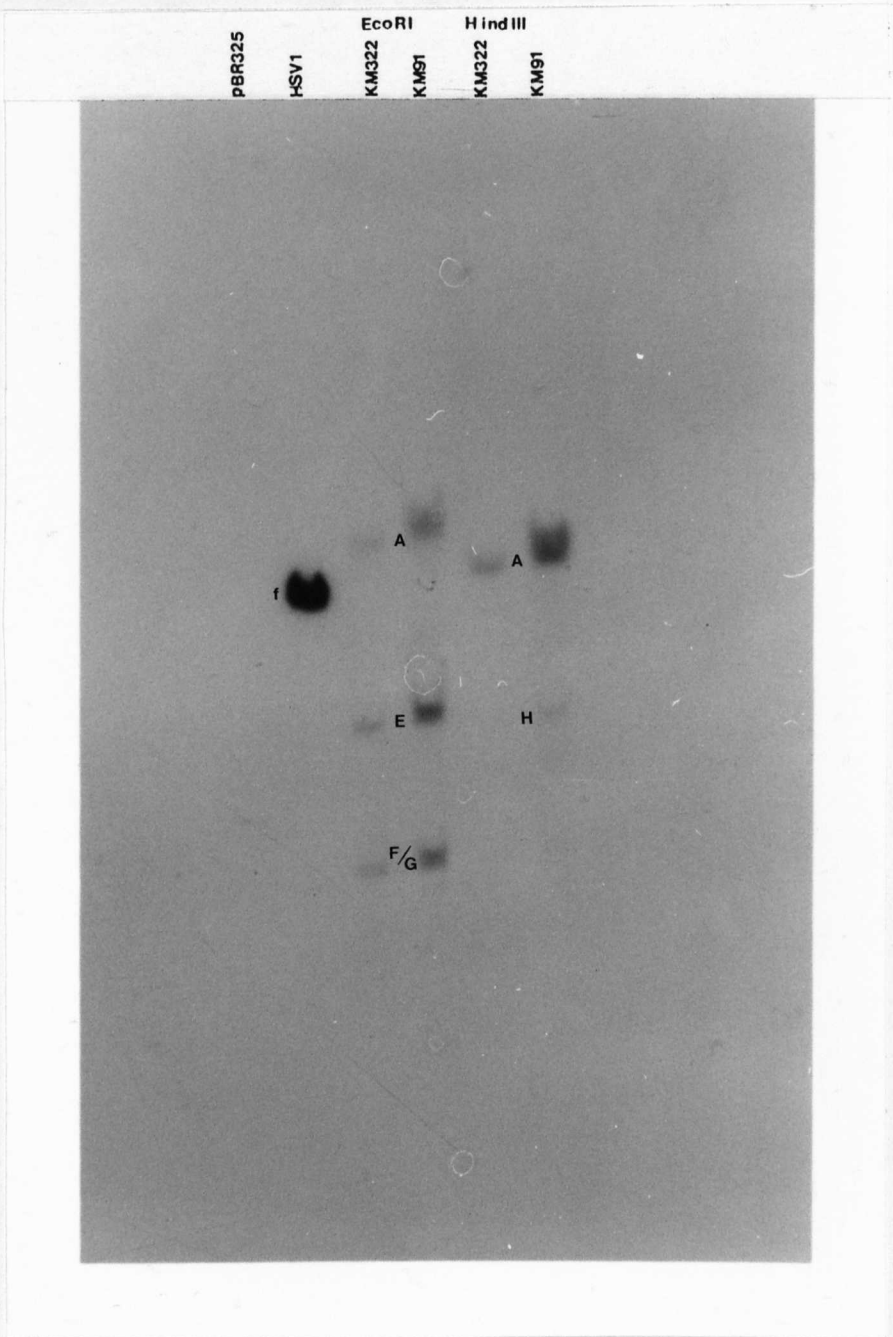


Figure 7.5 Hybridisation of HSV-1 EcoRI ek to αHVS DNA digested with HindIII and EcoRI.

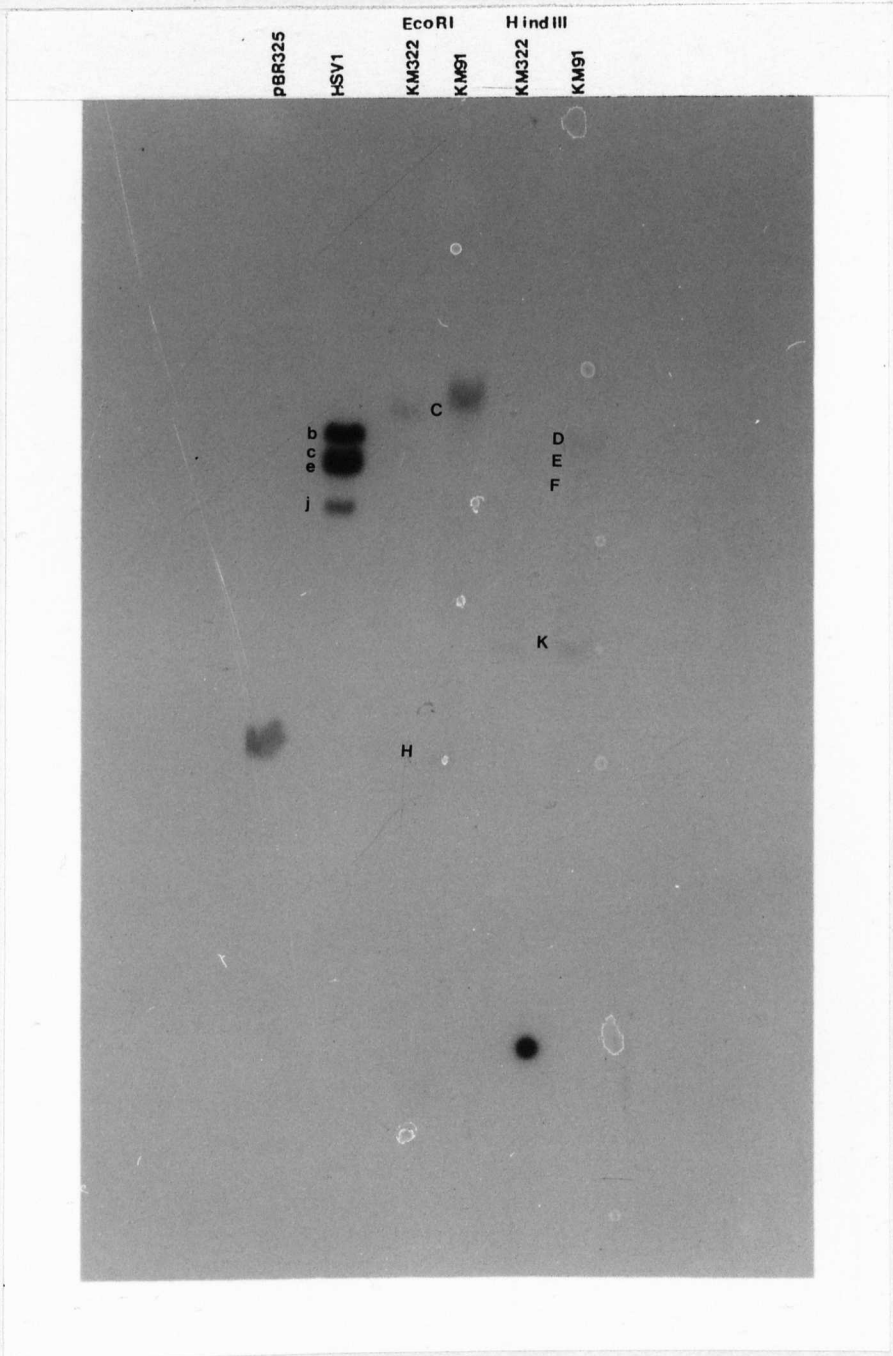


Figure 7.6 Hybridisation of HSV-1 EcoRI h to αHVS DNA digested with HindIII and EcoRI.

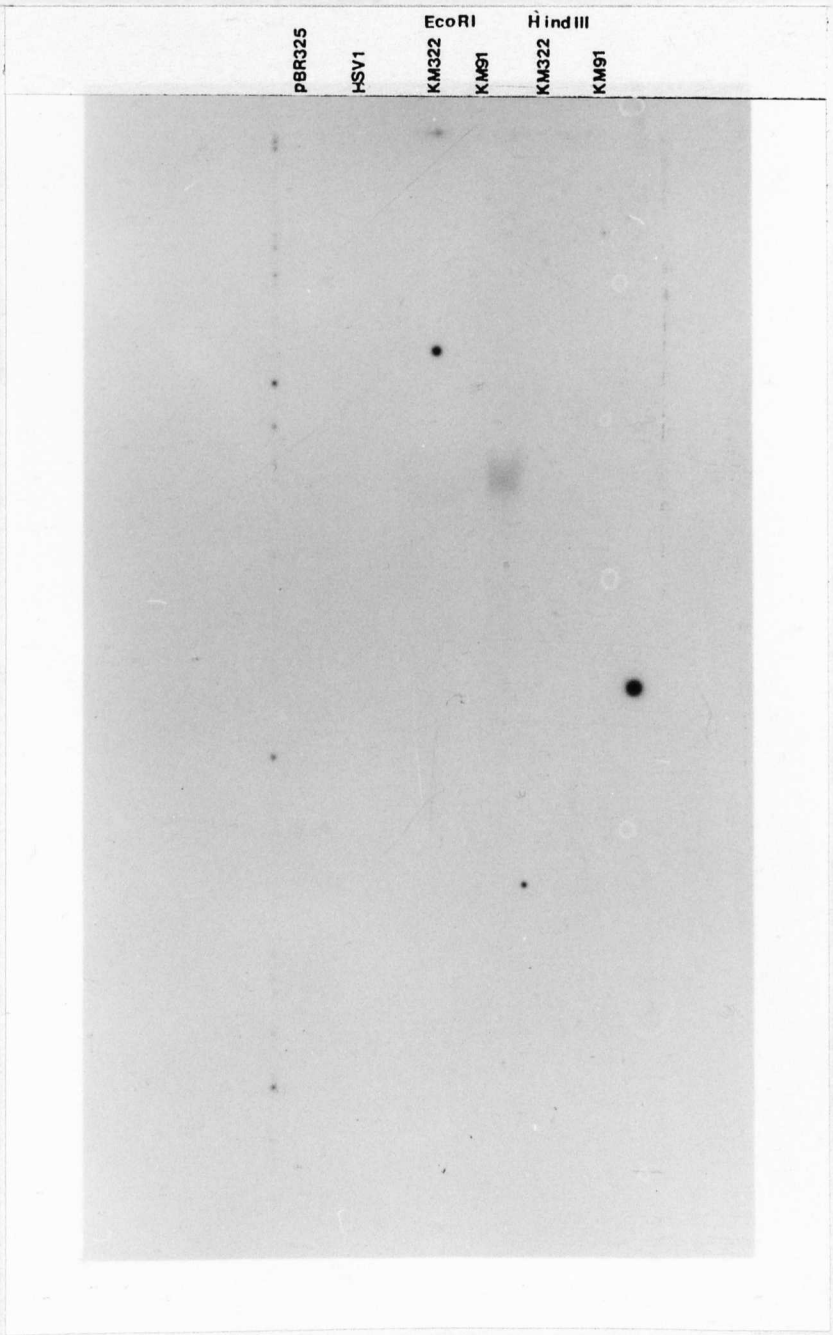


Figure 7.7 Hybridisation of HSV-1 pLPB1 to αHVS DNA digested with HindIII and EcoRI.

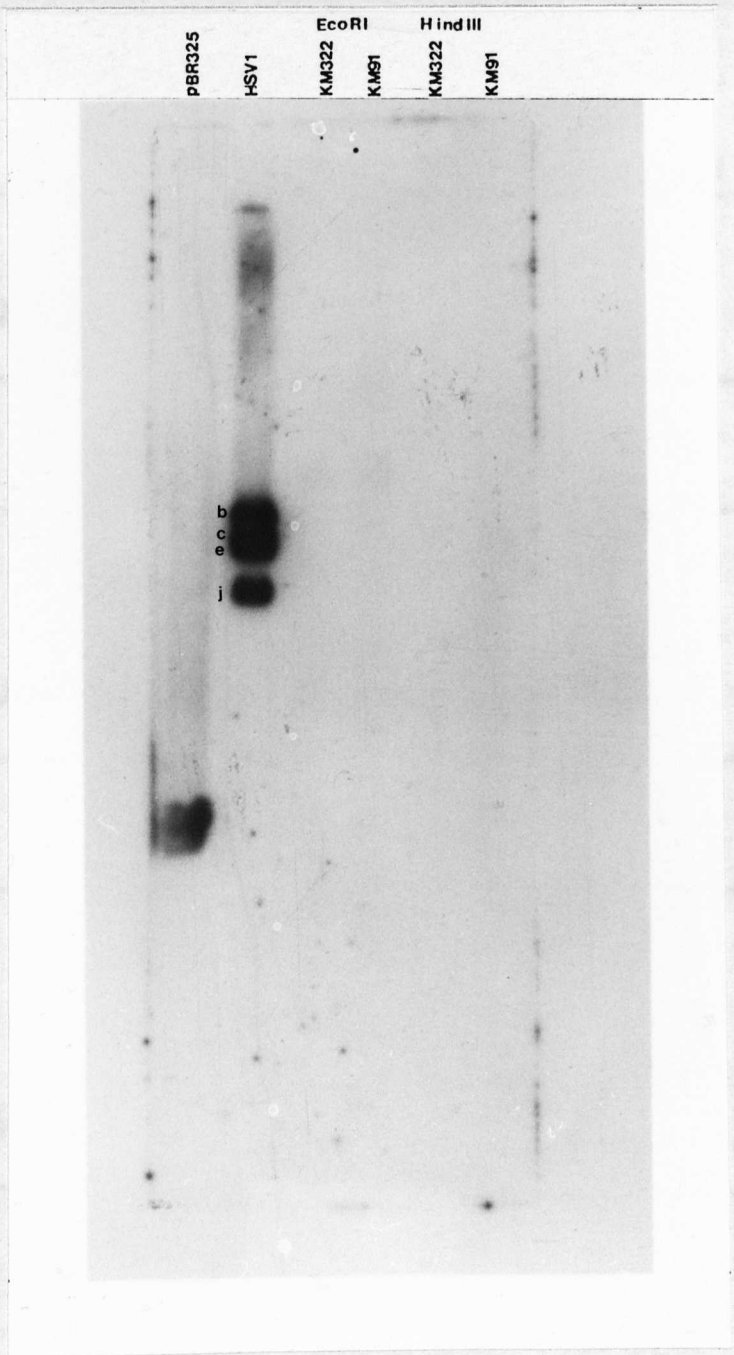
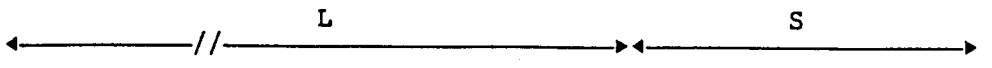
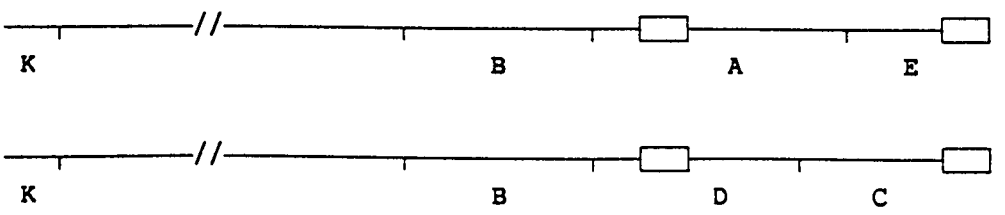


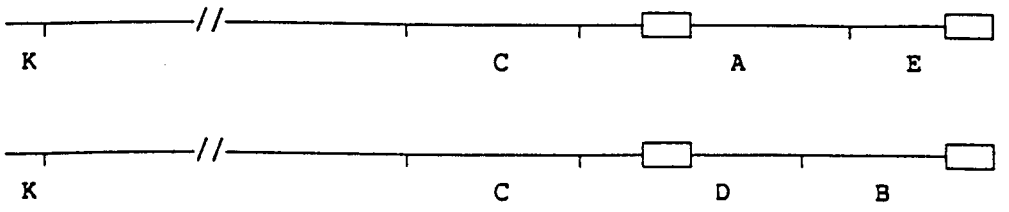
Figure 7.8 Postulated genome positions of specific αHVS (KM91 and KM322) HindIII and EcoRI fragments.



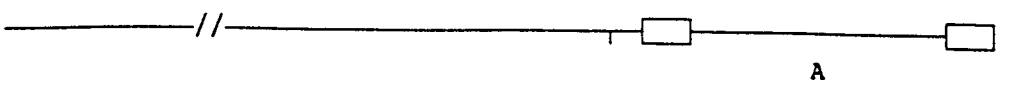
HindIII KM322



HindIII KM91



EcoRI, KM322 and KM91



7.4 Discussion

The results obtained from these hybridisation studies are qualitative rather than quantitative, and it is impossible to ascribe degrees of homology between the HSV-1 and αHVS sequences from them. However, the results are useful for identifying which fragments of αHVS, if any, are likely to contain sequences homologous to those of the HSV probes.

The results from all probes used show positive hybridisation only at relatively low stringencies. Hybridisation was performed in conditions such that most fragments hybridised to the probe and specificity was gradually increased by a series of washes. The strongest hybridisation observed did not withstand 0.5% SDS, 0.1 XSSC for 30 mins at 65^{o}C. This represents Tm-32^{o}C for homologous hybrids. Therefore, none of the HSV-1 fragments used hybridised to a significant extent to αHVS DNA. These results are in apparent disagreement with those of Desrosiers and Falk (1981) who found 16% homology between αHVS and HSV-1 DNA under conditions of Tm-34^{o}C. However, the results presented here apply to only 40% of the HSV-1 genome and therefore the two results are not necessarily mutually incompatible. The experiment was designed such that even low-stringency hybridisation that showed some degree of specificity, as determined by the negative control, might be informative as

to the distribution of specific genes within the αHVS genome. EcoRI n hybridised weakly to HindIII fragments B/C, G and I and/or J (22.41/21.05kb, 9.09kb and 7.27kb a 2M band) and to two EcoRI fragments B and E (32.35kb and 9.44kb) (figure 7.3). This HSV-1 fragment encodes part of the gH gene and the TK gene (table 7.1).

Glycoprotein H is generally well conserved amongst herpesviruses but shows no significant homology with αHVS sequences, at least for the portion of gH sequence present. However, it is possible that at least one of the fragments noted here may contain part or all of a gH equivalent for αHVS. The HSV-1 TK gene is also encoded by EcoRI n. Otsuka et al (1981) found no significant homology between the TK gene of αHVS and HSV-1. However, they have localised the αHVS TK gene within a 9.1kb HindIII fragment (Otsuka and Kit, 1984) and this fragment (HindIII G) hybridises to EcoRI n adding support to the specificity of fragments hybridising under low stringency conditions. All of the fragments hybridising to EcoRI n are from the U_L region of αHVS.

EcoRI f hybridised with HindIII fragments A and H (24.66kb and 8.63kb) and EcoRI fragments A, E and F, G (35.49kb, 9.44kb and a 2M fragment 4.95kb). This probe hybridised to αHVS under conditions of higher stringency than all other probes tested here (figure 7.4). This is particularly

significant since the EcoRI f fragment includes the genes for gB, DNA polymerase and the major DNA binding protein. These genes are particularly well conserved amongst herpesviruses (McGeoch, 1989; Davison and Wilkie, 1983). All hybridising fragments are U_L sequences in αHVS except for HindIII A and EcoRI A. This implies homology of HSV-1 U_L genes to sequences within the αHVS S segment. However, HindIII A sequences are also present in a second 0.5M fragment HindIII B (KM322) or C (KM91). Since the latter fragments do not hybridise to EcoRI f this raises the possibility of non-specific hybridisation to HindIII A and EcoRI A. This non-specificity may be due to the presence of repeat sequences in these αHVS fragments (see below).

EcoRI ek hybridised to HindIII fragments D, E, F and K (18.51kb, 16.00kb, 12.11kb and 5.77/6.01kb) and to EcoRI C and H (24.58kb and 4.31/4.51kb) and to the negative control (fig. 7.5). It is interesting to note that D and E are terminal HindIII fragments and that HindIII K and EcoRI H are variable fragments and are probably terminal or subterminal. EcoRI ek encompasses the U_L terminus, IR_L and most of IR_S. These results may imply homology between the terminal and junction sequences of αHVS and HSV-1, perhaps in IE gene sequences. However, DNA sequencing studies have shown that these regions are the least well conserved amongst herpesviruses. Davison and Wilkie (1983) found non-colinear

homology within the repeats and their junctions with unique sequences in their hybridisation experiments with a variety of alphaherpesviruses. They suggested these findings to result from the presence of multiple reiterations of simple sequences with high G + C content. This may explain the hybridisation found with EcoRI ek, and possibly EcoRI f, with αHVS repeat sequences. This latter explanation is favoured with EcoRI ek since the negative control also hybridised to the probe DNA at the same stringency. In addition there was no hybridisation between the probe and HindIII fragments A and C or EcoRI A, which contain identical sequences to the above mentioned fragments which hybridised to the probe. EcoRI h did not hybridise to αHVS DNA even at very low stringencies (Figure 7.6). This finding is in agreement with the fact that the S segment genes are generally not well conserved amongst herpesviruses (McGeoch, 1989) and in HSV encode genes which are for the most part dispensable for growth *in vitro* (Longnecker and Roizman, 1987; Longnecker *et al*, 1987; Weber *et al*, 1987). Table 7.1 illustrates the lack of homology of U_S genes of HSV-1 with VZV.

pLPB1 did not hybridise to αHVS, even at low stringency (Figure 7.7). pLPB1 encodes ICPO and the gene for the HSV-1 latency associated transcript (LAT). The proposed VZV equivalent to ICPO shows only limited homology to the HSV-1 gene (McGeoch, 1989). αHVS does not encode sequences homologous to LAT. Croen *et al* (1988) found that genes transcribed during VZV latency differed from those of HSV-1

and Rock et al (1987b) found that the major LATs of HSV-1 and BHV-1 did not cross-hybridise.

In summary, there were no significantly homologous sequences between αHVS and the selected HSV-1 DNA fragments. However, the results suggest that hybridisation under conditions of low stringency reflect a certain degree of specificity. Positive hybridisation of HSV-1 EcoRI n and EcoRI f to αHVS DNA was recorded under Tm-60^{o}C and Tm-55^{o}C conditions respectively. These U_L sequences of HSV-1 hybridised to U_L regions of αHVS. The strongest hybridisation was observed with an HSV-1 fragment coding for gB, DNA polymerase and DNA binding protein genes which have been shown to be well-conserved amongst other herpesviruses.

CHAPTER 8: DISCUSSION

Discussion

The primary aims of this thesis were to further characterise alphaherpesvirus saimiri, with particular emphasis on elucidation of the factors involved in producing the differences in neurovirilence in rabbits between strains KM322 and KM91. Neurovirulence determinants were investigated by comparing replication in cell culture, polypeptides and their antigenicity and the genomes of KM91 and KM322. These experiments also extended the characterisation of αHVS properties in cell culture (Daniel and Melendez, 1968; Mou et al, 1986; Leib, 1986), αHVS polypeptides (Leib, 1986; Desrosiers and Falk, 1981; Mou et al, 1986) and the αHVS genome (Leib, 1986; Desrosiers and Falk, 1981; Kit et al, 1980). In some cases experiments involved comparison of αHVS to the prototype alphaherpesvirus, HSV-1. Thus, polypeptide profiles and immunogenicity were compared between KM322, KM91 and HSV-1, and HSV-1 DNA probes were used to investigate genome homology between αHVS and HSV-1.

Previous work with KM322 has established the use of its inoculation into rabbits as a model system for alphaherpesvirus latency (McCarthy, 1972; Tosolini and McCarthy, 1975; Tosolini et al, 1981; Tosolini et al, 1982; Leib, 1986). This model was developed by an investigation

into KM322 latency in dorsal root ganglia by studying the effects of virus stimulating agents on reactivation in explant cultures.

The detailed results to these experiments have been discussed within each chapter and will not be reiterated here. Instead an overview of the findings reported in this thesis and how they relate to the KM91 model for human encephalitis and the KM322 model for alphaherpesvirus latency will be given.

Methylation of viral and eukaryotic DNA has been suggested as a means of controlling gene expression in a number of systems. Certainly microinjection experiments with isolated genes have shown that methylation of cytosine residues correlates with reduced transcription of those genes. This phenomenon is widely proposed as an important factor in switching off selected genes during the differentiation process. As a means of repressing herpesviral genes during latency, DNA methylation represents an attractive hypothesis.

The stimulating action of 5-azacytidine (5-azaC) on KM322 reactivation from latency in rabbit dorsal root ganglion explant cultures suggests a role for methylation of viral and/or host DNA in the control of alphaherpesvirus latency.

The search for the identity of a latency control mechanism

has so far failed to yield results. The fact that a number of different stimuli reactivate latent virus, without an apparent common mechanism, suggests that the latent state represents a complex interaction between viral and host determinants including factors such as cell phenotype, host immune status and unidentified viral genes. Thus, methylation is unlikely to be the sole mechanism controlling herpesvirus latency. However, these experiments suggest that it may well be involved, possibly only in limited regions such as gene promoter sequences rather than as genome-wide methylation. Such an hypothesis would explain why extensive DNA methylation was not found in latent HSV and PRV genomes (Dressler et al, 1987; Rziha et al, 1986).

It may well be that hypomethylation of the host cell genome is inducing the transcription of a number of otherwise silent genes. Such an effect might change physiological conditions within the neuron which is sufficient to reactivate latent virus. If such a case is true, rather than a direct induction of viral genes, an investigation into the effects of 5-azaC on the host cell might in itself prove informative with regards to herpesviral latency.

In addition, an important conclusion from experiments with 5-azaC is that this agent will be extremely useful in increasing the sensitivity of detection of latent αHVS in

rabbit ganglion explant cultures.

Other agents which have been used to increase reactivation of latent HSV were also tested in this system. Neither butyrate, dimethylsulphoxide or hexamethylene bisacetamide were able to increase reactivation of latent KM322. These results probably reflect the increased stability of KM322 latency relative to that of many HSV strains, a fact that is also evident from the lack of spontaneous recurrence of KM322 lesions in infected rabbits. These findings support the use of KM322 inoculation in rabbits as a model for herpes zoster in humans, where latency is also stable and prolonged (Straus, 1989).

Comparative studies on properties of KM91 and KM322 have revealed a number of interesting points and provided a firm basis for future investigation into this useful model for herpesvirus neurovirulence. It is now apparent that, in addition to an immunological difference between the two strains of αHVS, a variation in their *in vitro* and probably *in vivo* replication also exists. The fact that KM322 produced significantly lower yields than KM91 in both Vero and Neuro 2a cells explains why intracranial inoculation of KM322 allowed the survival of 17% of rabbits as compared to 100% fatality on intradermal inoculation of KM91 (Tosolini *et al*, 1982; Leib *et al*, 1988). Thus, even when KM322 is given

the advantage of direct introduction into the brain, which should allow the virus to spread rapidly before a significant immune response has time to develop, one rabbit out of six survived the infection.

This deficiency is not restricted to CNS structures, as has been shown to apply for HSV variants (Javier et al, 1988a; Stroop and Schaefer, 1989), since replication in Vero cells as well as in neuronal cells was reduced relative to that of KM91. KM322 produces smaller mean plaque sizes in Vero cells than KM91. It is likely that this observation and differing replication efficiencies are directly related. The cause of such a replication defect has not been determined, but variation in syncytial phenotype and the production of thymidine kinase have been discounted (Leib, 1986).

Plaque size and virus yield in vitro represent determinants of neurovirulence of KM322 and KM91 on intracranial inoculation into rabbits. These factors would also affect KM322 neurovirulence following peripheral inoculation were it not for the fact that co-inoculation of KM322 and KM91 elicits a rapid immune response in the rabbit which protects the animal from KM91 encephalitis. Such an effect cannot be explained by a replication defect in KM322, instead it implies that a positive property of the virus alerts an immune response not induced by KM91.

In agreement with the "protective effect" hypothesis, analysis of the immunogenic proteins of KM91 and KM322 has revealed differences between the two strains. These may be responsible for immunogenic differences *in vivo*. The variable polypeptides were within the range 37K to 47K molecular weight. Reference to similar studies with HSV suggest these to be a series of polypeptides antigenically related to a structural polypeptide involved in DNA packaging (Eberle *et al*, 1985; Bernstein *et al*, 1986; McKendall *et al*, 1988a and b). In HSV-1 this capsid protein is known as p40.

It may be that the differing replication efficiencies of KM91 and KM322 are directly related to variations in antigenic proteins. If this were the case and p40-related polypeptides were responsible for differing immunogenicity it would suggest that DNA packaging might be a rate limiting step in KM322 virion assembly. Genetic studies would be the most direct method of ascertaining whether plaque size, virus yield and immunogenicity are directly related in KM91 and KM322.

The genome of αHVS has previously been reported to exhibit two (Kit *et al*, 1980; Desrosiers and Falk, 1981) and four isomers (Leib *et al*, 1987b). Results from double and single restriction endonuclease (RE) digests suggest conclusively that the structure has two isomeric forms. Estimations for

the lengths of the short component (34kb, 22% of the genome), the long component (121kb, 78% of the genome) and the total genome (155kb) have been calculated from RE digests.

This information has been used to create partial RE site maps for αHVS DNA. Once additional hybridisation and fragment isolation studies have been performed and the organisation of RE sites within the long region is determined the analysis of the αHVS in rabbits model will be open to genetic manipulation studies. This will allow a more detailed characterisation of the viruses and their neurovirulence determinants. These investigations should focus upon the genetic differences between KM91 and KM322 defined by loss or gain of SalI, BglII and HpaI RE sites. Similarly, studies on neurovirulence determinants in HSV have concentrated on the identification of small regions of DNA responsible for pathogenicity, and work is in progress to identify the nature of these "neurovirulence genes" (Javier et al, 1988a; Thompson et al, 1989; Taha et al, 1989b; Rösen et al, 1986). The characterisation of neurovirulence determinants in αHVS (KM322 and KM91) would be an important contribution to the elucidation of why some strains of HSV-1 are more encephalitogenic than others.

The identification of at least three stable genetic differences between KM322 and KM91 and the determination of

replication efficiency and antigenic variation may all prove to be inter-related once mutational analysis of the αHVS genome has been undertaken. However, it is possible that these findings indicate a more extensive variation between KM91 and KM322 than has so far been determined (Leib, 1986).

Experiments comparing the αHVS genome and gene products with those of HSV-1 yielded apparently conflicting results. Southern hybridisation of HSV-1 DNA fragments with αHVS DNA in high stringency conditions indicated a lack of significant nucleotide sequence homology. However, SDS PAGE showed polypeptide profiles of αHVS and HSV-1 to be similar and Western blotting experiments suggested a high degree of antigenic cross-reactivity between proteins of the two viruses. These results are not directly comparable since hybridisation studies involved only 40% of the HSV-1 genome, only structural proteins rather than those of infected cells were analysed in SDS PAGE experiments and immunogenic polypeptides detected in Western blots represent only a minor subset of viral-specific gene products. In addition the finding that three out of three monoclonal antibodies tested were specific to αHVS suggests that antigenic differences between αHVS and HSV-1 are more extensive than suggested by experiments with polyclonal sera.

Otsuka et al (1981) found no significant homology by

hybridisation studies between the αHVS TK gene and that of HSV-1. However, Honess et al (1989b) detected 40% amino acid sequence homology between the predicted peptides of the two molecules. This anomaly reflects the general finding that homology determined by Southern hybridisation is generally lower than that revealed by comparison of nucleotide sequences and, to a certain extent, that fact that base changes within a codon may not change the identity of the amino acid encoded by it.

Under conditions of low stringency hybridisation was observed between U_L sequences of αHVS and HSV-1 sequences containing glycoproteins B and H, DNA polymerase and the major DNA binding protein genes. These results confirm the findings of others who have suggested that these genes are well conserved amongst herpesviruses (McGeoch, 1989). Of almost one hundred herpesviruses identified relatively few have been studied in detail. The characterisation of the αHVS genome, gene products and biological characteristics, and their comparison with properties of other herpesviruses, will contribute towards the determination of evolution and diversity within members of the Herpesviridae.

Characterisation of the protective response produced by KM322 in rabbits will contribute towards the incomplete knowledge on the immunobiology of alphaherpesvirus infections. Such

studies are of vital importance in the search for an HSV vaccine. Since both KM322 and KM91 produced lethal infections in the owl monkeys from which they were isolated (McCarthy and Tosolini, 1975b) it would be interesting and of importance to determine whether the immunological difference observed in rabbits extends to other host species. However, such studies are limited by the safety requirements when inoculating this virus due to its suspected virulence for man (Melendez, 1968; McCarthy and Tosolini, 1975a).

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The Effects of 5-Azacytidine, 12-*O*-Tetradecanoylphorbol 13-acetate and Sodium *n*-butyrate on Reactivation of Alphaherpesvirus Saimiri from Explant Cultures of Latently Infected Rabbit Dorsal Root Ganglia

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SUMMARY

The DNA hypomethylating agent 5-azacytidine greatly increased the reactivation of alphaherpesvirus saimiri-1 (αHVS) from latently infected rabbit dorsal root ganglia, although it inhibited the virus yield and plaque formation efficiency of αHVS in Vero cells. 12-*O*-Tetradecanoylphorbol 13-acetate (a protein kinase C activator) and sodium *n*-butyrate both had a stimulating action on replication in Vero cells but did not affect the release of αHVS from latently infected rabbit dorsal root ganglia.

Two isolates of alphaherpesvirus saimiri (αHVS) (formerly herpesvirus tamarinus) have been shown to produce fatal encephalitis (KM91) (Leib *et al.*, 1988) or to establish latency (KM322) in dorsal root ganglia (DRGs) (Tosolini *et al.*, 1982) when injected intradermally into rabbits. These isolates are very closely related in terms of restriction endonuclease digest patterns, polypeptide immunoprecipitation and growth characteristics (Leib *et al.*, 1987). Intradermal inoculation of rabbits in the flank with αHVS KM322 produces an initial skin lesion followed by ganglionitis as manifested by areas of hypoaesthesia (Tosolini *et al.*, 1982). This is followed by a period of prolonged latency during which spontaneous reactivation has never been observed (Tosolini *et al.*, 1981) although virus can be recovered by cocultivation of DRGs with a monolayer of permissive cells. However a very low proportion of ganglia serving the inoculated dermatomes yield reactivating virus by cocultivation (<20%), thus necessitating the sacrifice of a high number of rabbits to produce statistically significant results. In order to improve the sensitivity of detection of latent virus a number of agents known to stimulate other virus systems were incorporated into explant culture media. Since the molecular mechanisms involved in maintaining the latent state and in triggering reactivation are ill understood the agents used were selected for their different modes of action.

5-Azacytidine (5-AzaC) is a DNA hypomethylating agent. The level of methylation of cytosine in CpG dinucleotides in DNA has been implicated as a means of cellular and viral gene control in a number of systems (reviewed by Doerfler, 1983). Generally a high degree of DNA methylation correlates with gene inactivation whereas low levels of methylation are associated with active parts of the genome. Inverse correlations between DNA methylation and gene expression have been observed in a number of virus replication systems including those of herpesviruses. In an *in vitro* latency system Youssoufian *et al.* (1982) showed latent herpes simplex virus type 1 (HSV-1) DNA to be extensively methylated, but this was not the case in actively replicating virus. In gammaherpesvirus saimiri (a virus not closely related to αHVS) DNA is methylated in non-producing lymphoid cell lines but not in producing lines (Desrosiers *et al.*, 1979). Also, the experimental induction of a previously inactive thymidine kinase gene in HSV-1 is associated with concurrent demethylation of the gene promoter (Clough *et al.*, 1982;

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Ben-Hattar & Jiricny, 1988). A number of groups have used hypomethylating agents to increase reactivation from explanted tissue latently infected with HSV (Whitby *et al.*, 1987; Bernstein & Kappes, 1988; Stephanopoulos *et al.*, 1988).

The model used for this experiment extends the investigation to a different alphaherpesvirus, αHVS, which produces latency in the rabbit, and unlike other models does not involve spontaneous reactivation.

The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) exerts a variety of effects on cells, in particular it is a potent tumour promoter. In certain cells TPA may inhibit or stimulate differentiation, stimulate DNA synthesis, induce the production of various growth factors, hormones and other cellular secretions and induce proto-oncogenes (Yamamoto, 1984). TPA has been used in virus systems to enhance human immunodeficiency virus (HIV) replication in MT-4 cells (Harada *et al.*, 1986), to stimulate transcription from the simian virus 40 enhancer (Chiu *et al.*, 1987) and to induce Epstein–Barr virus (EBV) replication in producer lymphoblastoid cell lines (zur Hausen *et al.*, 1978; Laux *et al.*, 1988).

Sodium *n*-butyrate, like TPA, induces a wide variety of effects on cells. Generally it modifies gene expression without reducing protein synthesis but in specific cells it may prevent division and induce the production of certain proteins (Kruh, 1982). Butyrate has been used as a differentiation inducer in a number of cell lines in culture (Leder & Leder, 1975; Schneider, 1976). The agent was chosen for this experiment because of its stimulatory effect on reactivation of HSV from latently infected trigeminal ganglia (Hino & Sekizawa, 1986) and EBV replication in producer cell lines (Luka *et al.*, 1979; Saemundsen *et al.*, 1980). It has also been shown to activate HIV long terminal repeat-directed gene expression (Bohan *et al.*, 1987) and to permit HSV replication in otherwise restricted neuroblastoma cells in culture (Ash, 1986).

New Zealand white rabbits were inoculated intradermally in the flank with three 0·2 ml doses each containing 500 p.f.u. αHVS (KM322) as described previously (Tosolini *et al.*, 1982). After at least 1 month the rabbits were killed with an overdose of barbiturate. The spinal cord was exposed and six DRGs were dissected out from each side, spanning and over-reaching those supplying nerves to the infected dermatomes. Each ganglion was individually minced into approximately 0·5 mm^3 fragments, divided equally into test groups and placed onto confluent Vero cell monolayers in 16 mm diameter stoppered test tubes. These cultures were maintained at 37 °C in Earle's 199 medium (Gibco) containing 20% foetal calf serum, 2% sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin and either 50 μM-5-AzaC, 5 ng/ml TPA, 2 mM-sodium *n*-butyrate or no drug. In this way comparisons between drugs and controls were effected between fragments from the same DRG. The concentration of each drug was chosen as the highest not showing cytotoxicity in explant cultures. After 3 days all tubes were refed with drug-free medium and this was repeated every 7 days. The cultures were maintained for 8 weeks and monitored every day for cytopathic effect associated with reactivating virus. All isolates were confirmed as the input strain by restriction endonuclease analysis of viral DNA (Darville, 1983).

5-AzaC-treated cultures reactivated more rapidly (Fig. 1) and to a greater extent (Table 1) than controls and by 8 weeks post-explantation 42% of treated cultures had reactivated as compared to 16% of controls ($P < 0{\cdot}00001$). Neither TPA nor *n*-butyrate showed any significant difference in frequency (Table 1) or speed (Fig. 1*b* and *c*) of reactivation from controls. In all control cultures the timing of the appearance of c.p.e. around explanted ganglion fragments was consistent with reactivation of latent virus. Isolation of virus was more rapid in 5-AzaC-treated tubes, but in this well studied system where spontaneous reactivation in the intact rabbit has never been observed this is most unlikely to be detection of non-latent virus. It is more probable that this observation represents a stimulation of reactivation induced by 5-AzaC.

In order to investigate the effects of 5-AzaC, TPA and *n*-butyrate on productive KM322 infection, plaque formation efficiencies and virus yield were determined. Confluent monolayers of Vero cells in 16 mm diameter test tubes were infected with 2×10^2 to 5×10^2 p.f.u. KM322 in 199 medium (2% foetal calf serum) containing increasing concentrations of each drug. After 2 days, counts of microplaques were compared to those of drug-free controls. The supernatants from these cultures were then harvested, centrifuged to remove cellular debris and titrated on Vero cells to determine virus yield. An overlay was not used on tube cultures since previous

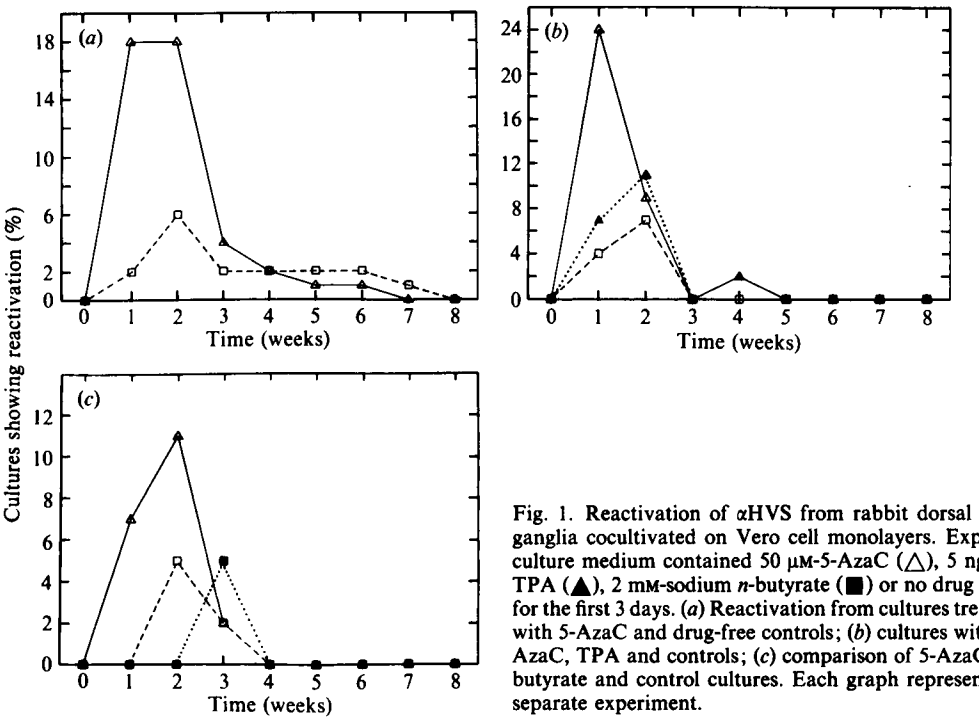

Fig. 1. Reactivation of αHVS from rabbit dorsal root ganglia cocultivated on Vero cell monolayers. Explant culture medium contained 50 μM-5-AzaC (△), 5 ng/ml TPA (▲), 2 mM-sodium *n*-butyrate (■) or no drug (□) for the first 3 days. (*a*) Reactivation from cultures treated with 5-AzaC and drug-free controls; (*b*) cultures with 5-AzaC, TPA and controls; (*c*) comparison of 5-AzaC, *n*-butyrate and control cultures. Each graph represents a separate experiment.

Table 1. *Number of explant cultures yielding reactivated virus**

	Drug	Control	*P* value
5-AzaC	57/132 43%	21/129 16%	<0·00001
TPA	8/45 18%	6/46 13%	NS†
n-Butyrate	2/44 5%	3/44 7%	NS

* Latently infected ganglionic fragments were cocultivated on Vero cell monolayers in medium containing 50 μM-5-AzaC, 5 ng/ml TPA or 2 mM-sodium *n*-butyrate. In each case fragments from the same ganglia were cultivated without drugs as controls.

† NS, Not significant.

observation has shown that no secondary plaque formation occurs within 48 h (Tosolini *et al.*, 1982) and supernatants were required for titration.

Increasing concentrations of 5-AzaC decreased both plaquing efficiency and virus yield (Fig. 2*a* and *b*). This may be due to cytotoxicity or possibly inhibition of a transient methylation of the herpesvirus genome during replication, as has been shown to occur in HSV-1 (Sharma & Biswal, 1977). TPA increased plaque formation efficiency and virus yield at low concentrations but inhibited viral replication at higher concentrations (5000 ng/ml) (Fig. 2*c* and *d*). The drug was cytotoxic at this level. Butyrate had no effect on plaque formation at low concentrations but was inhibitory at >2 mM. Conversely an increase in virus yield was seen with increasing concentrations of *n*-butyrate (Fig. 2*e* and *f*).

TPA and *n*-butyrate showed no significant effect on reactivation of αHVS, despite the fact that both drugs show stimulatory effects on αHVS replication in Vero cells at the concentrations used in explant cultures. It is noteworthy that Hino & Sekizawa (1986) have demonstrated increased reactivation of latent HSV from murine trigeminal ganglia using the same concentration (2 mM) of butyrate.

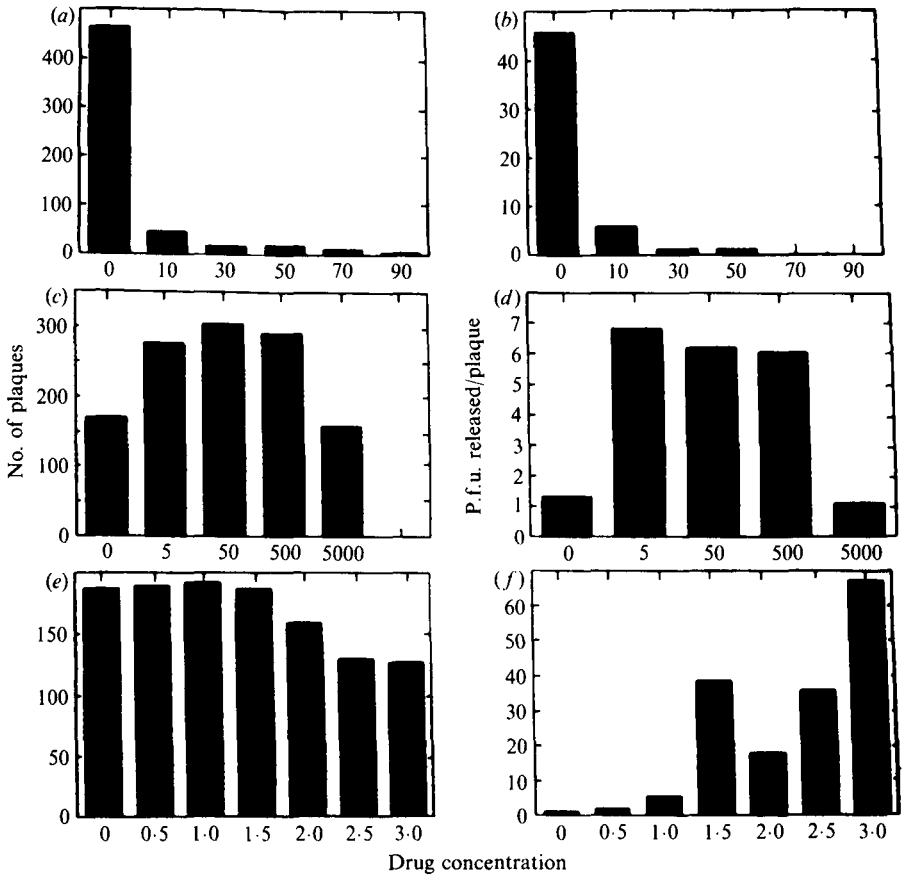

Fig. 2. Effects of 5-AzaC (μM; *a* and *b*), TPA (ng/ml; *c* and *d*) and *n*-butyrate (mM; *e* and *f*) on plaque formation efficiency and yield of αHVS (KM322) *in vitro*. Monolayers of Vero cells were infected with KM322 in medium containing increasing concentrations of each drug. After 2 days plaques were counted and supernatants were harvested, centrifuged and titrated on Vero cell monolayers.

The pleiotropic effects of TPA are thought to be mediated through stimulation of protein kinase C, a plasma membrane signal transducer (Nishizuka, 1984, 1986). The failure of TPA to affect reactivation rates may be due to a lack of the appropriate receptors on the neurons harbouring the latent virus, to the inability of protein kinase C activation or other TPA-induced effect to stimulate reactivation, or simply due to limits on the concentration used, imposed by cytotoxic effects in explant cultures. Clearly this drug is of no use in increasing the sensitivity of this cocultivation system.

The effect of *n*-butyrate varies according to cell type and the virus tested, for example it can stimulate EBV replication in P3HR-1 cells (Luka *et al.*, 1979) but inhibits adenovirus type 2 replication in 3T3 cells (Iseki & Baserga, 1983) and polyoma virus replication in mouse kidney cells (Wawra *et al.*, 1981). It has been proposed that *n*-butyrate modifies gene expression by inhibition of histone deacetylase and thus hyperacetylation of histones (Kruh, 1982).

5-AzaC is a cytosine analogue with the carbon-5 atom replaced by a nitrogen thus inhibiting methylation at this position when it is incorporated into DNA. An additional and more significant mode of action is by irreversibly binding to and inhibiting cellular methylases (Doerfler, 1983). Thus a 5% incorporation of 5-AzaC into DNA can cause an 80% drop in methylation (Taylor & Jones, 1982). The mechanism by which methylation regulates gene

expression is unknown but it has been suggested that it may be mediated by changes in chromatin structure (Kolata, 1985; Ben-Hattar & Jiricny, 1988).

5-AzaC induces a more rapid and greater incidence of reactivation than that seen in control cultures. The fact that the drug inhibits αHVS replication in Vero cells suggests that it is reactivation not subsequent replication that is being stimulated. That 5-AzaC stimulates reactivation does not directly prove a role for methylation in the control of latency since other modes of action for this drug have been reported (Reichman & Penman, 1973). However work with other hypomethylating agents would suggest that this is the case (Whitby *et al.*, 1987; Bernstein & Kappes, 1988; Stephanopoulos *et al.*, 1988).

Dressler *et al.* (1987) have produced evidence to suggest that the latent HSV-1 genome is not extensively methylated *in vivo*. Their analysis covered only 3% of CpG dinucleotides in the genome, but Ben-Hattar & Jiricny (1988) showed that a single CpG methylation in the thymidine kinase promoter segment resulted in a 20-fold reduction in transcription.

If the αHVS genome is almost totally suppressed during latency, except for limited transcription as seems to be the case for HSV, and since it resides in non-dividing nervous tissue it appears that any hypomethylation will be effected through DNA repair processes. Such unscheduled repair synthesis has been shown to occur in cultured neurons (Sanes & Okun, 1972).

The results of this paper confirm and extend those of Whitby *et al.* (1987), Bernstein & Kappes (1988) and Stephanopoulos *et al.* (1988) and suggest a role for methylation of host and/or viral DNA in the control of αHVS latency, as well as that of HSV-1 and HSV-2. The use of 5-AzaC in explant cultures also provides a useful tool for increasing the sensitivity of detection of latent virus.

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