THE CLONING AND EXPRESSION OF HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEIN C IN VACCINIA VIRUS

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by

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The Herpesviridae family contains numerous virus types, several of which can infect man. The virus which is most widely spread in terms of infection is herpes simplex virus (HSV), of which there are two serotypes (HSV-1 and HSV-2). Following a primary infection, the virus can establish a latent infection within neurons of sensory ganglia, where it remains throughout the lifetime of the host. HSV is able to reactivate from the latent state, and may produce clinical infection at the site of the initial virus invasion (recrudescent lesions). Although most HSV-1 infections are mild or subclinical, infection can lead to life-threatening illness. The search for an effective HSV vaccine has met with limited success to date. In recent years, vaccine research has turned toward the use of viral vectors, for the expression of individual virus proteins with a view to stimulating host immunity. A great deal of attention has been focussed on the vaccinia virus, which is able to accept large amounts of foreign DNA into it's genome with no loss of viability. Several HSV proteins have been expressed from recombinant vaccinia viruses, and this project outlines the cloning of DNA sequences encoding the HSV-1 glycoprotein C, and expression of that protein from a recombinant vaccinia virus. The HSV DNA sequences encoding the glycoprotein were cloned from an existing library, into plasmid vector pSC11. This plasmid allows insertion of gC sequences into the vaccinia virus genome by way of homologous recombination. The plasmid also provides a vaccinia virus promoter for the control of gC transcription during infection of cells with the resulting recombinant viruses. Recombinant vaccinia viruses produced on cloning of a 1.75kb NheI/SphI fragment containing the HSV-1 gC gene did not express the glycoprotein during infections. Vaccinia virus DNA polymerase is known to be sensitive to secondary structures within DNA, and the 5' non-coding region of the HSV gC gene is rich in GC basepairs, which could readily form such structures. Site-directed mutagenesis removed the 5' non-coding region (34 nucleotides) in an attempt to remove any such block to gC transcription. Vaccinia viruses containing the mutated sequences were able to express gC within infected cells, as determined by immunofluorescence. Mice inoculated with a gC-expressing recombinant vaccinia virus were able to induce HSV-neutralising antibodies and displayed 50% protection against a lethal HSV-1 challenge. The implications of gC-expressing vaccinia recombinant viruses, with respect to the search for an HSV vaccine and to the understanding of the role of gC during HSV infections is discussed.

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I would like to dedicate this thesis
to my family, for being there.

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION.

Herpes simplex virus (HSV) is a member of the Herpesviridae, a large family of viruses which share similar morphological and biological features. The characteristic properties of the family include a large array of virus-specified enzymes, virion synthesis and assembly within the nucleus of infected cells, an infectious cycle which leads ultimately to the death of the cell and an ability to cause silent longterm (latent) infections in their natural hosts. Some herpesviruses can reactivate from the latent state to produce recurring infections.

At present, there are seven herpesviruses known to naturally infect man. Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) have been recorded by physicians for many centuries, and varicella zoster virus (VZV), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) have also been known for many years. Two other human herpesviruses have recently been isolated. Human herpesvirus 6 (HHV-6, Salahuddin, et al., 1986) is often associated with HIV infections but is known to also cause disease in infants characterised by a high fever and a rash (Yamanishi, et al., 1988). The newly discovered human herpesvirus 7 (HHV-7, Frenkel, et al., 1990) has yet to be characterised. Herpesviruses are classified into three subfamilies; alpha-, beta- and gamma-herpesvirinae, on the basis of several biological features (see below; Roizman, 1982).

1.1.1. Alphaherpesvirinae.

The viruses of this subfamily display variable host ranges (from very wide to very narrow), a short replication cycle (18 to 24 hours) and a rapid rate of spread through the infected host. Alphaherpesvirinae, which include HSV (both serotypes) and VZV, usually establish latent infections within the neurons of sensory nerve ganglia.

1.1.2. Betaherpesvirinae.

These viruses, which include both human and murine CMV, have a very restricted host range - often only the species or genus to which their natural host belongs. The replication cycle is longer than for the alphaherpesvirinae (3 days in the case of CMV) and the infection progresses slowly in tissue culture. Infected cells frequently become enlarged (cytomegalia). The latent infections of this group generally target secretory organs, lymphoreticular cells and occasionally the kidneys and other tissues.

1.1.3. Gammaherpesvirinae.

EBV and HHV-6 are classified as gammaherpesvirinae, a group which are restricted in host range to the same family or order to which the natural host belongs. The length of the replication cycle of these viruses is very variable. Gammaherpesvirinae replicate specifically within T- or B-lymphocytes and produce latent infections within the lymphoreticular tissues.

1.2. CLINICAL SIGNIFICANCE OF HSV INFECTIONS.

1.2.1. Global Incidence.

HSV infection can be demonstrated by the presence of anti-viral antibodies, by clinical illness or by the isolation of virus from host tissues. The frequency of HSV infections within populations has been reviewed by Whitley (1985, 1990) and is greatly influenced by global location, age and socio-economic status. Poorer countries show a higher frequency of infection than developed countries, and sero-conversion occurs in younger age groups than in developed countries. Lower socio-economic classes have higher frequencies of infection than higher classes. In all populations studied, the incidence of infection rises with age such that elderly populations demonstrate almost 100% sero-conversion. This suggests that almost everyone is infected by HSV at some time in their life. HSV is not particularly contagious but is spread by close personal contact. No natural animal vectors have been found for HSV (although the virus will infect certain animals in the

laboratory) so that man is the only reservoir, by way of both clinically apparent and non-apparent infections (Whitley, 1985, 1990). This report is mostly concerned with HSV-1 and unless otherwise stated, HSV implies HSV-1.

1.2.2. Symptoms and Complications of HSV Infections.

HSV infections are often deemed to be of great biological interest but little clinical significance. This is not the case. Primary infection is usually without clinical signs and the virus establishes a latent infection within sensory nerve ganglia. Reactivation of latent virus within the ganglia may lead to the presence of virus at peripheral sites (recurrence). This in turn may lead to the development of herpetic lesions (recrudescence), but these are usually mild and infrequent; the occasional coldsore. However, HSV can produce a range of clinical signs, following both primary and recurrent infections, some of which can be fatal (see below; Whitley, 1985, 1990; Timbury, 1986). Of the two serotypes that infect man, HSV-1 is most commonly associated with infections of the oro-facial areas, whilst HSV-2 generally causes genital infections. However, any organ can be affected during a disseminated infection with either virus, and HSV-1 is responsible for up to one third of genital herpes infections (Whitley, 1985).

1.2.2.1. Skin involvement.

HSV infection can induce extensive vesicles in the skin. Symptoms range from minor skin eruptions to eczema. Bacterial superinfections may lead to life-threatening complications. Young children, and patients suffering from atopic disease or from burns are at special risk from severe HSV skin infections.

1.2.2.2. Gingivo-stomatitis.

Inflammation of the gums and oral mucosa may be accompanied by redness, multiple vesicles, painful ulcers and fever. The infection may spread to include other sites of the head and neck.

1.2.2.3. Conjunctivitis and keratitis.

Involvement of the eye is generally associated with primary infections that occur beyond the newborn age. The affected eye shows signs of photophobia and tearing, and the eyelids are often swollen, with vesicles and ulcers. Both the cornea and the conjunctiva are involved. Recurrences are common, involving both eyes on occasion, producing recrudescent ulcers, visual loss and possibly leading to rupture of the globe.

1.2.2.4. Encephalitis.

Involvement of the central nervous system (CNS) during HSV infections of man is rare. The onset of an encephalitic HSV infection is marked by fever, headaches and mental confusion. Infection of the front temporal lobes and acute necrosis of these tissues usually results in death. HSV invasion of the CNS can also produce a benign, self-limiting meningitis, but this is more common with HSV-2.

1.2.2.5. Neonatal infections.

New and unborn babies are at high risk of contracting HSV (types 1 and 2) from infected mothers, particularly at the time of birth from infected maternal genital secretions. However, infection can occur *in utero* via haematogenous routes. Clinical signs include vesicles, kerato-conjunctivitis, jaundice, seizures and dissemination of infection to other organs (lungs, liver and kidneys). Some cases progress to involve the CNS. Neonatal HSV infection has a high fatality rate.

1.2.2.6. Generalised infections.

In the normal adult, generalised HSV infection is rare. It is more frequent, however, in immunocompromised patients and is frequently a serious infection in AIDS sufferers, or those undergoing immunosuppressive therapy. In such cases, cutaneous dissemination can lead to frequent coldsores, which often cover the face and oesophagus, and may become necrotic. Progressive infections of the respiratory tract,

oesophagus and gastrointestinal tract are common. Recrudescence occurs at multiple sites and healing of lesions is slow.

1.2.2.7. Carcinomas.

Any virus which introduces foreign nucleic acid into a host cell is potentially oncogenic. Herpes viruses have been associated with cancers of several sites in man and animals. In recent years, attention has been focussed on the higher frequency of cervical cancers in women with genital HSV infections compared to uninfected women. Definitive proof that HSV infections can cause cancers is lacking, but the detection of HSV-2 RNA and proteins within cancerous cells taken from cervical carcinomas strongly links the presence of infection with the development of the cancers (McDougall et al., 1982).

1.2.3. Treatments Available for HSV Infections.

The search for safe, effective anti-viral agents against HSV has seen some success in the use of nucleoside analogues. The most widely used is acycloguanosine (9-[2-hydroxyethoxymethyl]guanine), or acyclovir (ACV). The molecule must be phosphorylated by the viral thymidine kinase (TK) for activity, and once activated can inhibit the viral polymerase and may be incorporated into the replicating virus DNA, preventing further elongation (Elion, et al., 1977; Fyfe, et al., 1978). The activity is specific for viral DNA replication, but shows a low toxicity to cellular processes. Intravenous, topical or oral administration of ACV has been shown to accelerate cutaneous healing, decrease viral shedding and reduce pain during HSV infections (Whitley, et al., 1990). The drug has high levels of activity, low toxicity in vitro and in vivo, and is active against other herpesviruses such as CMV, VZV and B-virus (Schaeffer, et al., 1978). Although unable to eliminate an established latent infection, high levels of ACV have been reported to prevent the establishment of latency in mice (Field, et al., 1979) and continuous usage of the drug is thought to inhibit reactivations from the

latent state (Klein, et al., 1981b). The occurrence of ACV-resistant strains, both natural isolates and selected strains (Field, et al., 1980; Field, 1982), does present a problem for the long-term use of the drug. Long-term use of ACV has also been observed to depress serum immunoglobulin levels (Erlich, et al., 1988) which may lead to immunosuppression over prolonged courses of treatment.

1.3. HSV INFECTIONS.

The course of HSV infection in man follows a characteristic pattern of events, outlined in Figure 1.1. and reviewed by Klein, (1982) and Corey and Spear, (1986). The initial entry and replication of the virus is termed the primary infection. Primary HSV-1 infections generally occur during infancy and are mostly subclinical. Primary infection with HSV-2 does not usually occur until adolescence or adulthood, except for cases of neonatal infection passed from an infected mother to her child at birth. HSV infections in man have been studied using a combination of patient-studies and laboratory animal models of the disease. The mouse, guinea pig and rabbit have been the most widely used models.

1.3.1. Virus Entry and Invasion of the Nervous System.

Exposure at mucosal surfaces or areas of abraded skin allows the virus to initiate an infection in the epidermal and dermal cells. The epidermis may suffer necrosis, leading to vesiculation, ulceration and scab formation (Corey and Spear, 1986). Local replication of the virus is followed by infection of nerve endings within the area and subsequent colonization of the related sensory ganglia. Virus can be detected in the ganglia of infected mice and guinea-pigs between 2 and 4 days after infection at peripheral sites (Klein, 1982; Corey and Spear, 1986). Since Johnson (1964a) first detected virus antigens in mouse CNS cells, virus has been observed within the cells of the ganglia, the Schwann cells surrounding the nerve fibres associated with the ganglia and within the nerve axons themselves (Rabin, et al., 1968; Cook and Stevens, 1973). The detection of virus

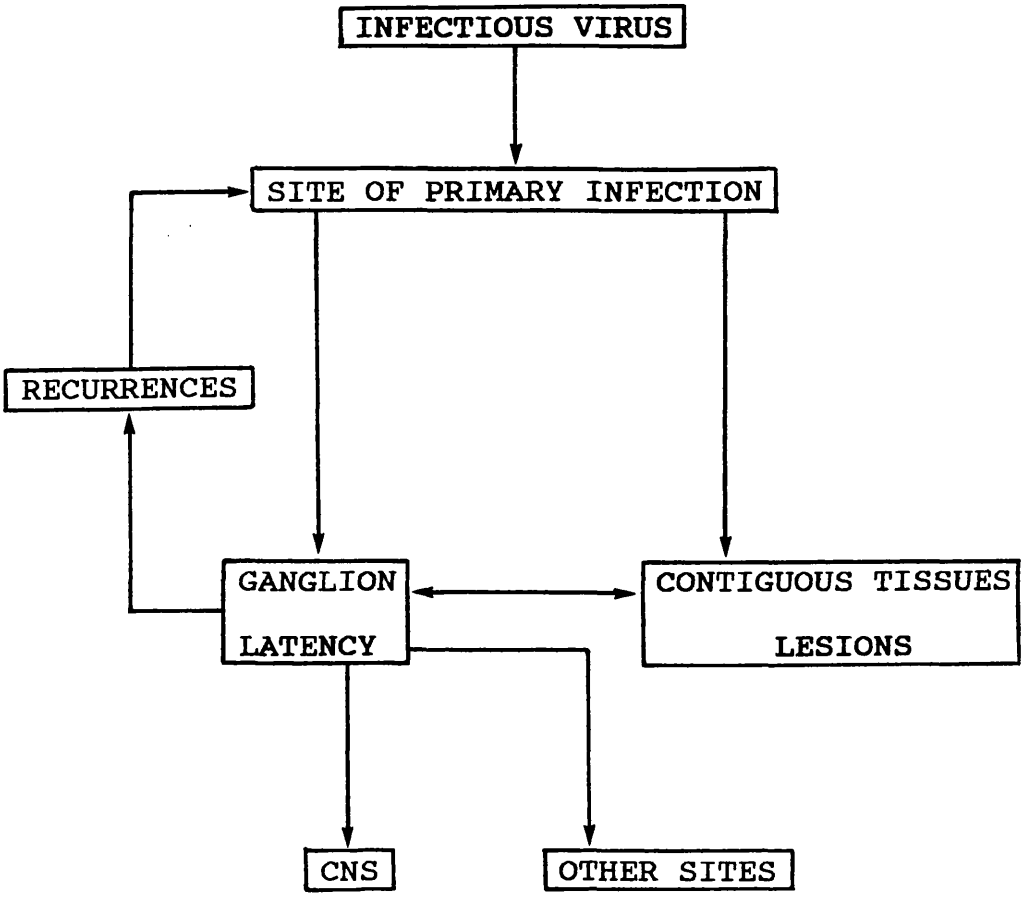


Figure 1.1. Representation of the events following invasion by HSV. Primary infection usually occurs via mucosal surfaces or at sites of damaged skin. The virus may replicate and spread to surrounding tissues, possibly producing herpetic lesions. Infection invariably results in colonisation of sensory ganglia, and the establishment of a latent infection within neurons. Virus may spread to the central nervous system (CNS) or to other body sites from infected ganglia. Latent virus may reactivate within ganglia and may give rise to recurrences and possibly herpetic lesion, usually at the site of primary infection. Adapted from Hall and Katrak, 1986.

within nerve axons, and the slow replicative cycle of HSV within Schwann cells (4 hours) suggested that virus travelled within the axons to the ganglia, rather than by sequential infection of adjacent Schwann cells. Margolis et al. (1989) were able to confirm the movement of virus via intra-axonal routes by following virus spread through the CNS in mice infected with HSV in the eye. Evidence suggests the virus travels as unenveloped nucleocapsids within the nerve axons (Cook and Stevens, 1973; Lycke, et al., 1984). However, reports of virus found within the bloodstream of HSV-1 infected mice and regional lymph nodes of HSV-2 infected guinea-pigs, and the ability of HSV to colonize ganglia even when sensory nerves had been severed (review, Klein, 1982) suggest that the virus can find alternative routes to the ganglion, possibly systemic or lymphatic.

HSV titres rise within the ganglia during the course of infection at the periphery. Locally applied antiviral treatments reduce ganglionic titres, presumably by arresting replication at the periphery. This suggests that the increase in viral titre within ganglia may be a consequence of continued virus supply from the peripheral site of infection, rather than replication of HSV within the ganglia (review, Klein, 1982).

1.3.2. Establishment of the Latent Infection.

Following colonization of the ganglia, virus spreads to other mucosal and skin surfaces via peripheral sensory nerves, in some cases affecting skin distant to the initial site of infection. Local spread of virus may also occur, allowing further mucosal extension of the infection. Host immune responses are usually capable of eliminating virus within the peripheral tissues following a primary infection. At this point, infectious virus cannot be detected within peripheral tissues or ganglia and the virus enters the latent stage of infection. Latent HSV infections are readily established within the mouse, and distribution of the virus within the mouse CNS is similar to that in human infections, making the mouse a useful model for studying latency.

1.3.3. The Site of the Latent Virus.

Stevens and Cook (1971a) were the first to demonstrate the presence of latent HSV in the sensory ganglia of mice. Following the resolution of the primary lesions, they could detect no infectious virus in homogenised ganglionic tissues. However, several days co-cultivation of ganglionic cells *in vitro* with permissive cells did produce virus, suggesting that virus was present within the ganglia during a latent infection, but that it required some form of stimulus to replicate. Similarly, latent virus has been demonstrated within the ganglia of guinea-pigs (Scriba, 1975) and from human trigeminal and sacral ganglia (Baringer and Swoveland, 1973; Baringer, 1974). Interestingly, both latent HSV-1 and HSV-2 could be recovered from the trigeminal and sacral ganglia, respectively, of a single human cadaver, suggesting an individual could be latently infected with both serotypes simultaneously. Baringer found latent virus only within the ganglia and proposed that the site of latency was the neuron, a cell unique to the ganglia. It has since been confirmed that these cells are indeed the site of latent HSV (Cook, *et al.*, 1974; McLennan and Darby, 1980).

However, in contrast to these reports, Scriba (1977) was able to recover latent virus both from nervous tissues and from skin tissues at the site of initial infection of guinea pigs infected with either HSV-1 or HSV-2. Also, latent virus has been demonstrated within the ear pinna (Hill *et al.*, 1980) or footpad (Al-Saadi *et al.*, 1983) of latently-infected mice. These non-neural sites of latent HSV may have important bearing on the human infection, although latent virus in man has only been isolated from sensory ganglia.

1.3.4. The State of the Latent Virus.

The inability to detect virus particles during latency initially suggested the virus was held in a completely static state, but this is now generally regarded as untrue. Rock and Fraser (1983) detected viral DNA in latently infected nervous tissues, and suggested that all genomic

sequences were present, except for the terminal fragments. The latent virus genome was proposed to be in some form other than the linear unit lengths found in virions (section 1.5.1.); as closed-circular or multiple-length concatenated forms. Viral RNA has also been detected in latently infected cells, although at very low levels, suggesting that limited transcription of viral genes occurs during latency. The most abundant viral RNA transcripts found in the ganglia of latently-infected mice (Stevens, et al., 1987; Deatly, et al., 1987, 1988) and humans (Gordon, et al., 1988; Krause, et al., 1988; Steiner, et al., 1988) are known as the latency-associated transcripts (LATs). These transcripts overlap the 3' end of the infected cell protein 0 (ICP0; molecular weight 110,000 daltons), but are derived from the opposite DNA strand to the ICP0 coding sequence. The ICP0, produced during the first stage of the replicative cycle, transactivates gene transcription during the later stages and is thought to be important for de novo protein synthesis from latent genomes and for reactivation to occur (Cai and Schaffer, 1989). Virus mutants lacking LAT sequences are able to establish latent infections (Javier, et al., 1988), but exhibit reduced frequencies of reactivation from latency, compared to wild-type viruses (Leib, et al., 1989a; MacLean, et al., 1989). These reports suggest that ICP0 and LAT are not essential for the establishment of latent infections, but are important during reactivation. The sequence of the LAT within infected mice has been determined (Wagner, et al., 1988a; Wechsler, et al., 1989) and has revealed two possible open reading frames (ORFs). The larger of the two contains an intron which, when removed by splicing, leaves the smaller ORF (Wagner, et al., 1988b). Whether the LAT produces polypeptides, and what role these peptides might have is yet to be determined.

Replication of the virus within the cells of the ganglia is not essential for latency (Coen, et al., 1989; Valyi-Nagy, et al., 1991). However, other virus functions have been shown to be important during latent infections. Virus mutants defective for ICP4 and ICP27 proteins, both

providing immediate-early functions, do not replicate at the primary site and fail to establish latent infections (Leib et al., 1989b). The viral ICP4, a regulatory protein, has been previously detected within latently-infected cells (Green, et al., 1981) and is thought to inhibit virus protein synthesis during latency, which would normally lead to a productive infection. Thymidine kinase (TK)-negative virus mutants can establish latent infections, although reactivation of TK-negative viruses from the latent state is hindered, compared to wild-type viruses (Coen, et al., 1989; Leist, et al., 1989). Like ICP0 and LAT functions, the TK protein appears to be important in reactivation, and has been detected in latently-infected cells (Yamamoto, et al., 1977).

1.3.5. Recurrent Infections.

The latent virus may reactivate from it's restricted state to produce fully infectious virus within the ganglia. This reactivation is generally followed by transport of the virus to a peripheral site where it may replicate, giving rise to a recurrent infection and possibly recrudescent lesions. The commonest site of recurrent infections in man is the skin (usually the face, neck or the anogenital areas) but lesions can occur at any body site (Wheeler, 1975). The first evidence of a recurrent infection is often a reported itching or burning sensation by the patient, at or near the site of recurrence, occasionally with neuralgic pain. The development of recrudescent lesions begins with a small group of vesicles containing large amounts of infectious virus. The vesicles reach maximum size in a few days and heal over a period of 1 to 2 weeks, usually without scarring. Recurrences can be asymptomatic, with virus shedding from the throat, in saliva and in tears, and infectious virus can be present in the skin without the development of clinical lesions. Reactivation, recurrence and recrudescence have been studied in the mouse, guinea-pig and rabbit.

1.3.6. The Mechanisms of Reactivation and Recrudescence.

Many cases of reactivation in humans (based on reports of recrudescent lesions) have been attributed to stimuli such as sunburn, fever, allergic reactions, menstruation, stress, sexual activity, trauma of the skin or nerves and immunosuppression. However, virus reactivation also occurs spontaneously, with no apparent identifiable stimulus. In the mouse, latent HSV can be reactivated by unusual agents such as UV-light, sellotape-stripping, dry-ice freezing and hair-plucking. It has been proposed that the latent virus reactivates within the ganglia at frequent intervals and travels to the skin (via nerves) perhaps as often as every few days. The virus is then thought to replicate within the skin to produce microfoci of infection within the epidermal cells (Blyth, et al., 1976; Hill and Blyth, 1976). It is thought that under normal conditions these foci are eliminated by the host defence mechanisms, but under the influence of stimulating agents, conditions within the skin may become altered such that virus multiplication is favoured and clinical symptoms rapidly develop.

Following reactivation, the virus undergoes a lytic replication cycle, which results in the death of the neuron (McLennan and Darby, 1980). The maintenance of latently-infected cells within the ganglia is thought to occur by a "round-trip". By this mechanism, reactivation (which destroys the latently-infected cell and neighbouring cells) and axonal transport of virus to peripheral tissues generates recurrence and possibly recrudescence, which leads to the recolonization of ganglia with virus, providing a new set of latently-infected cells.

1.4. THE REPLICATION CYCLE OF HSV.

The replication cycle of HSV within susceptible cells is illustrated in Figure 1.2. The initial interaction between the invading virus and the target cell involves attachment of the virus to the plasma membrane. The precise mechanism of attachment is unclear, but the cellular receptors involved are thought to be ubiquitous, as HSV can infect a

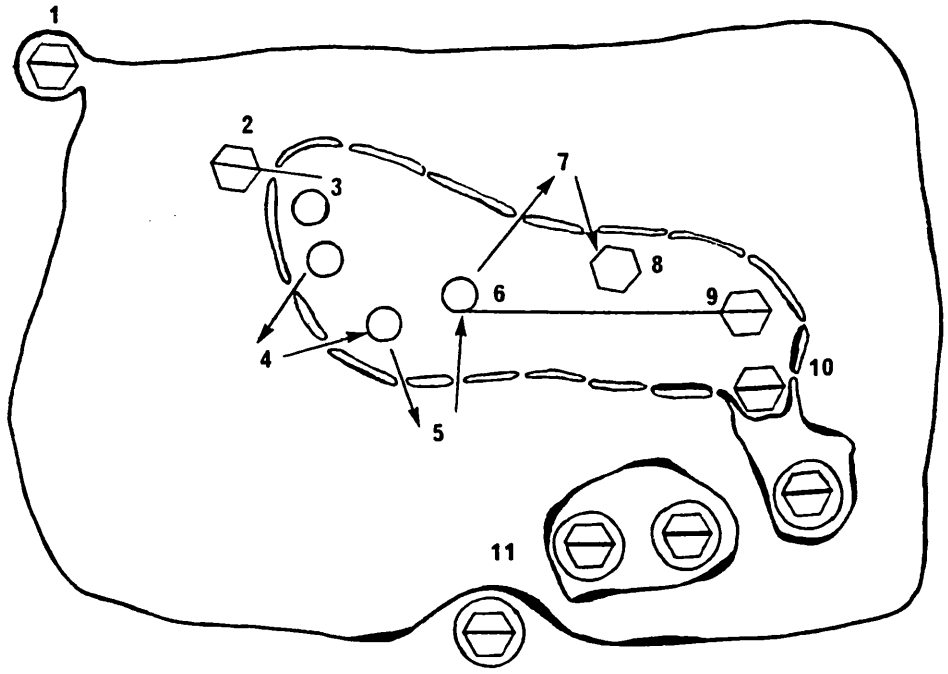


Figure 1.2. The replication cycle of HSV in susceptible cells. Virus attaches to the cell surface and fusion of the virion envelope with the plasma membrane (1) releases the nucleocapsid into the cell. Nucleocapsids become situated near nuclear pores (2) and virus DNA is released into the nucleus, where it immediately circularises (3). Transcription of immediate early mRNA is followed by synthesis of IE proteins within the cytoplasm (4), which return to the nucleus to initiate early phase transcription. Early mRNA is translated into early proteins (5) which then initiate DNA replication by rolling circle method (6) and late mRNA and protein synthesis (7). Structural proteins form empty nucleocapsids within the cell nucleus (8), which become filled with unit length viral DNA (9). Filled nucleocapsids gather below patches of the inner nuclear membrane which have become thickened and contain viral glycoproteins (10). Enveloped nucleocapsids move through the cell within the lumen of the ER and virus exits the cell following fusion of cellular membranes (11). Taken from Roizman and Sears, 1990.

variety of cells. Recent studies have identified heparan sulphate as a possible cell surface receptor molecule (WuDunn and Spear, 1989). Fuller and Spear (1985) found several antibodies specific to HSV envelope glycoproteins that were able to inhibit HSV adsorption to cells, suggesting the glycoproteins may mediate virus attachment to cells. Fusion of the viral envelope and the cell membrane allows penetration of the virus nucleocapsid into the cytoplasm. Glycoprotein B was the first viral component implicated in the penetration of attached virus into cells (Sarmiento, et al., 1979; Little, et al., 1981), later to be joined by gD (Fuller and Spear, 1987; Ligas and Johnson, 1988) and gH (Fuller, et al., 1989). The HSV glycoproteins are discussed more fully below (section 1.6.).

1.4.1. Viral RNA and Protein Synthesis.

The consequence of penetration is the release of the viral nucleocapsid into the cytoplasm of the host cell. The virus genome is released from the nucleocapsid and enters the cell nucleus, a process mediated by one or more virus structural proteins (Knipe et al., 1981; Batterson, et al., 1983). Once inside the nucleus, the viral DNA rapidly circularises and genes are transcribed by host enzymes (RNA polymerase II). HSV mRNAs have 3′ polyadenylated tails and 5′ cap structures identical to host mRNA transcripts, but they exhibit much lower splicing frequencies. Transcription of HSV genes and translation of the respective mRNA transcripts is organised into three principal phases (Honess and Roizman, 1974, 1975; Clements, et al., 1977), known as the immediate-early (IE), early (E) and late (L) phases. Immediate-early genes are transcribed between 2-4 hours following infection. No previous viral protein synthesis is required for IE transcription, but a virion component is necessary for the activation of IE promoter regions. Five proteins are synthesised during IE synthesis, which act as regulators of virus transcription. Two proteins in particular, ICP0 and ICP4, are involved in controlling transcription of genes within the early and late stages of virus replication (Everett, 1984, 1986; O'Hare and Hayward,

1985). Following the onset of early and late transcription, most IE genes are "switched off", but ICP4 is required throughout the virus replication cycle and transcription continues at a low level (Watson and Clements, 1980). Early genes are transcribed between 5-7 hours after infection and require previous synthesis of IE proteins. Most of the viral proteins involved in DNA replication are early proteins. Late genes are transcribed from 6 hours onwards and are mainly structural proteins. True late genes require previous IE and E protein synthesis and the onset of viral DNA replication, but several late proteins are synthesised without a requirement for DNA synthesis. The initiation and decline of each phase is tightly regulated in a cascade mechanism (Honess and Roizman, 1974, 1975; Clements, et al., 1977; Wagner, 1985).

HSV proteins are extensively processed, both during and after translation. Several modifications may be involved, including cleavage, phosphorylation, sulphation, glycosylation and poly(ADP) ribosylation (reviewed in Roizman and Sears, 1990). Host cell RNA and protein synthesis diminishes rapidly after infection, considerably more so with HSV-2 infections than with HSV-1, and host mRNA transcripts are degraded. This "shutoff" of host functions appears to be mediated by the interaction of a virion structural factor and a critical cellular component (Kwong and Frenkel, 1989).

1.4.2. Viral DNA Replication.

Synthesis of HSV DNA can be detected within cells approximately 3 hours post infection, and has been reviewed by Roizman and Sears (1990) and by Challberg (1991). Unlike other nuclear DNA viruses, herpesviruses specify a large number of enzymes involved in the replication of viral DNA. These enzymes fall into 2 categories, concerned with either nucleic acid metabolism or with viral DNA synthesis. Enzymes within the first category - thymidine kinase, ribonucleotide reductase, dUTPase and uracil-DNA glycosylase - are not essential for virus growth in dividing tissue cell culture.

Seven virus-specified gene products have been found to be essential for origin-dependent DNA synthesis within infected cells (Challberg, 1986; Wu, et al., 1988). The genes involved encode origin-binding and single-stranded DNA-binding proteins, DNA polymerase, a polymerase-accessory protein, and a helicase-primase complex. The products of these seven genes carry out the complete set of biochemical reactions predicted to be necessary for the replication of HSV DNA, which is outlined below (Challberg, 1991).

The HSV genome contains three origins of replication at which DNA synthesis can initiate (Vlasny and Frenkel, 1982). OriL is located within the long unique region, and oriS, of which there are two identical copies, is located within the repeated regions which border the short unique sequences. The origins consist of an extensive inverted repeat sequence surrounding a central core of 18 basepairs, which are exclusively AT pairs. The origin binding protein (U_L9 gene product), which is thought to exist as a homodimer (Challberg, 1991) binds to the origin sequences at a minimum of two sites (Olivo, et al., 1988; Weir, et al., 1989b; Weir and Stow, 1990). The protein is also thought to initiate unwinding of the parental strands, as it has been shown to contain helicase activity (Bruckner, et al., 1991; Challberg, 1991). Although the exact mechanisms are unknown, a replication fork is established at the position of U_L9-binding, and the six remaining gene products participate in DNA replication at this point. The helicase-primase is a complex of three peptides (U_L5, U_L8, U_L52) which unwinds the DNA at the replication fork in a 5′ to 3′ direction. The complex may also prime lagging-strand synthesis as the DNA is unwound (Crute, et al., 1988; 1989). A single-stranded binding protein (U_L29) facilitates the use of the ssDNA formed by the replication fork as templates for DNA polymerase activity and acts to destabilize the DNA helix (Challberg, 1991). The protein may also be involved in the organization of the proteins at the replication fork. The DNA polymerase exists as a heterodimer in which the two subunits are a polymerase (U_L30, 140K) and a double-stranded

DNA-binding protein (U_L42, 52K; Parris, et al., 1988; Gallo, et al., 1989) which increases the processivity of the polymerase and prevents its dissociation from the growing strand (Gottlieb, et al., 1990). Several different structural forms of virus DNA are found during the replication process. Available evidence suggests that HSV DNA circularises within the host cell nucleus and following generation of the replication fork, is subsequently replicated by a rolling-circle mechanism. This gives rise to concatameric structures of multiple copies of the genome in a head-to-tail orientation (Jacob, et al., 1979; Tooze, 1980; Jongeneel and Bachenheimer, 1981).

1.4.3. Assembly, Envelopment and Release.

Following replication, viral DNA is processed into unit length molecules and packaged into readily-assembled nucleocapsids within the cell nucleus. The mechanisms involved are not well understood, but presumably involve DNA-associated proteins that cut the concatamers at precise lengths, then insert and fold the DNA molecule into the nucleocapsid. The processing events occur within 2 hours of the onset of DNA replication and continue late into the infectious cycle. The full capsids move towards the outer edge of the nucleus and accumulate below the inner nuclear membrane. At the points of contact, the membrane becomes noticeably thicker when viewed with an electron microscope, and is seen to contain projections on it's outer surface. These are the viral glycoproteins. The nucleocapsids become totally enclosed within the altered membrane and enveloped virions leave the nucleus, contained within a vesicle formed from the outer nuclear membrane. The vesicles move through the cytoplasm towards the cell membrane, where they fuse with the plasma membrane, releasing the enveloped virus from the cell (review, Roizman and Sears, 1990). The Golgi is thought to be involved in this transportation and release. Treatment of infected cells with monensin (which affects Golgi functioning) prevents enveloped virions reaching the

cell surface by interfering with post-translational modifications within and transport through the Golgi (Johnson and Spear, 1982).

1.5. STRUCTURE OF THE HSV VIRION.

There are four distinct morphological virion features which are characteristic to the Herpesviridae. The electron-dense core contains the viral DNA genome associated with proteins and enclosed within an icosahedral nucleocapsid. Surrounding the capsid is an amorphous layer of variable size and unknown function, the tegument. The outermost coat of the virion is a lipid membrane envelope, usually acquired by the virion as it buds through the nuclear membrane of the infected cell. Several virus-specified glycoproteins project from the surface of the envelope (Tooze, 1980).

1.5.1. The HSV Genome.

The genetic material of the virus is contained within a double-stranded (ds) linear DNA molecule which is 152 kilobasepairs (kb) in length. The DNA molecule contains two components of unique sequence, the long and short unique regions (U_L and U_S, respectively), flanked by inverted repeat sequences, the long and short terminal/internal repeats (TR_L/IR_L and TR_S/IR_S, respectively). In addition, the genome possesses small direct repeat sequences, the a region, found in varying numbers at the termini and at the junction between the long and short regions (McGeoch, 1987). Preparations of HSV genomic DNA contain a mixture of four structural isomers, in equimolar quantities, differing in the relative orientations of the L and S segments about the junction. Conventionally, one isomer has been designated as the prototype for the purpose of mapping the viral genes. The precise mechanism by which these isomers are generated during the replicative cycle is unclear and may involve replication of all four isomers or of only one or two forms which subsequently undergo reiterations to produce the four relative orientation patterns (Jacob, et al., 1979; Jongeneel and Bachenheimer, 1981). The presence of all four isomers does not appear to be crucial to infectivity, as

viruses "frozen" in one particular orientation are infectious (Poffenberger, et al., 1983; Jenkins and Roizman, 1986). The complete sequence of the HSV genome has been determined (McGeoch, et al., 1985, 1988) and reveals 70 unique genes, of which two (the genes encoding ICP0 and ICP4) are present in two copies. The organisation of the HSV-1 genome and the position of all known HSV-1 genes are indicated in Figure 1.3. Coding sequences are found on both strands of the genome (McGeoch, et al., 1988). Immediate-early genes are clustered in and around the repeat regions of the genome, whilst the early and late genes are spread throughout the genome, except for several glycoproteins genes which are grouped within the short unique region.

1.5.2. The HSV Nucleocapsid.

The icosahedral nucleocapsid is approximately 100nm in diameter (Wildy, et al., 1960) and contains seven polypeptides ranging in size from 154 kilodaltons (kDa) to 12 kDa. The largest and the smallest capsid polypeptides (NC1 and NC7, respectively) show a high degree of homology between HSV-1 and HSV-2 nucleocapsids (Cohen, et al., 1980). Electron microscopy of extracted HSV nucleocapsids has shown them to consist of 162 hollow-cored elements (capsomeres) arranged in a regular fashion. The number and arrangement appears to be characteristic of the Herpesviridae.

1.5.3. The Tegument.

The tegument is defined as the material between the capsid and the envelope and appears as a fibrous layer when viewed with the electron microscope. Two viral proteins have been located within the tegument; the late protein Vmw65 which transactivates immediate-early transcription and the product of the U_L47 gene which is thought to modulate the activity of Vmw65. The tegument is present in most virions, but the thickness of the layer depends on the virus, the type of cell line and the location of the virion within the infected cell, as variations are seen between virions from different cells and even within the same cell (Tooze, 1980; Roizman, 1990). As the size of the tegument varies, so does

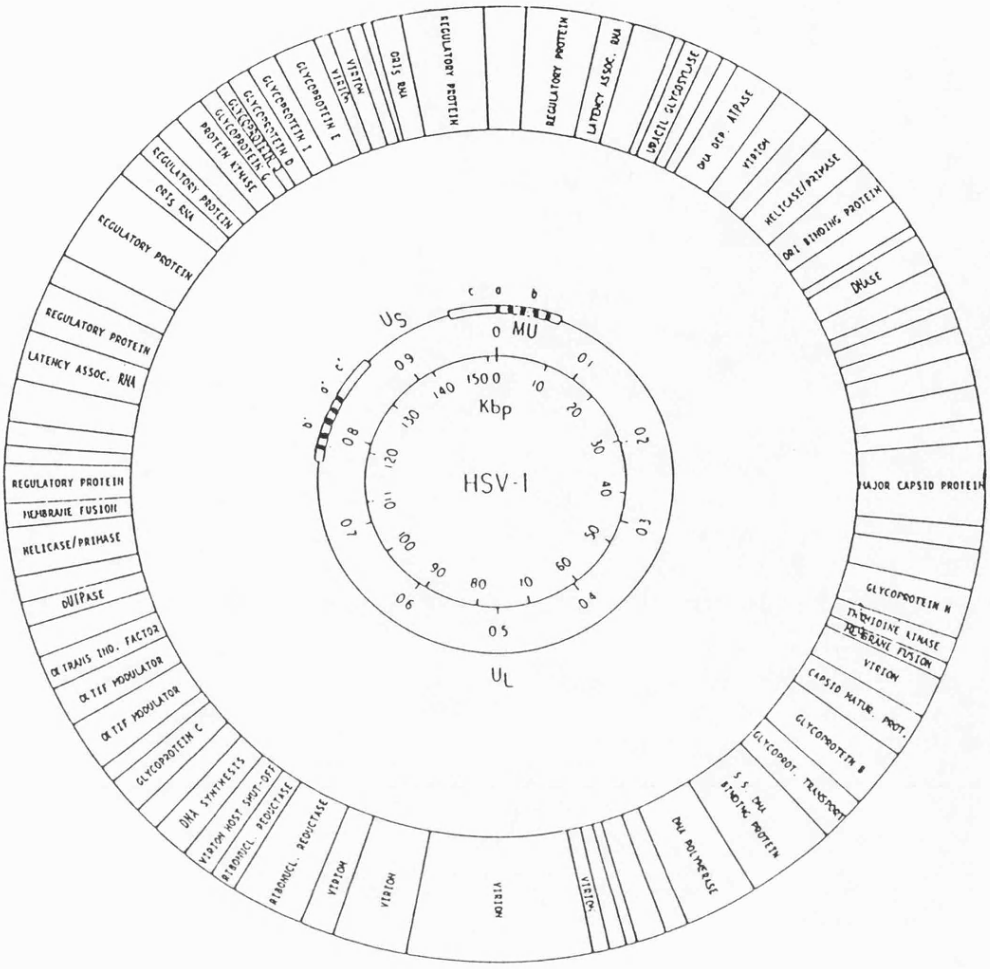


Figure 1.3. Functional organisation of the HSV-1 genome. Circles are described from the inside out. **Circle 1.** Map units (MU) and kilobasepairs (kbp). **Circle 2.** Sequence arrangement of HSV-1 genome, as a circularised version of the prototype arrangement. The letters a/a′ represent direct repeat sequences, letters b/b′ represent the long terminal and internal repeats, and letters c/c′ represent the short terminal and internal repeats. **Circle 3.** List of known functions of proteins specified by the genome. The position of each section corresponds to an open reading frame. Structural proteins are designated virion or glycoprotein. A large number of reading frames have not yet been assigned a functional protein. From Roizman and Sears, 1990.

the overall size of the virion, which accounts in part for the broad range of sizes (120-300nm) reported for the HSV particles. The tegumentis frequently distributed asymmetrically about the nucleocapsid.

1.5.4. The Viral Envelope.

Mature HSV particles are enclosed in an envelope of varying size and shape. The envelope is acquired by the virus as it exits the infected cell. The surface of the virion envelope displays projections when viewed by electron microscopy, of 8-10nm long (Wildy, et al., 1960). The projections are virally encoded glycoproteins, inserted into cellular membranes during the virus replication cycle. HSV contains at least eight glycoproteins, designated gB, gC, gD, gE, gG, gH, gI and gJ, which are involved in virus infectivity.

1.6. HSV GLYCOPROTEINS.

Enveloped viruses may contain one or more glycosylated proteins as important envelope constituents, specified by viral genes. The glycoproteins usually project from the surface of the virion envelope, and have also been found within the plasma membrane of infected cells. As a result of their situation within viral envelopes, glycoproteins are the first virion components to come into contact with the cell and as such are important in the infectious process and in the targetting of the virus to specific cells. The projection of viral glycoproteins from the surface of infected cells can influence the manner in which these cells interact with each other. More importantly, glycoproteins within plasma membranes are in contact with the mediators of host immunity and play a significant role in the induction of anti-viral responses. Seven glycoproteins (gB, gC, gD, gE, gG, gH and gI) have been identified within the HSV envelope and within HSV-infected cell membranes. DNA sequence data representing the viral genome has demonstrated the presence of an additional gene which may encode another glycoprotein, gJ, although the protein itself has not yet

been identified (Roizman, 1990). The roles of viral glycoproteins within infectious disease processes have been reviewed by Crumpacker (1980).

Glycoproteins are synthesised on membrane-bound polyribosomes and the growing amino acid chains are transported into the lumen of the RER. The proteins undergo a number of modifications during maturation, such as peptide cleavage, folding of the protein molecule and the addition of various prosthetic groups. These include sulphate groups, fatty acids and the addition of N-linked and O-linked oligosaccharides (Spear, 1985). N-linked oligosaccharide groups are added to the protein within the RER and are modified within the Golgi, whilst O-linked oligosaccharides are processed solely within the Golgi. 2-deoxy-D-glucose and tunicamycin inhibit the addition of carbohydrates to both cellular and viral proteins within cells. Treatment of HSV-infected cells results in significantly reduced production of infectious virus, and the few virions that are produced are unable to initiate further infection (Spivack, et al., 1982; Svennerholm, et al., 1982). Treatment of cells with monensin prevents the correct addition and processing of oligosaccarides within the Golgi, and as a result glycoproteins and virions are not transported to the plasma membrane (Johnson and Spear, 1982). The addition and modification of oligosaccharides within an infected cell happens sequentially, allowing detection of several non-glycosylated and partially glycosylated precursor forms of the virus glycoproteins. However, only fully glycosylated forms are found within virion envelopes and plasma membranes (Spear, 1976).

1.6.1. Glycoprotein B.

Early studies reported two closely associated glycoproteins in infected cells (gA and gB), but only gB was found within virions (Spear, 1976). The two proteins are now known to be the same polypeptide (Eberle and Courtney, 1980), differing only in the degree of glycosylation, and the name gA has been dropped from the nomenclature. HSV-1

glycoprotein B (gB-1) has a molecular weight of 126 kilodaltons (kDa) and is an essential protein for virion infectivity (Sarmiento, et al., 1979; Little et al., 1981). The genomic regions encoding both gB-1 and gB-2 have been mapped and sequenced (Bzik, et al., 1984, 1986; Pellet, et al., 1985). A large open reading frame within both sequences encodes a polypeptide of 903 amino acids, and the two gB molecules share 85% homology of amino acid sequence. Glycoprotein gB molecules spontaneously interact with each other to form stable oligomeric forms in infected cells (Sarmiento and Spear, 1979; Claesson-Welsh and Spear, 1986) and within the virion envelope (Zhu and Courtney, 1988). No other viral proteins appear to be necessary for this association, which occurs rapidly after gB synthesis. The molecular weights of these oligomers can vary, but the majority are 220 to 250kDa, implying that a dimer is the predominant form (Sarmiento and Spear, 1979).

Virus strains lacking a functional gB are unable to produce infectious progeny virus (Sarmiento, et al., 1979; Little et al., 1981; Cai, et al., 1988). In each case examined, the block in the infectious cycle occurred after the virus has adsorbed to the cell surface. The addition of poly-ethylene glycol (PEG), a fusogenic agent, allowed the attached viruses to enter the cells and initiate an infection. Glycoprotein B is therefore essential for HSV infectivity at the level of virus penetration into cells, causing fusion between the viral envelope and the plasma membrane. In addition to virus-cell fusion, some HSV strains (syncitial strains) cause cellular fusion, leading to the formation of polykaryocytes within infected monolayers. Defective gB synthesis abrogates this normal fusion activity by such strains, implicating the glycoprotein in cellular fusion activity (Manservigi, et al., 1977).

1.6.2. Glycoprotein C.

At 130kDa, the mature gC-1 molecule is the largest HSV glycoprotein. The smaller HSV-2 counterpart was initially thought to be a distinct protein, designated gF (75kDa).

However, gF was precipitated by monoclonal antibodies specific for gC-1 epitopes (Zweig, et al., 1983) which suggested a relationship between the two proteins. The gene for HSV-2 gF was finally mapped to a genome position colinear with that of gC-1 by Zezulak and Spear (1984), who proposed that gF be renamed gC-2. The genes for gC-1 and gC-2 have been sequenced (Frink, et al., 1983; Draper, et al., 1984; Swain, et al., 1985) and both encode a family of RNA transcripts related by splicing (Frink, et al., 1983; Swain, et al., 1985). The major unspliced RNA species directs synthesis of the glycoprotein, giving peptide chains of 513 amino acids for gC-1 and 480 amino acids for gC-2. The large size of the mature protein (130kDa) compared to the unglycosylated peptide (69kDa), indicates extensive glycosylation occurs during processing. In addition to nine potential sites for N-linked glycosylation, gC also contains a high proportion of O-linked carbohydrate residues (Olofsson, et al., 1983; Dall'Olio, et al., 1985). Most of the carbohydrates are clustered within the N-terminal half of the protein, where they affect the antigenicity of several gC-specific epitopes (Sjoblom, et al., 1987). It has recently been proposed that gC molecules can associate in oligomeric forms within the membrane of infected cells (Kikuchi, et al., 1990) and virions (Zhu and Courtney, 1988), but the associations are thought to be loosely binding compared to those in gB multimers.

Several strains of HSV-1 and HSV-2 have been found which do not express gC but are still viable in tissue culture (Zezulak and Spear, 1984). Glycoprotein C-negative strains have been shown to exhibit normal pathogenicity within mice (Johnson, et al., 1986; Sunstrum, et al., 1988) and gC-negative strains have even been isolated from man (Hidaka, et al., 1990), although this is rare. Several virus strains exist which contain a truncated gC gene, and produce truncated, gC-related proteins. These proteins lack the membrane-anchor region, causing secretion of the protein from infected cells, rather than its insertion within the virion envelope (Draper, et al., 1984; Holland, et al.,

1984; Homa, et al., 1986). The viability of these mutant strains suggests gC does not provide an essential function during virus replication. Glycoproteins homologous to gC have been found in other herpesviruses (Kitchinson, et al., 1986; Robbins, et al., 1986; Allen and Coogle, 1988), although the degree of homology to gC-1 is generally low. Fuller and Spear (1985), found that antibodies specific for gC were capable of inhibiting virus adsorption to cells. Glycoprotein gC has been recently demonstrated to be capable of attaching to cell surface heparan sulphate molecules, prior to virus penetration (Campadelli-Fiume, et al., 1990) and gC-negative virions bind to target cells at lower frequencies than wild-type virions (Herold, et al., 1991). These reports all suggest that gC may function at the level of adsorption and, although not crucial for virus replication, may act to enhance virus infectivity.

A more definitive role for gC has been established as a receptor molecule able to bind C3b, the third component of the complement cascade (Friedman, et al., 1984; Smiley, et al., 1985; McNearney, et al., 1987). Both gC-1 and gC-2 are able to bind C3b, but cell surface binding is not observed in HSV-2-infected cells (McNearney, et al., 1987; Seidel-Dugan, et al., 1988), suggesting other HSV-2 proteins may interfere with binding during infections. The binding of C3b by gC-1 on HSV-infected cell surfaces interferes with the both the classical and alternative pathways of complement activation (Fries, et al., 1986; Harris, et al., 1990), and results in protection from complement-mediated virus neutralisation and immune cytolysis of infected cells (McNearney, et al., 1987; Harris, et al., 1990; Hidaka, et al., 1991).

1.6.3. Glycoprotein D.

HSV gD-1 and gD-2 have molecular weights of 59kDa (52kDa unglycosylated precursor) and 56kDa (51kDa precursor), respectively (Cohen, et al., 1978; Eisenberg, et al., 1979, 1980). The genes encoding gD-1 and gD-2 have been sequenced (Watson, et al., 1982; Watson, 1983). Several antibodies

specific for gD were able to inhibit virus penetration into cells (Highlander, et al., 1987) and virus-induced fusion within an infected monolayer (Noble, et al., 1983), suggesting the glycoprotein was involved in membrane fusion. Fuller and Spear (1985) found antibodies to gD were able to inhibit both adsorption of the virus to cells, and penetration of attached virus into cells. However, only antibodies affecting fusion were strongly neutralising, suggesting gD is more important during penetration than virus attachment. This block to infectivity mediated by anti-gD antibodies could be removed by the application of PEG to infected cells (Fuller and Spear, 1987; Ligas and Johnson, 1988), supporting this theory. Nonetheless, gD has been implicated in binding to cell surface receptors (Johnson and Ligas, 1988). Glycoprotein D is therefore essential for infectivity, active during attachment and penetration of the virus into target cells.

1.6.4. Glycoproteins E and I.

Infection or transformation by HSV induces cell surface receptors able to bind the conserved region (Fc) of immunoglobulin G (IgG) molecules (Adler, et al., 1978). The receptor function has been associated with two glycoproteins, gE (Baucke and Spear, 1979) and gI (Johnson and Feenstra, 1987). Glycoprotein gE-1 is processed from a 65kDa precursor to a fully glycosylated protein of 80kDa (Bauke and Spear, 1979), whilst mature gE-2 is slightly larger, at 90kDa (Para, et al., 1982). Glycoprotein gI has an apparent molecular weight of 66kDa (Longnecker, et al., 1987; Sullivan and Smith, 1988). The precursor forms of gE-1 and gE-2 also demonstrate Fc-binding, indicating complete maturation of the glycoproteins is not essential for this particular function. Both gE and gI genes map to within the short unique region of the virus genome and both glycoproteins are dispensible for virus replication in vitro (Longnecker, et al., 1987; Neidhardt, et al., 1987). Johnson and colleagues (Johnson and Feenstra, 1987; Johnson, et al., 1988) were unable to demonstrate Fc binding by gE or gI alone, suggesting both glycoproteins are required for the

function and may complex together. However, expression of the individual glycoproteins within recombinant vaccinia virus vectors demonstrated that gE could bind to Fc without the aid of gI, but the binding was greatly increased in the presence of both glycoproteins (Bell, et al., 1990). HSV-specific antibodies bind to viral proteins via the Fab region of the antibody molecule. The binding of the IgG Fc region to the surface of HSV or infected cells prevents binding by Fab regions. This blocks antibody-mediated virus neutralisation and cytolysis of infected cells (Adler, et al., 1978; Dowler and Veltri, 1984). An antibody specific for a viral protein may bind via it's specific region to the antigen, whilst also binding to gE/gI via it's Fc region (Frank and Friedman, 1989). The result is a "bridged" antibody molecule, which does not present a free Fc region to the complement cascade.

1.6.5. Glycoprotein G.

The 128kDa HSV-2 glycoprotein (previously nominated gC-2) was shown to be distinct from other HSV-2 glycoproteins and to show no homology to gC-1. The map location for the coding sequence of this protein (Roizman, et al., 1984) overlapped that of a 92kDa glycoprotein detected by Marsden et al. (1984). The two proteins were determined to be the same protein, designated gG-2, and differing gel systems were blamed for the apparent discrepancies in size. A novel HSV-1 glycoprotein whose gene sequence mapped to a corresponding position to gG-2 on the HSV-1 genome (U_S4) was identified as gG-1 (Ackermann, et al., 1986; Frame, et al., 1986; Richman, et al., 1986), with a size between 56 and 68kDa. The gG-2 molecule has been shown capable of association into multimeric forms (Eberle and Courtney, 1982), a feature previously observed with gB. No apparent role for gG in HSV infections has been determined.

1.6.6. Glycoprotein H.

HSV-1 gH was characterised by Buckmaster and colleagues (1984) as a glycoprotein of 115kDa. The gH coding sequence maps close to the gene for TK and has been sequenced

(Gompels and Minson, 1986). Temperature-sensitive mutants unable to synthesise gH are not infectious, indicating the glycoprotein is essential for virus infectivity (Desai, et al., 1988). Antibodies specific for gH neutralise virus and inhibit plaque formation (Buckmaster, et al., 1984), block penetration of virus attached to cells, (Fuller et al., 1989) and block polykaryocyte formation by syncitial strains of HSV (Gompels and Minson, 1986). Glycoprotein H is important in membrane fusion events, resulting in the egress of enveloped progeny virions from infected cells, or possibly in the formation of intercellular junctions between infected and uninfected cells. Expression of gH-1 within a cell line or a recombinant vaccinia virus gave rise to a slightly altered protein, which was inefficiently transported to the cell surface (Gompels and Minson, 1989). The report suggested one or more other HSV proteins are required for the correct processing and transport of gH. However, a recent report has found baculovirus expression of gH capable of producing a glycoprotein of approximately authentic size which was transported to the cell surface (Ghiasi, et al., 1991).

1.6.7. Glycoprotein J.

The U_S5 gene of HSV is predicted to encode an eighth glycoprotein, gJ (Roizman, 1990), but as yet the protein has not been identified.

The clustering of glycoprotein genes within the U_S region of the genome has suggested evolutionary relationships between the genes. Genes U_S4 (gG), U_S5 (gJ?), U_S7 (gI) and U_S8 (gE) are thought to have arisen by duplication of the U_S6 (gD) gene (McGeoch, 1990). Glycoproteins gD, gG and gI each contain a related peptide domain spanning 3 closely-spaced cystine residues, which is also present in the predicted polypeptide structure for which the U_S5 gene encodes (McGeoch, 1990). Several features of the HSV-1 glycoproteins are outlined in Table 1.1.

TABLE 1.1.

PROPERTIES OF HERPES SIMPLEX VIRUS GLYCOPROTEINS.

GLYCOPROTEIN	MOL.WT. (KDa)	POSSIBLE ROLES DURING INFECTION
gB	126	Attachment to cellular receptors, virus entry via fusion of envelope and cell membrane, fusion of cell membranes
gC	130	Attachment to cellular receptors? Penetration of virus into cells? Regulation of gB-mediated fusion? C3b-receptor and inhibitor of complement-mediated immune responses
gD	59	Fusion of envelope and cell membrane (virus entry), attachment?
gE	80	IgG Fc receptor and inhibitor of antibody-mediated responses
gG	56-68	Unknown
gH	115	Membrane fusion, virus egress from cell, formation of intercellular bridges?
gI	6 6	Augmentation of gE Fc-binding function
gJ	??	Unknown

See text for references

1.7. HSV GLYCOPROTEINS AND HOST IMMUNE RESPONSES.

The herpesvirus glycoproteins, situated on the surface of virions, are involved in the primary interactions between the virus and host cell. This, and their location on the surface of infected cells means they are likely to be involved in immune recognition by host defences. HSV induces both natural and adaptive immune responses within an infected host.

1.7.1. Natural Responses to HSV Glycoproteins.

Natural (non-specific) responses to HSV invasion and cellular damage form the first line of defence against the virus (review; Lopez, 1984). Cellular responses include phagocytic macrophages and natural killer (NK) cells, which ingest and destroy foreign particles and lyse HSV-infected cells, both in the presence and absence of HSV-specific antibodies. Mature macrophages are also able to limit HSV spread as they are non-permissive for HSV replication (Johnson, 1964b; Stevens and Cook, 1971b). Several cell types produce interferon (IFN) proteins in response to virus infection, which induce a block on virus replication within uninfected cells. IFN can also enhance the activities of both macrophages and NK cells. Torseth and Merigan (1986) have reported correlations between high IFN titres and longer lesion-free intervals within patients suffering HSV recurrent infections. More recently, Hendricks and colleagues (1991) reported that exogenously produced IFN was able to protect mice against HSV corneal disease.

The cytolysis of HSV-infected cells by NK cells is greatly influenced by the presence of virus glycoproteins on the cell surface. The loss of gB and/or gC expression on the infected cell surface results in a significant drop of NK activity (Bishop, et al., 1983). Later work also suggested that NK cells were able to recognise specific viral glycoproteins on the surface of HSV-infected cells (Bishop, et al., 1986). Glycoprotein C influences natural responses to HSV and to HSV-infected cells through it's ability to bind the complement protein C3b. This binding blocks the

activation of the complement cascade and therefore antibody-independent, complement-mediated neutralisation and cytolysis of HSV and HSV-infected or transfected cells (McNearney, et al., 1987; Harris, et al., 1990; Hidaka, et al., 1991).

1.7.2. Antibody Responses to HSV Glycoproteins.

Antibodies are produced in response to both primary and recurrent HSV infections. Antibody responses involve several effector pathways, including neutralisation of the virus and lysis of infected cells by macrophages, NK cells or complement proteins (Norrild et al., 1984; Norrild, 1985). Antibodies have been implicated in restricting the spread of HSV to the nervous system of the infected host (McKendall, et al., 1979; Kapoor, et al., 1982; Glorioso, et al., 1984) and possibly in the prevention of zosteriform spread of virus within the skin (Simmons and Nash, 1985). Whatever the exact role of antibodies in HSV infections, they appear to have limited effects on virus replication at the peripheral site of invasion. Antibody responses have also been proposed to play a role in the maintenance of the latent state and the prevention of recurrences (Stevens and Cook, 1974). A large proportion of anti-HSV antibody responses specifically recognise the viral glycoproteins, although antibodies do recognise capsid proteins and even non-structural proteins (Showalter, et al., 1981; Eberle and Mou, 1983; Eberle, et al., 1985; Kuhn, et al., 1987). Cells infected with HSV mutants which do not express surface glycoproteins are resistant to immune lysis (Glorioso, et al., 1980), implicating them in the stimulation of important responses to infections. Antibodies can be generated following immunisation with glycoprotein preparations (Mertz, et al., 1984; Blacklaws, et al., 1987; Blacklaws and Nash, 1990) and glycoprotein-specific antibodies have been shown to protect mice against lethal virus infections (Dix, et al., 1981; Balachandran, et al., 1982; Kumel, et al., 1985).

Glycoprotein D is a type common antigen and stimulates antibodies capable of recognising both gD-1 and gD-2 (Cohen, et al., 1978; Pereira, et al., 1980) and which can protect mice against both HSV-1 and HSV-2 infections (Long, et al., 1984; Mishkin, et al., 1991). Loss of the correct folding of mature gD can cause a dramatic reduction in the levels of recognition by several antibodies (Cohen, et al., 1988; Sodora, et al., 1989), demonstrating the importance of conformational epitopes. However, synthetic peptides representing linear gD epitopes are capable of inducing protective antibody (Cohen, et al., 1984; Eisenberg, et al., 1985; Watari, et al., 1987; Weijer, et al., 1988), suggesting continuous epitopes are also important for gD immunogenicity.

Glycoprotein gB stimulates mostly type-specific antibodies, despite the high degree of homology between gB-1 and gB-2 (Eberle and Courtney, 1989). However, several type-common epitopes within gB-1 and gB-2 can stimulate antibodies capable of protecting mice from homologous and heterologous HSV challenge (Dix, 1990). In addition to conformational epitopes generated by peptide chain folding, the formation of gB dimers also generates conformational epitopes, which are lost when the monomers dissociate (Chapsal and Pereira, 1988; Eing, et al., 1989). Glycoprotein B has been shown to stimulate anti-HSV antibody (Eberle and Courtney, 1980; Kino, et al., 1986).

Antibodies directed against glycoprotein C are usually type-specific (Pereira, et al., 1980; Dix, et al., 1981). However, a small peptide representing a gC-1 epitope has stimulated antibody with type-common reactivity towards HSV-1 and HSV-2 infected cell extracts (Zweig, et al., 1984). A second type-specific epitope within gC-1 was found to gain gC-2 specificity following the substitution of a single amino acid (Kimmel, et al., 1990). It may be possible to generate type-common responses from gC, a typically type-specific protein. The use of monoclonal antibody resistant (mar) virus mutants, has mapped the epitopes of gC to within

two spacially distinct regions (Marlin, et al., 1985; Wu, et al., 1990). Region I, within the C-terminal portion of the protein, contains epitopes which express antigenicity in the absence of their carbohydrate molecules, but the N-terminal region II contains epitopes which are altered in antigenicity by the removal or modification of carbohydrate residues (Sjoblom, et al., 1987; Olofsson, et al., 1991). The binding of C3b by gC can protect both virus and infected cells from antibody-dependent, complement-mediated neutralisation or cytolysis, although the effect is less marked than against antibody-independent mechanisms (McNearney, et al., 1987; Harris, et al., 1990).

Glycoproteins gE, gG and gI have been shown to induce low levels of virus-neutralising antibodies (Para, et al., 1982; Ackermann, et al., 1986; Sullivan and Smith, 1988). The ability of gE to bind the Fc region of IgG molecules has, like gC, been found to confer a degree of resistance towards immune cytolysis mediated by HSV-specific antibodies (Adler, et al., 1978; Dowler and Veltri, 1984). HSV type-specific and type-common neutralising antibody has also been detected with specificity towards gH (Buckmaster, et al., 1984; Gompels and Minson, 1986).

1.7.3. T-Lymphocyte Responses to HSV Glycoproteins.

The importance of T-lymphocytes during HSV infections was first demonstrated in immunosuppressed mice by Oakes (1975). Resistance to subcutaneous HSV infection could be restored to these mice by passive transfer of HSV-sensitised spleen cells, but not with antibody. Kapoor and colleagues (1982) found immune lymphoid cells could eliminate HSV from the ear pinna and nervous system of infected nude mice, whilst neutralising antibody had no effect on virus spread within the ear. T-lymphocyte responses to HSV in mice have been reviewed by Nash, et al. (1985). There are four subsets of T-lymphocytes, the cytotoxic T cells (CTL), helper cells (TH), suppressor cells (TS) and delayed hypersensitivity cells (TDH). CTL cells were shown to be involved in HSV immunity (Pfizenmaier et al., 1977; Larsen, et al., 1983)

and their activity is generally restricted to recognition of herpes antigens in conjunction with Class I Major Histocompatibility Complex (MHC) self antigens (Sethi, et al., 1983; Yasukawa, et al., 1983). However, incidences of Class II-restricted anti-HSV CTL activity has been reported in mice and in humans (Yasukawa and Zarling, 1984; Schmid, 1988; Kolaitis, et al., 1990). A second subset of T cells was implicated in HSV resistance by Howes, et al. (1979), restricted in activity by Class II recognition. These are the TDH cells (Nash, et al., 1980; 1981c; 1982). The TDH response varies in mice with the route of inoculation. An intravenous injection of HSV into mice will induce a state of tolerance towards TDH responses, but leaves CTL responses intact (Nash, et al., 1981a; Nash and Ashford, 1982). The unresponsiveness of TDH cells to intravenous injection of virus is mediated by TS cells, which are specific for HSV (Nash, et al., 1981b; Nash and Gell, 1983; Schrier, et al., 1983a).

Anti-HSV T-cell responses have specificity for virus glycoproteins (Lawman, et al., 1980; Carter, et al., 1981; Yasukawa and Zarling, 1985). Confirmation of this has come from the ability of envelope and glycoprotein preparations to stimulate anti-HSV T-cell responses (Zarling, et al., 1988). Purified or cloned glycoproteins B, C and D have all been shown able to stimulate CTL (Zarling, et al., 1986a; Witmer, et al., 1990; Hanke, et al., 1991), TDH (Schrier, et al., 1983b; Blacklaws, et al., 1987) and TH (Chan, et al., 1985; Torseth, et al., 1987) responses against HSV. There have been several reports suggesting that gC is a predominant T-lymphocyte antigen for HSV. Schrier and colleagues (1983b) demonstrated the ability of purified gC to protect mice against HSV-1 challenge did not involve antibody mechanisms, but correlated with TDH responses. The specificity of CTL responses within the mouse were investigated by Glorioso, et al. (1985). A number of CTL clones were developed and the majority demonstrated specific for gC. More recent work supported the implications of these reports that gC is an immunodominant antigen. Cell lines

expressing gC on their surface were lysed by CTL from HSV-infected mice, whilst cell lines expressing gB, gD or gE were not lysed by the HSV-specific CTL (Rosenthal, et al., 1987). Non-glycosylated and non-structural proteins have also been shown to stimulate anti-viral CTL for HSV infections (Martin, et al., 1988).

1.8. VACCINE DEVELOPMENT FOR HSV INFECTIONS.

The development and application of vaccines represents the most cost-effective means of preventing and controlling infectious diseases. A number of vaccines for animal herpesvirus infections are already used regularly in veterinary medicine (review; Parks and Rapp, 1975) and the search for a human vaccine has been progressing for some time (Watson and Enquist, 1985; Hall and Katrak, 1986; Dix, 1987). Primary HSV infection almost inevitably leads to a latent infection, which may reactivate and produce recrudescence. Therefore merely priming the recipient to clear the primary infection is not sufficient to avoid recurrent herpes. The vaccine must either prevent invasion altogether, prevent invasion of the nervous system, or prevent recrudescence. The obvious difficulty lies with the ability of the virus to become latent with very little replication within the periphery and none at all in the ganglia. However, replication at peripheral sites is necessary, and this at least provides a starting point for vaccine design. The lack of knowledge concerning the maintenance of latency and the causes of reactivation mean that a vaccine which might prevent recrudescence following the establishment of a latent infection is harder to envisage at the present time.

1.8.1. Live Virus Preparations.

For most diseases, previous exposure to the infecting agent can prime the host's immune system to ameliorate disease. The course and severity of HSV-1 and HSV-2 infections have been modified in several animal models by prior infection with the homologous virus, although the extent of protection varies and neither HSV-1 nor HSV-2 have

been able to prevent latent infections following subsequent virulent challenge. Live virulent viruses obviously contain the risk of disease, and attenuated viruses are more likely vaccine candidates, particularly virus strains with genome deletions. These are less able to revert to virulence than are attenuated strains with a complete genome. Roizman and colleagues (1982) engineered HSV deletion mutants which showed markedly reduced neurovirulence in mice, yet protected the animals against lethal challenge. Recombination between HSV-1 and HSV-2 produced a deletion mutant virus which had reduced virulence in guinea pigs, rabbits, mice and owl monkeys (Meignier, et al., 1988). However, protection against a subsequent challenge was not absolute and virus shedding was as high as from non-immunised animals.

Live HSV vaccines have the potential to produce latent infections, to revert to virulence, and to recombine with other infecting strains to produce an active infection. These problems, and the association of HSV replication with the formation of cancers, means that the use of live vaccines seems unlikely for the prevention of HSV infections in man. Nevertheless, live attenuated VZV vaccines have been tested in man (Gershon, 1985; Diaz, et al., 1989), with some degree of success.

1.8.2. Inactivated Virus Preparations.

Methods of virus inactivation include treatment with heat, phenol, formaldehyde, UV-illumination, or various combinations of these. Inactivated virus preparations may be immunologically active and induce immunity to HSV challenge (reviewed by Hall and Katrak, 1986). Some inactivated vaccines have been tested in man but with little success (Frank, 1938; Anderson, et al., 1950) and although free from the danger of disease, inactivated vaccines still contain viral DNA, a putative carcinogen.

1.8.3. Subunit Preparations.

The earliest subunit vaccines were crudely extracted from infected cells, using various detergents and/or sonification to disrupt the virus and centrifugation to disrupt the cells and remove any remaining whole virus particles. Several such preparations have been able to induce protection against virus-induced neurological illness and death, or at least significantly reduce the incidence and severity of cutaneous infections (Dix, 1987). Subunit vaccines have been found to stimulate levels of protection in mice (Klein, et al., 1981a) and rabbits (Cappel, 1976) comparable to whole virus preparations. However, there was no resistance towards latent infections. An HSV-2 subunit preparation derived from infected cells has induced protective immunity against HSV-2 challenge in mice (Skinner, et al., 1980a, 1980b), guinea-pigs (Welch, et al., 1988; McBride, et al., 1989) and monkeys (Skinner et al., 1982a). This and other HSV-2 subunit vaccines have been tested in human volunteers (Skinner, et al., 1982b, 1982c; Mertz, et al., 1984; Zarling, et al., 1988), but without placebo controls. A DNA-free subunit vaccine developed by Kitces, et al. (1977) was able to reduce acute disease, CNS invasion and the incidence of latency following subsequent challenge in mice. Similarly, Cappel tested a nucleic acid-free HSV-2 subunit vaccine within animal systems and also in human volunteers suffering frequent recurrences (Cappel, et al., 1980, 1982, 1985). Immunised mice and rabbits experienced some protection against HSV-2 challenges. In humans, transient immune responses were raised against HSV-2 and volunteers experienced fewer and less severe recurrences than non-vaccinated individuals. However, a double-blind, placebo-controlled study in man using a subunit HSV-2 preparation revealed patients receiving both vaccine and placebo experienced reduced recurrences (Kutinova, et al., 1988). The response to the placebo casts doubts on the efficacy of the preparations tested to date. In addition, the exact composition of crude subunit preparations is generally uncertain, which causes further problems regarding safety.

1.8.4. Glycoprotein Preparations.

Affinity-purified HSV glycoproteins B, C and D have been shown to induce protective immunity towards subsequent viral challenge (Chan, 1983; Schrier, et al., 1983a; Long, et al., 1984; Chan, et al., 1985; Dix and Mills, 1985; Roberts, et al., 1985; Mishkin, et al., 1991). Glycoprotein D provides type-common immunity, protecting against either HSV-1 or HSV-2 challenge (Long, et al., 1984; Dix and Mills, 1985) whilst gC provides only type-specific immunity (Schrier, et al., 1983b). None of the glycoproteins were able to prevent the establishment of ganglionic latency following infectious challenges.

Chemically synthesised peptides which represent specific epitopes of virus antigens have been used in successful immunisations against viruses such as influenza, hepatitis B and foot-and-mouth disease (review; Dix, 1987). A range of synthetic peptides representing various epitopes of gB (Mester, et al., 1990) or gD (Eisenberg, et al., 1985; Watari, et al., 1987; Geerligs, et al., 1990) have been shown to protect mice against subsequent HSV challenge. However, the full range of immunogenic sites are not represented by these peptides, and conformational epitopes are not represented by short linear molecules, resulting in lower protection than that attained using full length glycoproteins (Eisenberg, et al., 1985). The efficacy of glycoprotein preparations in general are influenced by various factors, such as the type of preparation, the site of immunisation and the adjuvant used (Stanberry, et al., 1987, 1989). Neither crude or purified subunit preparations replicate within the immunised host, so that large and repeated doses of the immunogen need to be administered.

1.8.5. Foreign Expression Vectors.

Large quantities of purified, DNA-free herpesvirus glycoproteins have been synthesised using prokaryotic, eukaryotic and viral expression vectors. For example, immunoreactive forms of HSV-1 gC (Amann, et al., 1984) and gD (Watson, et al., 1982; Kocken, et al., 1988) have been

expressed in bacterial culture by Eschericha coli, fused with the bacterial ß-galactosidase protein and gD has recently been expressed by a Salmonella strain (J. Bowen, personal communication, PhD. thesis). However, bacteria do not possess the metabolic pathways necessary for the correct modification of cloned glycoproteins. Eukaryotic vector systems can produce native glycoproteins, and gB derived from a yeast vector was shown to stimulate anti-HSV antibody in immunised guinea pigs (Nozaki, et al., 1985). Several HSV glycoproteins have been expressed in mammalian cell lines. Full-length and truncated (secreted) forms of gB and gD have been synthesised from transfected cell lines and have been able to protect mice and guinea pigs from lethal infections (Berman, et al., 1984; Blacklaws, et al., 1987; Pachl, et al., 1987; Stanberry, et al., 1987, 1988; Lasky, 1990; Manservigi, et al., 1990).

The use of viral vectors for the expression of HSV proteins has the advantage of using a replicating system. Inoculation of an animal host with a live, recombinant viral vector expressing an HSV protein would allow that protein to be presented to the host immune system in a manner analogous to the presentation during an HSV infection. A viral vector is also capable of maintaining the antigen presentation for some time (until the immunised host clears the invading vector), which eliminates the need for large or frequent doses. Small DNA viruses were first to be explored as vectors, as they were easily manipulated in vitro. However, packaging constraints limited the amount of foreign DNA which could be inserted, and small DNA viruses have been largely superceded as expression vectors by large DNA viruses. The most widely used viral vector of recent years, and the vector of choice for this project, is vaccinia virus.

1.9. RECOMBINANT VACCINIA VIRUS VECTORS.

Almost 200 years after Edward Jenner first used vaccinia to protect subjects against smallpox, the virus is once again of great significance in vaccine research. Numerous

reviews have been presented on the subject of vaccinia virus as a cloning and expression vector (Paoletti, et al., 1984b, 1987; Mackett and Smith, 1986; Moss, et al., 1988; Piccini and Paoletti, 1987; 1988; Smith, 1987). The vaccinia genome, a linear dsDNA molecule, is not infectious when stripped of its proteins, which means foreign genes cannot be cloned directly in vitro, but require the use of plasmid vectors as intermediates. Special plasmids have been designed which possess vaccinia DNA sequences (generally the TK gene) interrupted by restriction enzyme sites, which allow cloning of foreign genes into the viral sequences (Boyle, et al., 1985; Chakrabarti, et al., 1985). The cloning of genes into the body of the virus gene allows homologous recombination between plasmid and virus DNA, thereby introducing foreign genes into the virus genome. The resulting recombinant viruses can be selected on the basis of their TK-negative phenotype. The large size of the vaccinia genome (187kb) and the lack of packaging constraints means large stretches of foreign DNA can be inserted without significantly affecting viability. To date 25kb of foreign DNA has been successfully cloned into infectious vaccinia vectors (Smith and Moss, 1983). Venkatesen et al. (1981) proposed that vaccinia, which replicates within the cytoplasm of infected cells, has evolved unique transcription recognition signals that differ from prokaryotic and mammalian signals. The virus encodes it's own transcriptional enzymes which do not recognise prokaryotic or eukaryotic signals, only those of it's own genes (Mackett, et al., 1982; Cochran, et al., 1985a). This means that foreign genes must be cloned under the influence of vaccinia promoter regions if they are to be transcribed by a recombinant virus. The most commonly used vaccinia promoters have been the P7.5 and the P11 promoters, which control the transcription of a 7.5 kDa protein (Venkatesen, et al., 1981) and an 11 kDa protein (Bertholet, et al., 1985), respectively. The P11 promoter acts late in the infectious cycle, whilst the P7.5, which has been shown to contain two transcriptional start sites, allows mRNA synthesis at both early and late times during the infection (Mackett, et al., 1984; Cochran, et al., 1985b). Fuerst and

colleagues (1986, 1987) developed a vaccinia recombinant which expressed the bacteriophage T7 RNA polymerase under the control of the P7.5 promoter. This recombinant was able to drive high levels of gene expression from plasmids or from a second vaccinia recombinant possessing the second gene under the control of T7 promoters. The levels of expression of the second gene were higher than the levels attained using vaccinia promoters alone.

Selectable marker genes are often cloned alongside the gene of interest (both controlled by individual vaccinia promoters) to provide easier selection of recombinant proteins. Markers have included genes specifying a varying plaque size in tissue culture (Rodriguez and Esteban, 1989), resistance to neomycin (Franke, et al., 1985) or to mycophenolic acid (Falkner and Moss, 1988). The gene encoding the firefly luciferase enzyme, which can generate visible light, has also been used (Rodriguez, et al., 1988). The method of selection for recombinant viruses within this project was developed by Chakrabarti and colleagues (1985), and cloning of foreign genes into the vaccinia genome was via the plasmid vector pSC11. The plasmid contains the E. coli ß-galactosidase gene, under the control of the vaccinia virus P11 promoter region. A unique SmaI site allows cloning of the foreign gene under the control of the P7.5 promoter region. The bacterial enzyme metabolises the substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), developing a blue-coloured product which allows easy identification of recombinant viruses within an infected cell monolayer.

One of the advantages of using the vaccinia virus expression system is that the foreign proteins are faithfully synthesised, modified and transported through the infected cells, compared to within their natural environment (Smith, et al., 1983a, 1987; Rice, et al., 1985; Ball, et al., 1986; Zhao, et al., 1987; Falkner, et al., 1988; Winokur, et al., 1991). Because they are faithfully represented, and because the vector itself infects the host,

many proteins have been expressed by recombinant vaccinia viruses which have induced good immune responses. For instance animal hosts have been protected against infection with viruses such as influenza (Smith et al., 1983b), rabies (Kieny, et al., 1984; Wiktor, et al., 1984), respiratory syncytial virus (Elango, et al., 1986), Hepatitis B (Moss, et al., 1984) and vesicular stomatitis virus (Mackett, et al., 1985b), following immunisation with recombinant vaccinia viruses expressing a single protein from the infecting agent. Several other foreign proteins have induced immune responses in vivo, against the pathogens they represent, including the malarial parasite Plasmodium knowlesi (Smith, et al., 1984) and the human immunodeficiency virus (Hu, et al., 1991).

1.9.1. HSV Proteins Expressed by Vaccinia Recombinants.

Vaccinia virus recombinants have been used to express herpesvirus proteins. The HSV TK gene was one of the first proteins to be expressed by recombinant vaccinia viruses (Mackett, et al., 1982; Panicali and Paoletti, 1982) and since that time all the known HSV glycoproteins have been expressed (Table 1.2.). Glycoprotein D was the first HSV glycoprotein expressed within a vaccinia virus (Paoletti, et al,. 1984a) and has been shown capable of inducing both antibody and T-cell responses against HSV (Zarling, et al., 1986b; Martin, et al., 1987; Martin and Rouse, 1987). Mice and guinea pigs vaccinated with gD-expressing viruses were protected against infection with both HSV-1 and HSV-2, and were also capable of averting HSV-1 latent infections (Cremer, et al., 1985; Wachsman, et al., 1987). HSV gB expressed by vaccinia virus has induced MHC-restricted anti-HSV CTL responses in mice (McLaughlin-Taylor, et al., 1988) and has given protective immunity to lethal HSV challenge following vaccination (Cantin, et al., 1987). Glycoprotein C has been expressed by vaccinia recombinants and has been shown to stimulate neutralising antibody and T-cell proliferation responses (Weir, et al., 1989a; Allen, et al., 1990), which were sufficient to protect mice against a lethal HSV infection. HSV glycoproteins gE, gG, gH and gI

TABLE 1.2.

HERPESVIRUS PROTEINS EXPRESSED BY RECOMBINANT VACCINIA VIRUS VECTORS.

VIRUS	PROTEIN	REFERENCES
HSV	TK	Mackett, et al., 1982; Panicali and Paoletti, 1982
	gB	Cantin, et al., 1987; McLaughlin-Taylor, et al., 1988
	gC	Allen, et al., 1990; Weir, et al., 1989
	gD	Cremer, et al., 1985; Martin, et al., 1987; Moss and Notkins, 1986; Paoletti, et al., 1984a; Perkus, et al., 1985; Wachsman, et al., 1987; Zarling, et al., 1986b
	gE	Blacklaws, et al., 1990; Bell, et al., 1990
	gG	Blacklaws, et al., 1990; Sullivan and Smith, 1987
	gH	Blacklaws, et al., 1990; Forrester, et al., 1991
	gI	Blacklaws, et al., 1990; Bell, et al., 1990; Sullivan and Smith, 1988
CMV	gB	Britt, et al., 1988; Cranage, et al., 1986
	gH	Cranage, et al., 1988
	pp89	Koszinowski, et al., 1988; Volkmer, et al., 1987
EBV	gp340	Mackett and Arrand, 1985; Morgan, et al., 1988

have also been expressed within vaccinia viruses, but only gE, gG and gI have shown any immune stimulation (Sullivan and Smith, 1987, 1988; Blacklaws, et al., 1990). The failure of gH to stimulate immunity when expressed in a vaccinia virus is thought to be due to a need for other HSV proteins for the correct maturation of gH (Forrester, et al., 1991).

1.10. STUDY PLAN.

HSV contains several envelope glycoproteins, of which gC is particularly interesting. The viability of strains lacking gC both in vitro and in vivo (Johnson, et al., 1986; Sunstrum, et al., 1988; Hidaka, et al., 1990) suggests the glycoprotein is dispensible for virion infectivity. However, the protein may act to augment the essential role of other HSV glycoproteins during virus attachment and penetration into cells (Campadelli-Fiume, et al., 1990; Herold, et al., 1991). The prominant role of gC appears to be in protection of the virus from immune elimination, by binding to the C3b protein and inhibiting complement-mediated neutralisation and cytolysis (Fries, et al., 1986; McNearney, et al., 1987; Harris, et al., 1990; Hidaka, et al., 1991). Despite the inhibition of these immune effector pathways, gC is capable of stimulating high levels of specific immunity. Both antibody and T-lymphocyte responses are generated towards gC and the glycoprotein has been proposed as a major antigen for the stimulation of anti-HSV T-lymphocyte responses (Schrier, et al., 1983b; Glorioso, et al., 1985; Rosenthal, et al., 1987). The glycoprotein, and synthetic peptides representative of gC epitopes have been used successfully as immunising agents (Schrier, et al., 1983b; Roberts, et al., 1985), confirming it's role in the stimulation of valuable immunity.

Interest in gC has lead to the expression of the glycoprotein in various vectors. Initial attempts to express the protein within bacteria produced some unexpected results. Fusion of the gC gene with bacterial ß-galactosidase gene did not give rise to any detectable gC peptides, but the presence of the gC DNA was linked to

inhibition of bacterial growth (Amann, et al., 1984). Fusion with both ß-galactosidase and the lambda bacteriophage repressor proteins did produce immunoreactive gC peptides, but the levels detected decreased with increasing size of gC DNA portions cloned (Amann, et al., 1984). Several cell lines have been manipulated to express gC from plasmids transfected into the cells and maintained by various selection pressures. The gC has been expressed under the control of HSV promoters by superinfection with a gC-negative strain (Arsenakis, et al., 1986, 1988), and under inducible (Friedman, et al., 1989) or constitutive promoters from foreign viruses (Seidal-Dugan, et al., 1988; Ghosh-Choudry, et al., 1990). Friedman and colleagues (1989) reported the occurrence of bizarre syncitial shapes in cells expressing gC over prolonged periods, and continued stimulation of transfected cell lines containing gC-plamids resulted in either the death of the cell line or an abolition of gC expression. These curious findings lead to the propsal that gC may be toxic to cells, a generally felt, but unproven feature of the glycoprotein. However, the constitutive expression of gC by Ghosh-Choudry, et al. (1990) did not appear to have any adverse effects on the gC-expressing cell lines for 20 passages.

The aim of this project has been to clone and express HSV-1 gC within a recombinant vaccinia virus vector. Glycoproteins gB (Cantin, et al., 1987), gD (Cremer, et al., 1985; Zarling, et al., 1986b; Martin, et al., 1987; Martin and Rouse, 1987; Wachsman, et al., 1987) and gG (Sullivan and Smith, 1987) had previously been cloned into vaccinia viruses, demonstrating the ability to stimulate HSV-specific immune responses and protection against HSV infections with such constructs. The coding sequences for the entire HSV-1 (strain 17) glycoprotein C were excised from a KpnI genebank (Davison and Wilkie, 1983) and cloned into plasmid vector pSC11 (Chakrabarti, et al., 1985). Following homologous recombination of cloned sequences into the vaccinia virus genome via virus TK sequences within pSC11, recombinant viruses were selected on the production of blue colour in

the presence of X-gal. Figure 1.4. outlines the mechanisms involved in the generation of recombinant vaccinia viruses. The expression of gC from vaccinia P7.5 promoter during recombinant virus infection of cells was determined, and gC-producing recombinant vaccinia virus was inoculated into mice. The ability of the recombinant virus to stimulate HSV-specific immune responses, and the protective quality of such immunisations against HSV infections were examined.

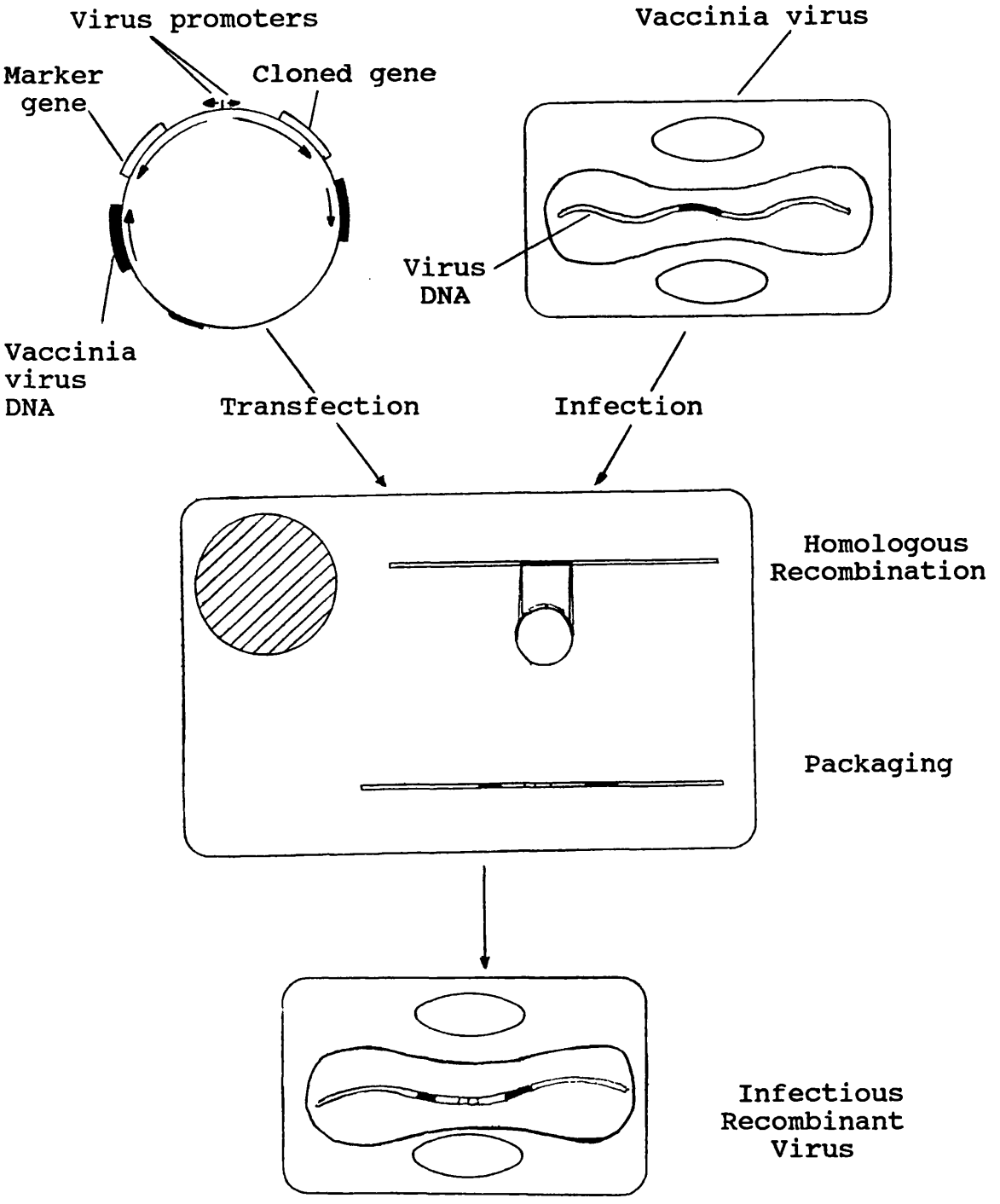


Figure 1.4. Mechanisms for the generation of recombinant vaccinia viruses. Plasmid vector containing foreign genes under the control of vaccinia promoters is used to transfect vaccinia-infected cells. Homologous recombination occurs between virus DNA sequences within the plasmid and the viral genome. The resulting recombinant DNA is packaged into infectious recombinant vaccinia viruses. From Mackett and Smith, 1986.

CHAPTER 2

MATERIALS AND METHODS

2.1. MEDIA AND CHEMICALS.

L-broth :
15g bacto-tryptone
5g bacto-yeast extract
5g NaCl
Adjusted to pH 7.2, made up to 1 litre, autoclaved.

L-agar :
As above, with 15g Difco agar per litre.

B-agar :
10g bacto-peptone
8g NaCl
15g Difco agar
Made up to 1 litre, autoclaved.

B-Top agar :
As above, with 6g Difco agar per litre.

Minimal (M9) agar :
6g Na_2HPO_4
3g KH_2PO_4
0.5g NaCl
1g NH_4Cl
Adjusted to pH 7.4, made up to 1 litre
15g Difco agar
Autoclaved, allowed to cool to 50^{o}C, and the following added;
2ml of 1M $MgSO_4$
10ml of 20% (w/v) glucose
0.1ml of 1M $CaCl_2$

Tissue culture media : Minimal Essential Medium (MEM; Gibco, BRL) supplemented with 2mM L-glutamine, or Leiboweitz (L-15) medium (Gibco, BRL) supplemented with 300mg/ml L-glutamine. Both media supplemented with 5% foetal calf serum, 100μg/ml penicillin and 100 units/ml streptomycin (Gibco). MEM maintained at equilibrium with 5% CO_2 at 37°C during culture.

Ampicillin : (Boehringer Mannheim) Stock solution of 50mg/ml in distilled water (dH_2O). Stored at -20°C.

Chloramphenicol : (Boehringer Mannheim) Stock solution of 170mg/ml in 100% ethanol. Stored at -20°C.

Tetracycline : (Boehringer Mannheim) Stock solution of 25mg/ml in ethanol. Stored -20°C.

Isopropyl-thio-ß-galactoside (IPTG) : (Boehringer Mannheim) Stock solution at 0.1M in dH_2O. Stored at -20°C.

5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) : (New England Biolabs) Stock solution of 20mg/ml in dimethyl formamide. Stored at -20°C.

TE : 10mM Tris-HCl (pH 8.0)
1mM EDTA (pH 8.0).

PBS : 8g NaCl
2g KCl
1.15g $Na_2HPO_4.2H_2O$
2g KH_2PO_4
Made up to 1 litre.

Lysosyme : (Sigma Chemical Company) Stock solution of 80mg/ml in dH_2O. Stored at -20°C.

Ribonuclease A (RNase A) : Stock solution of 10mg/ml in dH_2O, heated in a boiling waterbath for 10 mins. Stored at -20°C.

Phenol : Melted at 60°C, and equilibrated with 0.1M Tris-HCl pH 7.5. Stored at -20°C.

Chloroform : Used as a working solution of chloroform : isoamyl alcohol (24 : 1).

Ethidium Bromide (EtBr) : Stock solution of 10mg/ml in dH_2O. Stored at 4°C.

Antisera/antibodies : HSV-1 gC-specific monoclonal antibody 7542 kindly donated by Dr. A. Cross, MRC Virology Unit, Glasgow.

Centrifuges/rotors : Sorvall RC-5B centrifuge and Sorvall SS-34 rotor were used unless stated otherwise.

2.2. BACTERIAL STRAINS AND VECTORS.

E. coli JM 101 : Strain is *rec* A^- , contains F-plasmid and a deletion within the chromosomal *lac* Z (ß-galactosidase) gene. For full genetic background, see Maniatis, *etal*. (1982). Bacteria were maintained on B-agar and stored long-term in L-broth containing 20% (v/v) glycerol at -20°C.

E. coli RZ 1032 : Strain is tetracycline resistant and deficient in dut and ung gene functions. For full genetic background, see Kunkel, et al. (1987). Maintained on minimal (M9) agar containing 12.5μg/ml tetracycline and stored long-term as above.

KpnI genebank : Vector library containing the entire genome of HSV-1 (strain 17), fragmented by restriction enzyme KpnI, within plasmid pAT153 (Davison and Wilkie, 1983). HSV-1 (17) gC coding sequences are contained within the KpnId fragment.

pUC18 : Ampicillin-resistant plasmid containing the N-terminal of the E. coli lacZ gene, controlled by lacI and interrupted by a multiple cloning site polylinker.

pSC11 : Ampicillin-resistant plasmid containing the E. coli lac Z gene under the control of the vaccinia virus P11 promoter region. The plasmid also contains a unique SmaI site situated immediately downstream of vaccinia virus P7.5 promoter sequences. These regions are flanked by vaccinia thymidine kinase sequences (Chakrabarti, et àl., 1985).

M13mp19 : Bacteriophage vector containing E. coli lacZ and lacI regions interrupted by a multiple-cloning site polylinker, as described for pUC18.

2.3. CELL LINES AND VIRUS STRAINS.

CV-1 : African Green monkey kidney cells. Maintained at 37°C in either MEM (with 5% CO_2) or in L-15 medium. Long-term storage

of cells was in medium containing 10% DMSO and 20% serum, at approximately $5x10^6$ cells/ml, under liquid nitrogen.

Vaccinia virus : Wildtype strain Lister, stored at -80°C, supplied by Dr. M. Mackett, Manchester.

HSV-1 : Wildtype strain 17, stored at -80°C, supplied by Dr. K. Blake, PHLS, CAMR.

2.4. DNA MANIPULATION ENZYMES.

Enzymes were used as described below, according to manufacturer's instructions or in accordance with Greenaway and Dale (1983) or Maniatis et al. (1982). The concentration of DNA solutions was determined by the measurement of optical density at 260nm wavelength. Solutions were diluted 100-fold in TE buffer. Absorbances were determined using a Pye-Unicam SP8-300 UV/VIS spectrophotometer zeroed with TE, and calculations were adjusted to account for the dilution factor. An absorbance of 1.0 at this wavelength corresponds to a concentration of 50μg/ml of double-stranded (ds) DNA.

2.4.1. Restriction endonucleases.

Supplied (with reaction buffers) by New England Biolabs or Bethesda Research Laboratories (BRL). Restriction enzymes were used at a concentration of 1 unit/μg DNA. Digestion of DNA was achieved by incubation in 20-30μl volumes, in the appropriate reaction buffer, for 1 hour at 37°C or at 30°C for SmaI. Following restriction enzyme digestion, DNA fragments that required further manipulations were extracted as follows: magnesium ions in the reaction buffer were chelated with an equal molarity of EDTA and digests were mixed with 0.4 volumes of 5M sodium acetate and 2 volumes of iso-propyl alcohol. DNA was allowed to precipitate at room temperature for 10 min and was pelleted for 10 min in a bench microfuge. The DNA pellet was resuspended in an appropriate volume of TE.

2.4.2. E. coli DNA polymerase I (Klenow enzyme).

Supplied by BRL. Restriction-digested DNA fragments were resuspended in 25μl TE and mixed with 3μl of nick-translation buffer (NTB : 0.5M Tris-HCl (pH 7.2), 0.1M $MgSO_4$, 1mM dithiothreitol, 500μg/ml BSA). Cohesive fragment ends were converted to blunted ends by incubation with 1 unit of Klenow enzyme in the presence of dATP, dCTP, dGTP and dTTP (1μl of a 2mM solution of all four dNTP's in TE) at 37°C for 30 min. The reaction was terminated with 2μl of 0.5M EDTA.

2.4.3. Bacterial Alkaline Phosphatase.

Supplied by BRL. Terminal phosphate groups were removed from linearised vector DNA by 1-2 units of enzyme per μg of DNA, added directly to digested DNA (no extraction required). The reactions were incubated at 68°C for 30 min and the DNA immediately extracted twice with phenol, once with chloroform, and ethanol-precipitated.

2.5. CLONING OF DNA SEQUENCES.

2.5.1. Separation of DNA Fragments in Agarose Gels.

Complete digestion of DNA by restriction endonucleases was confirmed by electrophoresis through 0.8% agarose gels. DNA fragments required for cloning procedures were separated in 0.6% low-melting-point (LMP) agarose gels. Agarose was melted by heating in B-buffer (90mM Tris-HCl, 90mM boric acid, 2.5mM EDTA) and allowed to cool to approximately 60°C prior to pouring. For 0.8% agarose gels, ethidium bromide (EtBr) was added to the gels at this stage to a final concentration of approximately 0.5μg/ml. DNA samples were mixed with loading buffer (10% Ficoll, 0.05% bromophenol blue, 0.005% Xylene cyanol FF, 0.05% orange G) and electrophoresis was carried out in BRL horizontal gel apparatus at 40V (LKB Macrodrive powerpack). Standard 1kb ladder DNA fragments (BRL) were used as molecular weight markers (see Appendix 4). Following electrophoresis, DNA fragments in LMP-agarose gels were visualised by UV-illumination (450nm wavelength) following staining in B-

buffer containing 1μg/ml EtBr. The required fragments were excised in blocks of agarose, which were melted at 65°C for 5 min in 3ml dH_2O/g of gel and stored at 4°C.

2.5.2. Ligation of DNA Fragments.

DNA fragments were ligated within the matrix of the melted LMP agarose. Prepared vector and insert DNA fragments were mixed at a ratio of approximately 1 to 10, in the presence of 0.1 volumes of ligation buffer (0.5M Tris-HCl (pH 7.4), 0.1M $MgCl_2$, 0.1M dithiothreitol, 10mM spermidine, 100mM ATP) and 5 units of T4 DNA Ligase (BRL). Ligation was allowed to proceed at room temperature for 16-22 hours.

2.5.3. Preparation and Transformation of Competent Cells.

A single colony of E. coli JM101 was taken from a B-agar plate, inoculated into 10ml of L-broth and incubated overnight with shaking at 37°C. Fresh L-broth (100ml) was inoculated with 100μl of overnight culture and cells were grown to an optical density of between 0.2-0.25 at 600nm. 50ml of cells were centrifuged at 5,000rpm (4°C) for 10 min. The cell pellet was resupended in 10ml of ice-cold 0.1M $CaCl_2$ and maintained on ice for 30 min. Cells were pelleted as above and resuspended in 1ml of ice-cold 0.1M $CaCl_2$. Competent cells could remain on ice for up to 24 hours and could be stored for up to 1 month at -80°C in 0.1M $CaCl_2$ containing 20% (v/v) glycerol. Ligation reactions were mixed gently with 100μl of competent cells and incubated on ice for 30 min. The cells were heat shocked at 42°C for 1 min and returned to ice for a further 5 min. L-broth was added up to a final volume of 1ml, and the cells incubated at 37°C for 1 hour. Aliquots of transformed cells were spread onto agar plates containing 50μg/ml ampicillin and, if required, 0.1 μM IPTG and 40μg/ml X-gal. Transformed colonies were allowed to develop overnight at 37°C.

2.6. SCREENING OF RECOMBINANT PLASMIDS.

2.6.1. Colony Growth on Nylon Membranes.

Colonies of transformed bacteria were cultured in 100µl of L-broth containing 50 µg/ml ampicillin, in 96-well assay plates (Nunc) for several hours at 37°C. 84mm-diameter circular Hybond-N nylon membranes (Amersham International plc) were numbered with waterproof ink and laid onto the surface of agar plates containing ampicillin. Bacterial cultures were transferred onto the nylon membranes so that each 96-well plate inoculated two nylon membranes. The agar plates were incubated overnight at 37°C to allow colonies to develop on the surface of the nylon membranes. Cultures were stored within 96-well plates at -20°C, following the addition of 100µl of 50% glycerol in dH_2O per well.

2.6.2. Probing of Colonies for Recombinant Plasmids.

Nylon membranes were removed from agar plates and placed (colony-side up) onto Whatmann 3MM filter paper soaked in 0.5M NaOH, 0.15M Na acetate for 10 min. Membranes were transferred to filter paper soaked in 1.0M Tris-HCl (pH 7.5) and finally onto filter paper soaked in 1.5M NaCl, 0.5M Tris-HCl (pH 7.5), 1mM EDTA for 15 min. The membranes were blotted with tissue to remove bacterial cell debris and allowed to dry. DNA was fixed to the membranes by UV-illumination of Saran wrapped-membranes for 5 min (325nm wavelength). Non-specific hybridisation of DNA was blocked by incubating membranes with 1mg of denatured herring sperm DNA (Boehringer Mannheim). The DNA was boiled for 5 min with 0.1 volumes of 1M NaOH, allowed to cool and neutralised with 0.1 volumes of 1M Tris-HCl (pH 7.0). Membranes were incubated in sealed plastic bags containing denatured DNA in prehybridising solution (Appendix 1) at 37°C for at least 1 hour. Radioactive probe (section 2.6.3.) was added to the membranes using a hypodermic needle and syringe to pierce the bag (which was then firmly sealed with tape) and allowed to hybridise to the membranes at 37°C overnight.

2.6.3. Synthesis of Radiolabelled DNA Probe.

DNA restriction fragments were radioactively labelled with 5'-[α-^{32}P]-deoxyadenosine triphosphate ([α-^{32}P]-dATP, Amersham International plc) by the method of Feinberg and Vogelstein (1984). DNA was excised from an LMP-agarose gel and placed in a boiling waterbath with 3ml dH_2O/g of gel for 7 min. A 50μl aliquot of DNA was mixed carefully with 10μl of oligolabelling buffer (Appendix 1), followed by 50μCi of [α^{32}P]-dATP and finally 2-4 units of Klenow enzyme. The reaction was allowed to proceed for 3-4 hours at room temperature. Incorporation of radioactive label into the DNA fragment was determined as follows. A small volume (2μl) of the radio-labelled DNA solution was spotted onto two pieces of DE-81 filter paper and allowed to dry. One filter was washed 5 times with 5% sodium phosphate and allowed to dry. Both filters were immersed in 4ml of liquid scintillant and the radioactivity measured as counts per minute (cpm) using a Minaxi Tri-carb 4000 series liquid scintillation counter. Incorporation of radiolabel into the DNA molecule was determined by comparison of washed to unwashed filters. A level of incorporation of $1x10^6$ cpm or greater was acceptable for use as described above.

2.6.4. Autoradiography of Probed Membranes.

Radioactive solutions were removed from membranes at room temperature with 3 washes in 2 x SSC (Appendix 1), 0.1% SDS. Membranes were washed at 65^{o}C for 1 hour in 0.1 x SSC, 0.1% SDS and allowed to dry. Dry membranes were placed between plastic sheets and exposed to Hyperfilm ß-max X-ray film (Amersham International plc) in light-sealed cassettes for 24 to 72 hours. Film was developed using Kodak LX-24 developer and FX-40 fixative, both diluted as directed by manufacturer. The autoradiographs were rinsed well with tapwater and allowed to dry completely before handling.

2.7. PLASMID PREPARATION.

2.7.1. Small Scale Preparation.

Cultures from 96-well plates were used to inoculate 2ml L-broth containing 50μg/ml ampicillin, and were incubated overnight at 37°C. Cultures were harvested into 1.5ml eppendorf tubes and cells pelleted for 2 min in a bench microfuge (high speed). The supernatants were discarded and the cell pellets were resuspended in 350μl of STET buffer (8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50mM EDTA, 10mM Tris-HCl (pH 8.0)). Lysosyme stock solution was diluted to 10mg/ml with 10mM Tris-HCl (pH 8.0) and 25μl was rapidly mixed into each tube. The tubes were placed in a boiling water bath for 40 seconds and immediately clarified in a bench microfuge for 10 min. The pellets were discarded and supernatants were precipitated at -70°C for 15 min with 40μl of 2.5M ammonium acetate and 420μl of isopropyl alcohol. Tubes were centrifuged at high speed for 10 min and DNA pellets were resuspended in 50μl of TE and stored at -20°C. Plasmids were digested with restriction enzymes and treated with 1μl of RNase A stock solution prior to agarose gel electrophoresis.

2.7.2. Large Scale Preparations.

Cultures from 96-well plates were used to inoculate 10ml aliquots of L-broth containing 50μg/ml ampicillin overnight at 37°C. The 10ml cultures were used to seed 500ml of fresh broth, grown for a further 24 hours at 37°C. In the case of pUC-based plasmids, yield was increased by chloramphenicol-amplification, added to a final concentration of 170μg/ml at approximately 8 hours post inoculation. Cells were harvested by centrifugation from two 250ml aliquots at 5,000rpm, 4°C for 10 min (Sorvall GS 6x500ml rotor) and each pellet was resuspended in 2.6ml of 25% (w/v) sucrose, 10mM Tris-HCl (pH 8.0) on ice. Cells were lysed on ice for 5 min with 400μl of freshly prepared 20mg/ml lysozyme in 0.25M EDTA and mixed with 2.6ml of 0.25M EDTA for 5 min. Using a 10ml pipette, 4ml of Brij-DOC solution (1% (w/v) Brij 58, 0.4% sodium deoxycholate, 10mM Tris-HCl (pH 8.0), 1mM EDTA) was vigorously introduced into each tube. The suspensions were

held on ice for 30 min and cell debris was pelleted by centrifugation at 15,000rpm (4°C) for 45 min. The volume of each clarified supernatant was accurately measured and caesium chloride was added to a concentration of 0.95g/ml followed by 100µl of 10mg/ml EtBr. The solutions were transferred to 11.5ml polyallomer tubes (Sorvall, DuPont), balanced in pairs to within 0.02g with caesium chloride (0.95g/ml in TE). Tubes were crimp-sealed, loaded in balanced pairs into a Sorvall TFT 80.13 rotor and plasmid DNA was banded at 71,000rpm (18°C) for 18 hours using a Sorvall OTD-75B ultracentrifuge. The tube was pierced with a syringe needle and plasmid DNA was harvested into a syringe, using a second needle inserted just below the visible (lower) band, bevel upwards. The needle was removed from the syringe prior to emptying the contents into a tube, to prevent shearing forces damaging the DNA. Plasmid DNA was extracted three times against CsCl-saturated isopropanol and dialysed against 3 changes of TES (10mM Tris-HCl, 1mM EDTA, 100mM NaCl) over several hours then against TE overnight. Plasmids were stored at 4°C.

2.8. CLONING WITH BACTERIOPHAGE M13mp19.

2.8.1. Competent Cells for Bacteriophage Transformations.

A single colony of *E. coli* JM101 from a B-agar plate was used to inoculate 10ml L-broth, which was incubated at 37°C for 4-5 hours. A secondary culture was seeded with 50µl of the initial culture and incubated for 2-3 hours to provide indicator cells. The initial culture was pelleted at 5,000rpm (4°C) for 10 min and the cells were resuspended in 4ml ice cold 10mM NaCl. Cells were immediately centrifuged as before and resuspended in 4ml of 100mM $CaCl_2$, on ice for 30 min. The cells were again pelleted and finally resuspended on ice in 1ml ice cold 100mM $CaCl_2$. These cells could remain viable overnight if maintained on ice. DNA ligation reactions were mixed with 100µl of competent cells on ice for 1 hour and heat shocked at 42°C for 2 min. Transformed cells were rapidly mixed with 200µl of indicator cells, 50µl of X-gal stock solution, 20µl of IPTG stock and

3ml of B-Top agar (warmed to 42°C). The agar was mixed gently, poured quickly onto a B-agar plate and allowed to set. Plates were incubated at 37°C overnight.

2.8.2. Preparation of Replicative Form Bacteriophage DNA.

Isolated plaques were picked from the bacterial lawn, using sterile toothpicks, into 5ml L-broth containing 50µl of a fresh (5 to 6 hours growth) E. coli JM101 culture and incubated at 37°C overnight with vigorous shaking (200rpm). Cells were pelleted in a bench microfuge for 3 min and resuspended in 100µl of ice-cold, freshly-made lysis mix (2% (w/v) glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA, 4mg/ml lysosyme) on ice for 30 min. A freshly made solution of 0.2M NaOH, 1% SDS was added (200µl/tube) for a further 5 min on ice followed by 150µl of 3M potassium acetate (60ml of 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml dH_2O) for 5 min on ice. Cell debris was pelleted in a bench microfuge for 2 min and the supernatant was precipitated with cold (-20°C) absolute ethanol for 1 hour at -20°C. DNA was pelleted in a microfuge for 10 min, resuspended in 50µl dH_2O and stored at -20°C.

2.8.3. Preparation of Single-stranded Template DNA.

An overnight E. coli JM101 culture was used to seed fresh L-broth (50µl cells to 5ml broth) which was inoculated with a single, isolated plaque from a transformed B-agar plate. Cultures were incubated at 37°C for 5-6 hours with vigorous shaking (200rpm). The supernatants were clarified twice in microfuge tubes and incubated with 0.2 volumes of 2.5M NaCl, 20% PEG 6000 at room temperature for 60 min. Bacteriophage was pelleted by centrifugation in a microfuge, drained and centrifuged again briefly for 10 seconds to remove any remaining buffer. The pellets were resuspended in 100µl of 1.1M sodium acetate (pH 7.0) and DNA was extracted with one volume of phenol, followed by one volume of chloroform. DNA was precipitated at -20°C overnight with 5 volumes of absolute ethanol, pelleted for 5 min in a microfuge, and stored in 50µl dH_2O at -20°C.

2.9. SEQUENCE ANALYSIS OF CLONED DNA.

Cloned DNA regions were sequenced using the Sequenase enzyme kit (Version 2.0, United States Biochemical Corporation), following techniques described in the manual provided. Briefly, 7μl of single-stranded template DNA was annealed to 1μl of oligonucleotide primer in the presence of reaction buffer (2μl of 5-times concentrate, as supplied) by heating to 65^{o}C for 2 min and allowing to cool slowly to room temperature in a waterbath. Template-primer mixes were incubated with 1μl of 0.1M dithiothreitol, 2μl of Sequenase labelling solution diluted in ice-cold TE (1 in 5 regular reactions, 1 in 15 for reactions to sequence close to the annealed primer), 0.5μl of [α-^{35}S]-dATP (Amersham International plc) and 2μl of Sequenase enzyme (diluted 1 in 8 in supplied buffer). Polymerase reactions were allowed to proceed at room temperature for 3-5 min and were terminated by incubating 3.5μl aliquots of the reaction mix with 2.5μl of each of the four supplied dideoxynucleotide solutions (ddATP, ddCTP, ddGTP or ddTTP) at 37^{o}C for 3-5 min. Finally, 4μl of the supplied STOP buffer was added to each reaction mix and the tubes stored on ice. Prior to electrophoresis through a 6% polyacrylamide gel (Appendix 2), samples were incubated for 1-2 min in a boiling water bath. Electrophoresis was run at 40W (26mA) in 1x TBE buffer (Appendix 2) for 1-4 hours and gels were fixed with 10% methanol, 10% acetic acid for 10 min. The fixed gels were washed with dH_2O, vacuum dried onto Whatmann 3MM filter paper at 80^{o}C for 1 to 2 hours and exposed to X-ray film overnight. The sequencing mixtures could be stored for up to 1 week at -20^{o}C.

2.10. SITE DIRECTED MUTAGENESIS.

The procedure outlined below is derived from Kunkel, *et al*. (1987).

2.10.1. Passage of Bacteriophage Through Cells.

A 10ml volume of L-broth containing 12.5μg/ml tetracycline was inoculated with a single colony of *E. coli* RZ1032 from a minimal (M9) agar plate, and incubated for

several hours at 30°C with shaking. Fresh L-broth (5ml) was inoculated with 50µl of this culture and a single bacteriophage plaque from a tranformed B-agar lawn (section 2.8.1.), and incubated at 37°C overnight with shaking. Bacteriophage were harvested as described (2.8.3.) but were resuspended in 20µl of dH_2O following PEG-precipitation. The procedure was repeated, infecting 5ml L-broth with 50µl of cells as above and half the bacteriophage preparation.

2.10.2. Preparation of Suicide Template.

A large volume of L-broth (500ml) containing 12.5µg/ml tetracycline was inoculated with 5ml of a fresh E. coli RZ1032 culture and 10µl of bacteriophage suspension (2.10.1.). The culture was incubated at 37°C for 5-6 hours and the cells were pelleted at 5,000rpm (Sorvall GS 6x500ml rotor), at 4°C for 10 min. The supernatant was incubated with 0.2 volumes of PEG/NaCl buffer (150g PEG 8000, 146g NaCl, to 1 litre with dH_2O) on ice for 1 hour. Precipitated bacteriophage was pelleted at 5,000rpm for 15 min, drained well and resuspended in 5ml of 100mM Tris-HCl (pH 8.0), 300mM NaCl, 1mM EDTA, in a 15ml Corex tube. Tubes were vigorously vortexed, maintained on ice for 1 hour and centrifuged as above to remove residual debris. The supernatant was extracted twice with phenol and twice with chloroform, and the DNA was precipitated with 0.1 volumes of 3M sodium acetate (pH 5.0) and 2 volumes of ethanol at -20°C overnight. DNA template was pelleted by centrifugation, resuspended in 100µl TE and the DNA concentration of the solution was determined (2.4.1.).

2.10.3. Phosphorylation and Annealing of Primers.

Mutagenesis primers P-MUT1 and P-MUT2 (Appendix 3) were phosphorylated prior to annealing to suicide templates. In a 20µl reaction volume, 1µg of primer was incubated with 2µl of kinase buffer (0.5M Tris-HCl (pH 7.5), 0.1M $MgCl_2$, 50mM dithiothreitol, 10mM ATP) and 2 units of T4 Polynucleotide Kinase. The reaction was allowed to proceed at 37°C for 1 hour and was terminated with 3µl of 100mM EDTA at 65°C for

10 min. The phosphorylated primers were annealed to 1μg of template DNA in the presence of 10x SSC by heating in a waterbath to 70°C and slowly cooling to room temperature.

2.10.4. In vitro DNA Synthesis and Ligation.

The reactions were performed in a volume of 100μl containing the primer-template mix, 20mM HEPES (pH 7.8), 2mM DTT, 10mM $MgCl_2$, 500μg each of dATP, dCTP, dGTP and dTTP, 1mM ATP, 2.5 units of T4 DNA polymerase and 2 units of T4 DNA ligase. All components were mixed on ice, the enzymes being added last. The reaction was incubated on ice for 5 min and then at 37°C for 2 hours. EDTA was added to a final concentration of 15mM to terminate the reactions. The resulting DNA solutions were used to transform competent E.coli JM101 cells (section 2.8.1.).

2.11. PRODUCTION OF RECOMBINANT VIRUSES.

The generation of recombinant vaccinia viruses was achieved following the methods of Mackett et al., 1985a.

2.11.1. Mammalian Cell Culture.

CV-1 cells were maintained in MEM or L-15 medium as described (section 2.3.) in 75ml culture flasks (Nunc). When monolayers were confluent (2-3 days), cells were passaged by washing twice with PBS containing 0.02% EDTA and once with 0.25% trypsin (Gibco-BRL), 0.02% EDTA in PBS at 37°C for 1-2 min. The cells were released by tapping the flask and the cell suspension was divided into 4 flasks of equal size containing fresh medium. The flasks were incubated at 37°C.

2.11.2. Growth of HSV-1 Virus Stocks.

HSV-1 (strain 17) stocks were prepared from infected monolayers. Just-confluent monolayers of CV-1 cells grown in 225ml flasks (Nunc) were washed with PBS and infected with virus at a multiplicity of infection (moi) of 0.01 plaque forming units (pfu)/cell. Virus was allowed to adsorb to the cells for 1 hour at 37°C, then the inoculum was replaced with a minimal volume of fresh culture medium (to just cover cells). The infected monolayer was harvested after 2 days

incubation, or when 100% cytopathic effect (cpe) was observed. Cells were scraped into the culture medium, and virus was released by 8 cycles of sonification (15 sec) and cooling on ice (15 sec). Cell debris was removed by centrifugation at 5,000 rpm (4°C) for 30 min and the resulting virus suspension stored at -80°C.

2.11.3. Growth of Vaccinia Virus Stocks.

CV-1 cells were infected with vaccinia virus (strain Lister) at an moi of 0.01 pfu/cell in PBS for 1 hour at 37°C. The inoculum was removed and replaced with fresh culture medium and the cells were incubated at 37°C for 48 hours, until the monolayer displayed major cpe, but had not disconnected from the wall of the culture vessel. The infected monolayer was harvested into the culture medium and the cells were pelleted at 1,000rpm (Chillspin benchtop centrifuge) for 5 min. For crude virus stocks, infected cells were resuspended in 1ml fresh medium and virus was released from the cells by rapid freeze-thawing, three times. For purified virus stocks, infected cells were harvested into 10mM Tris-HCl (pH 9.0) and virus was released by 8 cycles of sonification (15 sec) and cooling on ice (15 sec). Cell debris was pelleted at 1000rpm, washed with 10 mM Tris-HCl (pH 9.0), and the supernatants pooled. The supernatant was incubated with 0.025% trypsin at 37°C for 30 min with frequent vortexing and virus was pelleted at 25,000g (4°C) using an AH-629 swing-bucket rotor (36ml buckets) in an OTD-75B ultracentrifuge for 80 min. The virus pellet was resuspended in 2ml of 1mM Tris-HCl (pH 9.0) and incubated with trypsin as before. Virus was banded through a 20-40% sucrose gradient in 1mM Tris-HCl (pH 9.0) at 18,750g for 45 min. The virus band was collected, diluted 1 in 3 in 1mM Tris-HCl (pH 9.0) and virus was pelleted at 25,000g for 1 hour. Virus was finally resuspended in 1ml of 1mM Tris-HCl (pH 9.0). Viral stocks were stored at -80°C.

2.11.4. Virus Titration.

CV-1 cells were grown in 1.5cm-diameter wells (24-well plates, Nunc). Monolayers were infected for 1 hour at 37°C with 1ml volumes of virus, serially diluted in PBS. Each dilution was tested in quadruplicate. Virus inocula were removed and replaced with 1ml fresh medium and the cells were incubated without disturbance at 37°C for 48 hours. The infected monolayers were viewed under a low-power microscope and the plaques in each well counted. Virus titre was calculated from an average count for all four wells containing the same dilution and expressed as plaque forming units per ml (pfu/ml).

2.11.5. Calcium-Phosphate Precipitation of Plasmid DNA.

A 20μg sample of plasmid DNA, made up to 420μl volume with 1mM Tris-HCl (pH 7.5), 0.05mM EDTA was vigorously vortexed whilst slowly adding 60μl of 2M $CaCl_2$. Still vortexing, 480μl of HEPES-buffered saline (50mM HEPES (pH 7.12), 280mM NaCl, 1.5mM Na_2HPO_4) was added and the DNA was allowed to precipitate at room temperature for 30 min.

2.11.6. Transfection of Infected Cells.

CV-1 cells were grown in 25ml flasks (Nunc) until just confluent. Medium was removed, the monolayer washed with PBS and inoculated with 0.05 pfu/cell of vaccinia virus in 4ml PBS. The virus was allowed to adsorb for 30 min at 37°C, monolayers were washed three times with PBS and fresh culture medium was added. The infected cells were incubated at 37°C for 90 min then washed three times with serum-free medium. Calcium phosphate-precipitated plasmid DNA was added to the monolayer for 30 min at 37°C and 10ml of fresh medium then added. After incubation at 37°C for 48 hours, the cells were harvested and virus released into 1ml of fresh medium by freeze-thawing. The transfected viral stocks were titrated and stored at -80°C.

2.11.7. Plaque Purification of Recombinant Virus.

CV-1 cells were grown in 6-well plates (34mm-diameter well, Sterilin). Confluent cell monolayers were washed with PBS and inoculated with transfected virus stocks, diluted between 10^{-2} to 10^{-7} in 500μl volume of PBS. Virus was allowed to adsorb to the cells for 1 hour at 37^{o}C, and was replaced by a solid overlay of 1 ml fresh medium and 1ml of 1.2% SeaPlaque LMP agarose (SeaKem) in PBS, mixed at 37^{o}C. The cells were incubated at 37^{o}C for 48 hours and 1ml of 0.6% SeaPlaque LMP agarose in PBS containing 900μg/ml X-gal was added. The cells were incubated at 37^{o}C until blue colouration developed, about 3-4 hours. Virus plaques were picked using a sterile Pasteur pipette, in a plug of the overlaying agarose, into 250μl of PBS. Virus was released by freeze-thawing and stored at -80^{o}C. Subsequent rounds of plaque purification were carried out as described, using serial dilutions between 10^{-1} to 10^{-3} in PBS.

2.12. DETECTION OF CLONED SEQUENCES IN VIRUS DNA.

2.12.1. DNA Dot-Blot Hybridisation.

CV-1 cells were grown in 1.5cm-diameter wells (24-well plates, Nunc) and inoculated with 50μl of plaque-purified recombinant viruses, with 1x10^{3}pfu vaccinia virus or with 1x10^{3} pfu HSV-1, in a total volume of 200μl PBS. Virus inocula were allowed to adsorb for 1 hour at 37^{o}C and were replaced with 1ml fresh medium. The cells were incubated at 37^{o}C for 48 hours. Most of the medium from each well was removed, leaving approximately 100μl into which the monolayer was harvested using a syringe plunger. The suspended cells were collected onto a Hybond-N nylon membrane (Amersham International plc) using a minifold dot-blot apparatus (Scheicher and Scuell, Inc.) attached to a vacuum. The nylon membrane was treated as described for DNA hybridisation using a radioactively-labelled DNA probe and for autoradiography (sections 2.6.2. to 2.6.4.).

2.12.2. Southern Blot Hybridisation.

CV-1 cells were grown in 75ml flasks and infected with virus (as above) in 2ml PBS when just confluent. When the cell monolayers showed approximately 80% cpe, the medium was removed and the infected monolayers were harvested into 1ml PBS containing 0.02% EDTA. A proportion of resuspended cells (200µl) were pelleted at low speed in a bench microfuge for 5 min and resuspended TE containing 30% (w/v) sucrose. The cells were lysed by incubation with 1% SDS and 100mM ß-mercaptoethanol for 30 min at 4°C. Following lysis, all procedures were performed using wide-bore pipettes, to minimise shearing of the DNA extracts. The lysed cells were incubated at 37°C for 2 hours with 500µg/ml Proteinase K (Sigma Chemical Company). DNA was extracted once with an equal volume of phenol, and once with an equal volume of chloroform. For total cell DNA extracts, the aqueous phase was often below the solvent phase. The DNA preparations were precipitated overnight at -20°C with 0.1 volumes of 5M NaCl and 2.5 volumes of ethanol. DNA was resuspended in 50µl TE and stored at 4°C. Restriction of DNA with HindIII was performed using one quarter of the DNA sample, and digested samples were electrophoresed through a 0.8% agarose gel. The gel was immersed in 0.2M HCl for 15 min and DNA was denatured by three washes (30 min each) with 1.5M NaCl, 0.5M NaOH. The gel was neutralised by three washes (30 min each) in 10mM Tris-HCl (pH 7.2), 1mM EDTA, 1.5M NaCl. A piece of Whatmann 3MM filter paper was cut to the required size and laid onto the gel, ensuring no air bubbles were trapped between the gel and the filter paper. Excess gel was trimmed away using a scalpel and the gel was inverted onto a filter paper wick on a glass plate. The ends of the wick were submerged in a reservoir of 20x SSC buffer (Appendix 1), and was completely soaked with the solution. Again no air bubbles were left between the layers. A piece of Hybond-N nylon membrane (Amersham International plc) was cut to size and laid onto the gel, ensuring no air bubbles were trapped. Two pieces of Whatmann 3MM paper soaked in 2x SSC were laid on top of the nylon membrane, followed by several dry papers and a stack of dry tissues, to 4-5cm high. The papers were

weighted down under a glass plate and the DNA was transferred onto the nylon membrane overnight by capillary action. The membrane was removed, air-dried and the DNA crosslinked onto the membrane by UV-illumination for 5 min. The membrane was probed as described previously (sections 2.6.2. to 2.6.4.).

2.12.3. End-Labelled Molecular Weight Markers.

Approximately 1μg of lambda DNA (BRL) was digested to completion with Hind III restriction enzyme. The digested DNA was mixed with 2μl of reaction buffer (330mM Tris-acetate (pH 7.9), 660mM potassium acetate, 100mM magnesium acetate, 5mM DTT, 1mg/ml bovine serum albumin) and the volume brought up to 19μl with dH_2O. 1μl of a 2mM solution of dCTP, dGTP and dTTP was added, with 2μCi of [α-^{32}P]-dATP (Amersham International plc) and the reaction incubated at 37^{o}C for 5 min with 2.5 units of T4 DNA Polymerase. 1μl of 1mM unlabelled dATP was added, the reaction was continued for a further 10 min and stopped by heating to 70^{o}C for 5 min. The radiolabelled DNA was stored at -20^{o}C.

2.13. DETECTION OF CLONED PROTEINS IN INFECTED CELLS.

2.13.1. Indirect Immunofluorescence.

CV-1 cells were grown on sterile round glass coverslips (13mm-diameter) in 1.5cm wells. Confluent monolayers were inoculated with virus in 200μl PBS or were mock infected with PBS only, for 1 hour at 37^{o}C. Inocula were removed and replaced with 1ml fresh medium and cells were incubated for 24 hours at 37^{o}C. Medium was removed and the monolayers washed with PBS, prior to fixing with acetone for 5 minutes. The fixed coverslips were drained and used immediately. Coverslips were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min and incubated for 1 hour with anti-gC1 monoclonal antibody 7542 diluted 1 in 5000 in PBS. Coverslips were washed 5 times with PBS for 5 min each then incubated for 1 hour with isofluorothiocyanate- (IFTC)-conjugated rabbit anti-mouse IgG (a gift from Dr. S. Clark, CAMR) diluted 1 in 200 in PBS. The conjugate was removed and the coverslips washed as previously. The coverslips were

then inverted onto a drop of 90% glycerol in dH_2O on a glass microscope slide and viewed under UV-illumination using a Leitz microscope, at x40 magnification.

2.14. IMMUNOLOGICAL PROCEDURES.

2.14.1. Inoculation of Inbred Mice.

BALB/c mice (kindly donated by Dr. J.R. Stephenson, CAMR) were inoculated with virus at the base of the tail. Virus inoculum was placed onto the tail and a 23-gauge syringe needle was used to scarify the area until the inoculum was adsorbed. This procedure was used for both the initial virus immunisation and for he subsequent HSV-1 challenge.

2.14.2. Cardiac Puncture of Mice.

Mice were placed in an ether chamber until no response was detected on touching the eye. The heart was punctured with a 21-gauge syringe needle, and blood withdrawn into a disposable 1ml syringe. Sera from five mice in each inoculation group were pooled in Microtainer serum separator tubes (Becton Dickinson), which were centrifuged at 6500rpm (bench microfuge) for 5 min to separate cells and serum. Serum samples were stored at -20°C.

2.14.3. Virus Neutralisation Assay.

Sera were diluted two-fold in 200µl volumes of PBS, to 1/256. Half each diluted sample was heat inactivated at 56°C for 30 min. Samples were incubated with 20pfu HSV-1 (17) at 37°C for 2 hours, and CV-1 cells in 1.5cm wells were infected with adsorbed virus. After 48 hours incubation, virus plaques were counted and neutralisation titres determined.

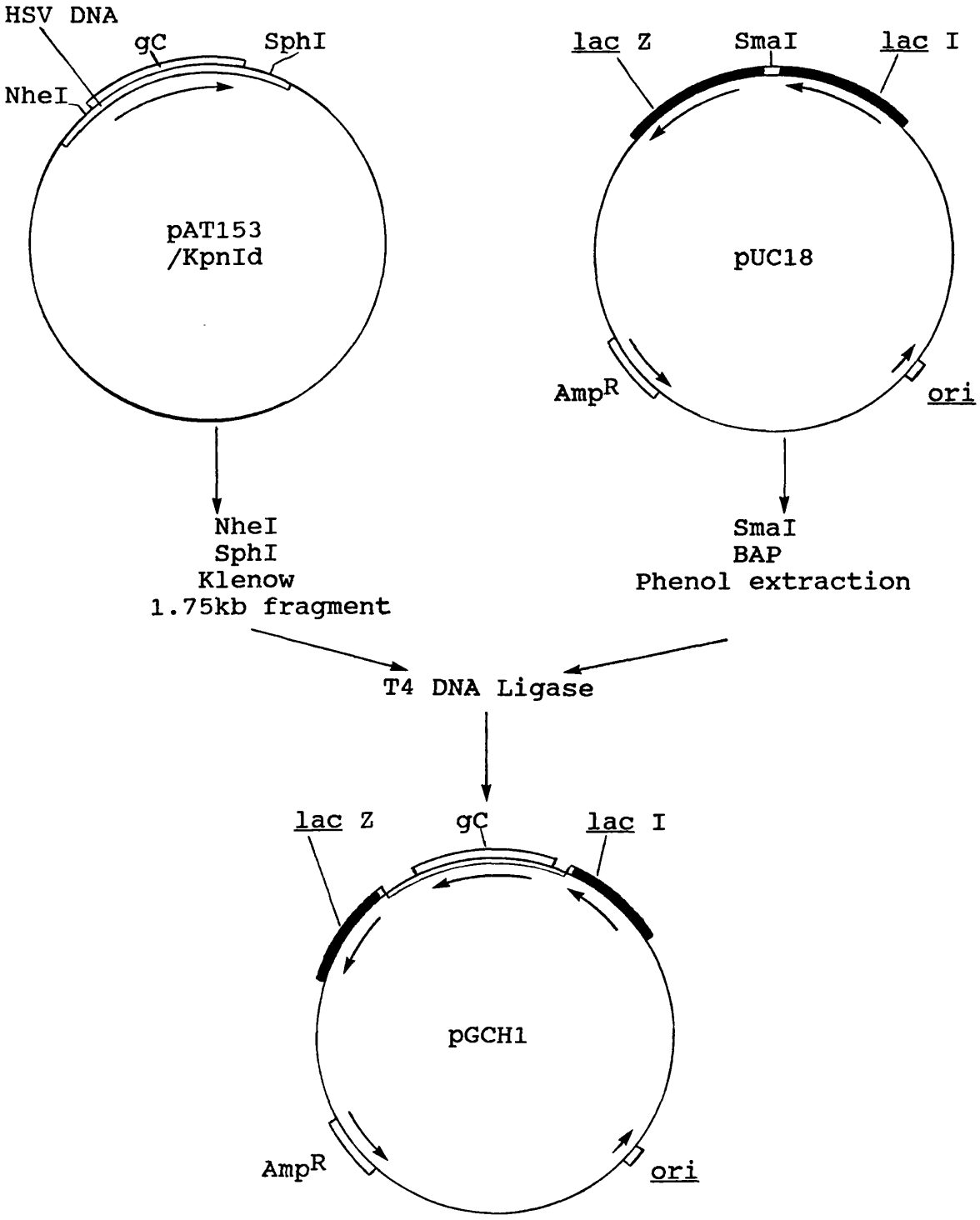


Figure 2.1. Cloning strategy to insert HSV-1 gC coding sequences into plasmid pUC18.

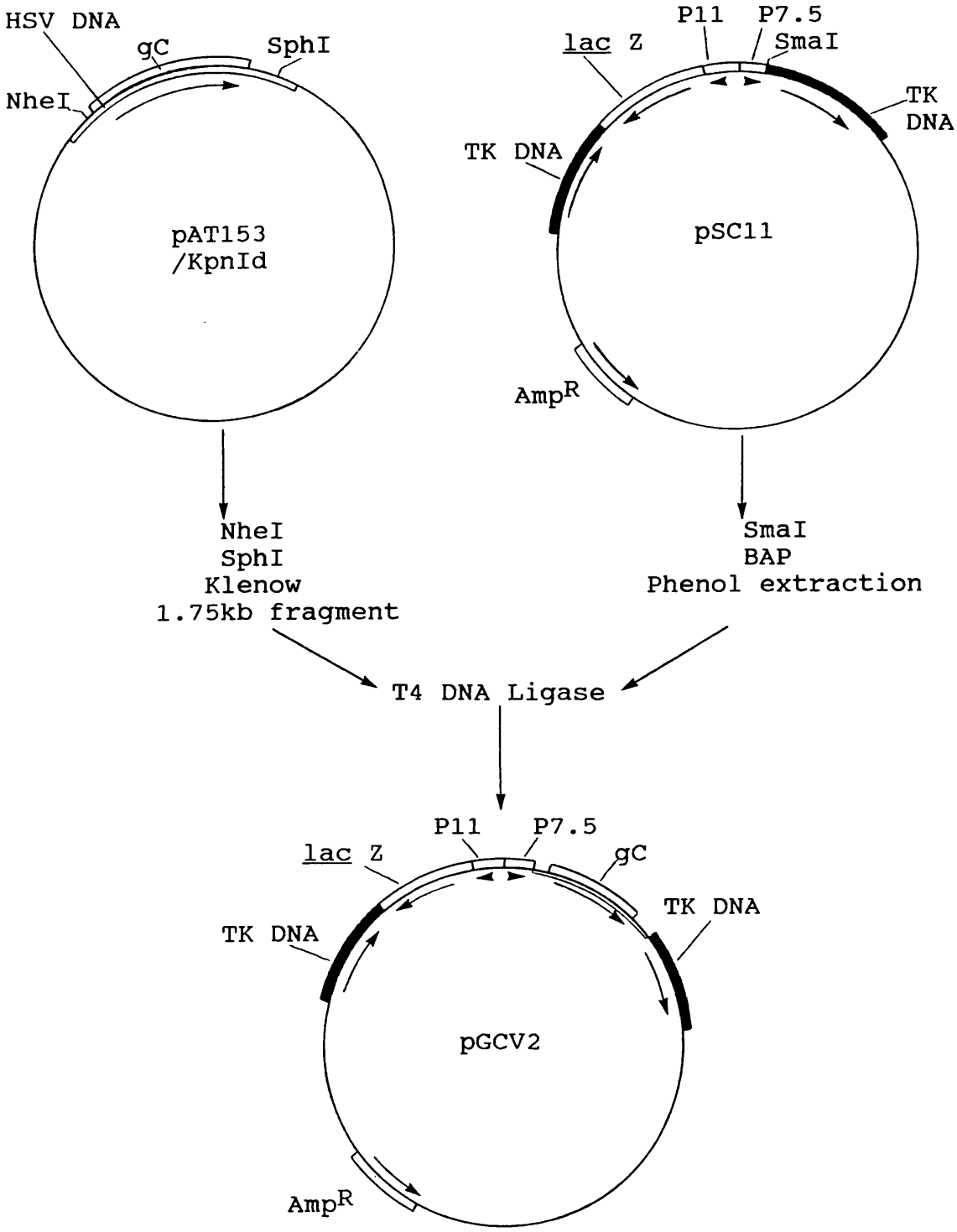


Figure 2.2. Cloning strategy to insert HSV-1 gC coding sequences into plasmid pSC11.

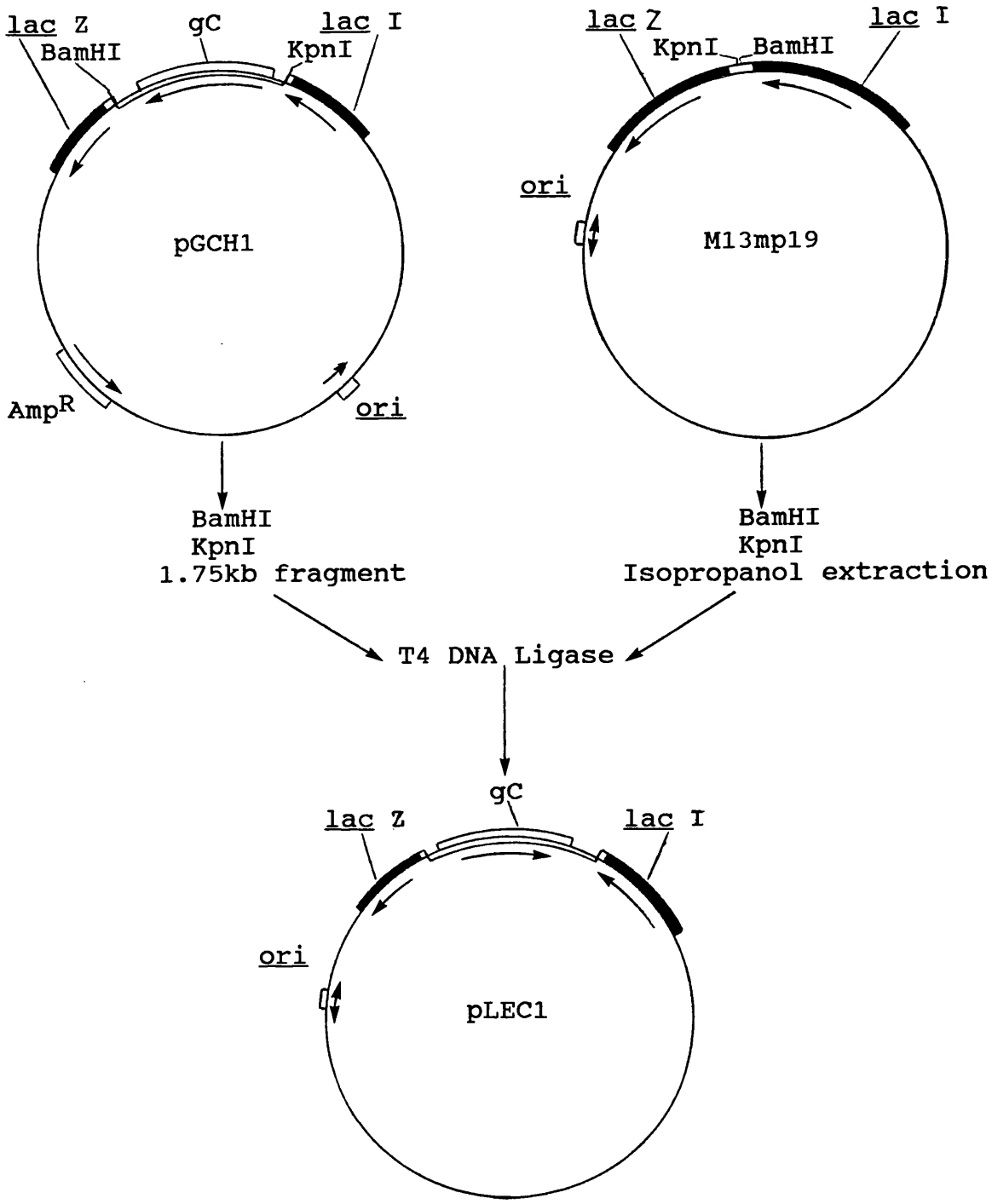


Figure 2.3. Cloning strategy to insert HSV-1 gC coding sequences into bacteriophage M13mp19.

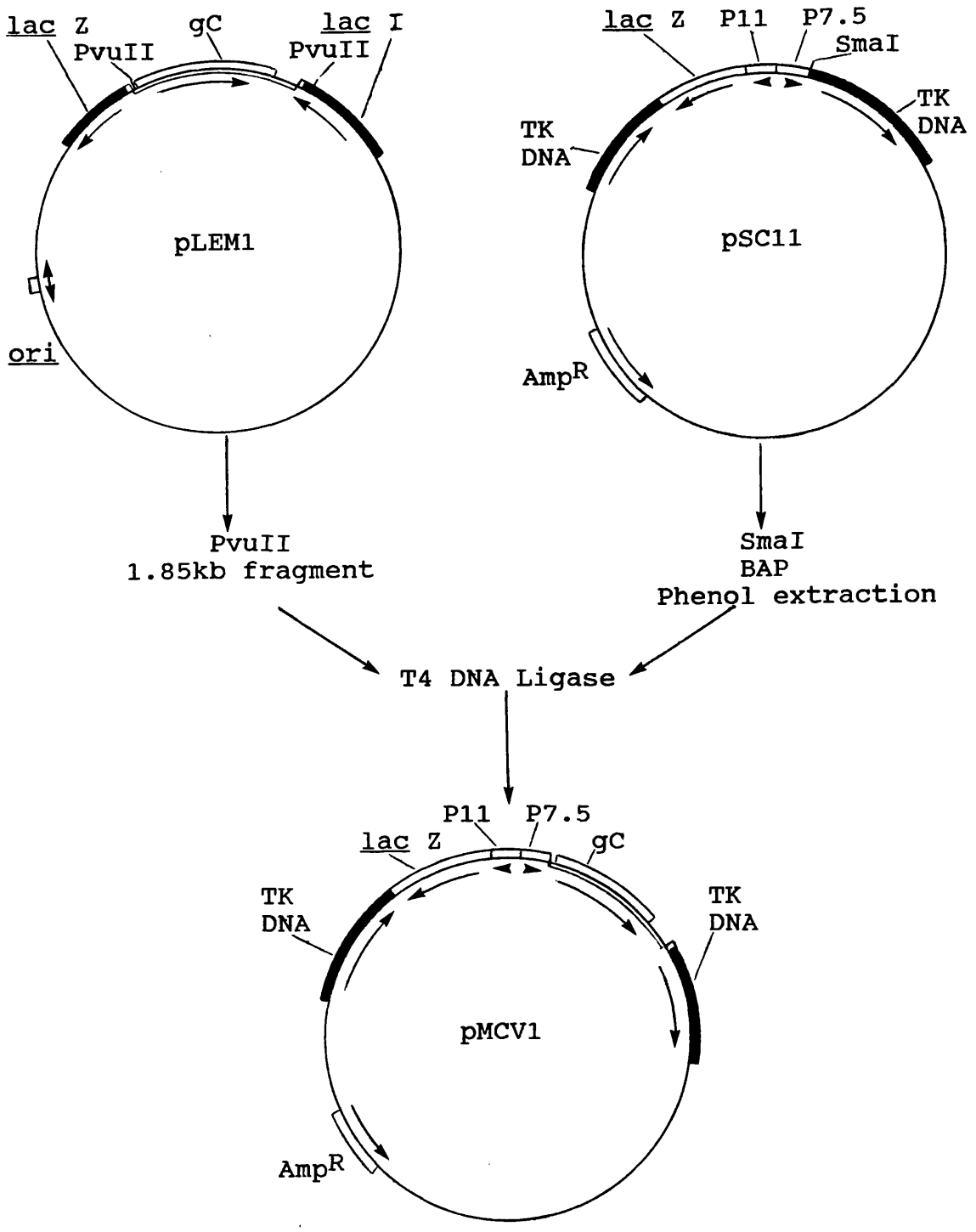


Figure 2.4. Cloning strategy to insert manipulated HSV-1 gC coding sequences into plasmid pSC11.

CHAPTER 3

RESULTS PART 1

CLONING OF HSV-1 gC SEQUENCE

3.1. GENERATION OF RECOMBINANT PLASMID VECTORS

The genome of HSV-1 (strain 17) was fragmented with KpnI restriction endonuclease and a genomic library constructed within the plasmid vector pAT153 (Davison and Wilkie, 1983). Each fragment was designated a letter; fragment KpnIa being the largest, KpnIb the next largest and so on. The nucleotide sequence encoding the complete glycoprotein C (gC) gene is found within the KpnId fragment (approximately 14 kilobasepairs (kb) in length) and the plasmid vector containing this fragment (referred to here as pAT153/KpnId) was used as the source of gC DNA sequences within this study.

3.1.1. Sequence Data for HSV (KOS) gC Gene.

At the time of cloning, sequence data was not available for HSV-1 strain 17, but HSV-1 strain KOS sequences were available, through the Genbank database. The sequences within and around the HSV-1 (KOS) gC gene were examined as a guide to restriction enzyme sites which would allow excision of the entire gC gene, preferably within a single DNA fragment. The data indicated that digestion with endonucleases NheI and SphI would produce a DNA fragment of 1756 base-pairs (bp) in length, containing the complete gC coding sequence of 1536bp. In the KOS strain NheI is situated 34bp upstream from the gC translation initiation codon whilst SphI cuts at a point 186bp downstream from the termination codon. Figure 3.1. illustrates the HSV-1 (KOS) sequences examined and indicates the position of the NheI and SphI sites, with respect to the gC coding region. Several other restriction enzyme sites within the gC gene are also indicated, which were used for orientation of this region when cloned into plasmid or bacteriophage vectors. Figure 3.2. illustrates the plasmid vector pAT153/KpnId, based on sequence information of the KpnId region of the HSV (KOS) genome (Genbank database) and the plasmid pAT153 (Maniatis, et al., 1982). The data indicate that digestion of the plasmid would only yield one fragment of size 1.75kb, and that this fragment would contain the entire gC gene from the cloned KpnId region.

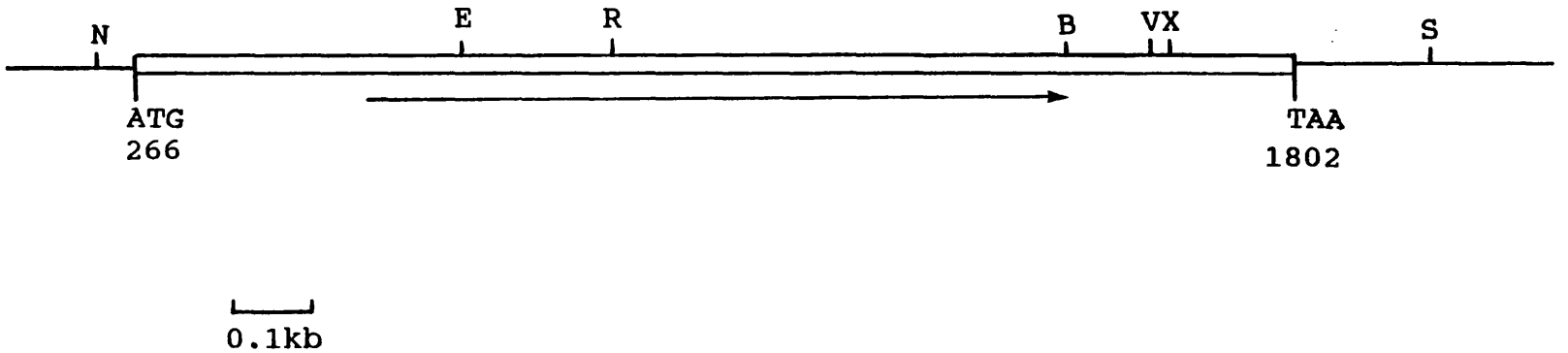


Figure 3.1. Restriction endonuclease sites within and around the HSV-1 (KOS) gC coding region. The portion of the virus genome containing the glycoprotein gC is represented. The gC coding sequences are depicted by the open box. The position of the initiation (ATG) and termination (TAA) codons of the gC gene are shown, as is the direction of transcription of the gene (arrow below coding region). The points at which several restriction enzymes cut are indicated. Enzymes include BglI (B), EcoRI (E), EcoRV (V), NarI (R), NheI (N), SphI (S) and XbaI (X). Information is from Genbank Database, accession number J02216 (Frink, et al., 1983; Draper, et al., 1984). Nucleotide numbers (as depicted in database information) are indicated below the representation.

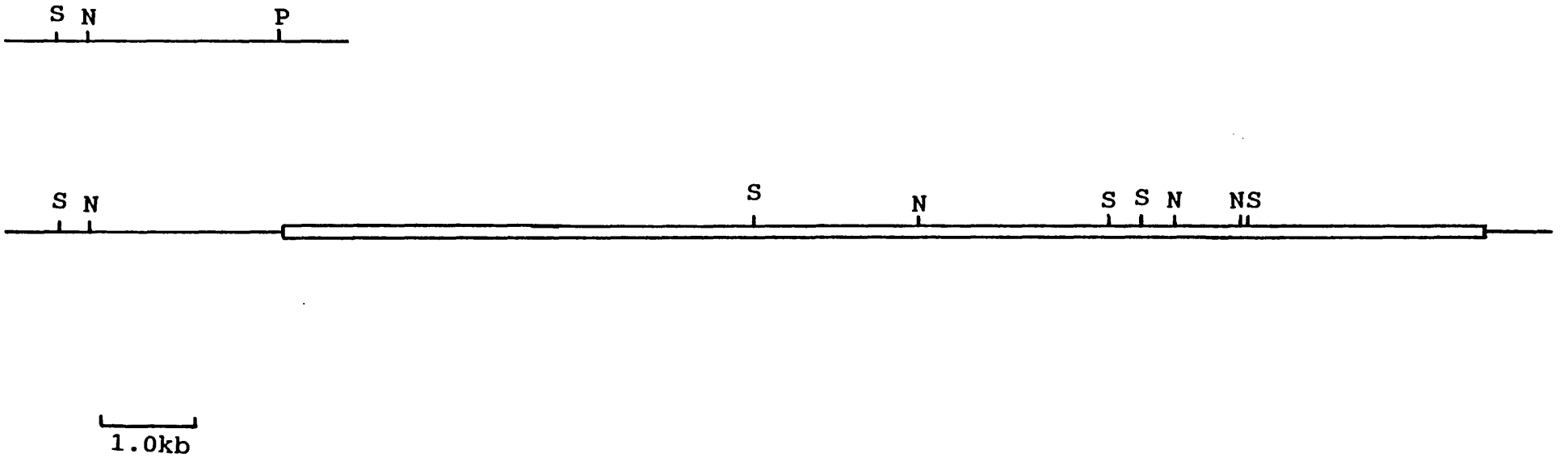


Figure 3.2. Restriction maps of plasmids pAT153 (top) and pAT153/KpnId (bottom). The HSV-1 (strain 17) KpnId genomic region (open box) was cloned into the PstI site (P) of the pAT153 plasmid (single line). The position of restriction enzymes NheI (N) and SphI (S) are indicated. Digestion of pAT153 with these enzymes generates two fragments of sizes 3.3kb and 0.3kb. An identical digest of pAT153/KpnId would generate several DNA fragments, with sizes 7.0kb, 3.7kb, 2.1kb, 1.75kb, 0.65kb, 0.35kb, two fragments of 0.3kb and one of 84bp. Only one fragment is generated of size 1.75kb and this fragment contains the entire HSV-1 gC gene (closed box). Information is from the Genbank database (accession number D00317) and from Maniatis et al., 1982).

Digestion of pAT153/KpnId with NheI and SphI resulted in several DNA fragments of varying sizes, separated by agarose gel electrophoresis (lane a; Figure 3.3.). One of the fragments (illustrated with an arrow) migrated to a position approximating to 1.8 kb and was taken to be the 1.75kb fragment predicted by KOS sequence data to contain the gC gene. Digestion of the parental plasmid pAT153 with NheI and SphI gave fragments of sizes 3.3kb and 0.3kb (lane b), but no fragment of 1.75kb. The fragments obtained by the digests agreed with the information illustrated in Figure 3.2. and confirmed that the 1.75kb fragment of pAT153/KpnId was from inserted viral sequences, and not of plasmid origin. The 1.75kb fragment from KpnId was chosen for the cloning of HSV-1 (17) gC gene.

3.1.2. Construction of pGCH plasmids.

These plasmids were generated by the insertion of the HSV-1 gC gene into the SmaI site of the parental vector pUC18. Plasmid vector pAT153/KpnId (10μg) was digested with 12 units of NheI and 10 units of SphI. A higher ratio of enzyme to DNA was used for NheI as the enzyme was found to cut DNA at a poor rate. Restriction enzyme NheI generates 3′ recessed termini when it cuts DNA, whilst SphI generates 3′ overhanging termini. Following digestion with these enzymes, the DNA was treated with Klenow enzyme. This enzyme contains a 5′-3′ DNA polymerase activity, which allows recessed 3′ termini to be filled in, and a 3′-5′ exonuclease activity, which removes 3′ overhangs. The enzyme was therefore used to simultaneously fill in the NheI-generated 3′ recessed termini and remove the SphI-generated 3′ overhanging termini. The DNA fragments were separated by electrophoresis through LMP agarose. The 1.75kb fragment was excised from the gel and eluted as described in Chapter 2. Following Klenow treatment, this blunt-ended fragment was expected to contain the gC gene, 34bp upstream and 182bp downstream of the coding sequence; a total of 1752bp. The recipient plasmid, pUC18, was digested with SmaI (which generates blunt-ended DNA fragments) and treated with alkaline phosphatase. This enzyme removes the 5′ phosphate groups

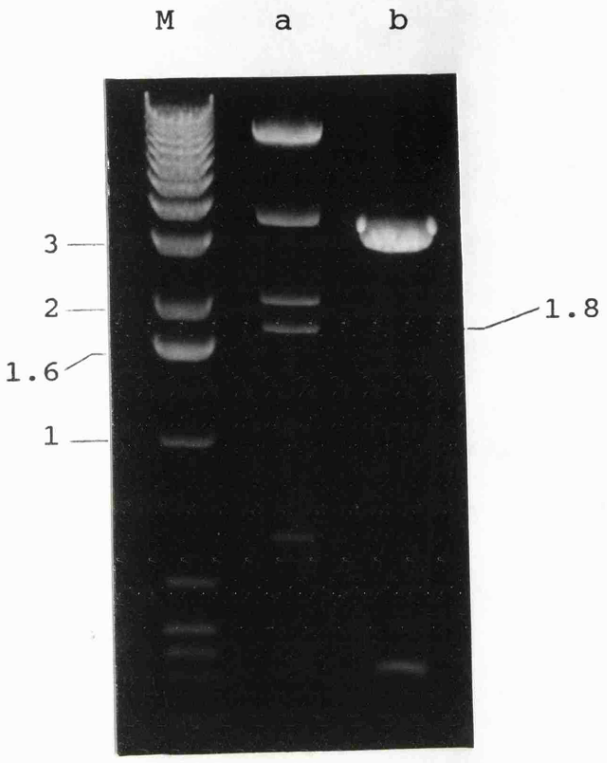


Figure 3.3. Agarose gel separation of restriction enzyme digests of pAT153 plasmids. Plasmids pAT153/KpnId (lane a) and pAT153 (lane b) were digested with restriction enzymes NheI and SphI. Arrow indicates the position of the 1.75kb fragment chosen for cloning of the HSV-1 gC gene sequence. Molecular weights are in kb. Markers (M) are 1kb ladder (see Appendix 4).

from DNA termini. DNA ligase enzymes are capable of generating phosphodiester bonds between the 5′ phosphate group of one DNA fragment and the 3′ hydroxyl group of a second fragment. The action of the phosphatase enzyme, by removing the 5′ phosphates from vector DNA fragments, prevents self-ligation of the pUC18 plasmid in the presence of ligase. Ligation with the 1.75kb fragment is allowed, as the fragment contains 5′ phosphate groups. An 80μl volume (approximately 0.25μg) of the 1.75kb eluted from LMP agarose was ligated into 0.5μl (approximately 0.2μg) of prepared pUC18 in a 100μl volume, using the enzyme T4 DNA Ligase. Competent *E. coli* JM101 cells were transformed by the ligation products, and selected on L-agar containing containing ampicillin, IPTG and X-gal. Parental plasmid pUC18 contains a copy of the *E. coli* gene encoding the enzyme ß-galactosidase (*lac*Z gene) which, in the presence of the inducing agent IPTG, is able to utilise the chromogenic substrate X-gal, resulting in a blue-coloured product which is visible on agar plates within colonies of bacteria containing the plasmid. Insertional inactivation of the pUC18 copy of the *lac*Z gene following ligation into the plasmid polylinker region gives rise to white colonies on agar plates containing IPTG and X-gal, compared to the blue (parental) colonies. In this instance, both white and blue colonies were present on agar plates inoculated with competent bacteria transformed with ligation products. The presence of blue colonies indicated that incomplete alkaline phosphatase activity had allowed pUC18 self-ligation to occur. The white colonies signified inactivation of the plasmid *lac*Z gene, most likely due to insertion of the 1.75kb DNA fragment present within the ligation reaction. From 2 agar plates inoculated with transformed bacteria, 14 white colonies were present amongst 154 blue colonies.

Eight white colonies were cultured for small plasmid preparations in order to analyse potential recombinant plasmids by restriction endonuclease digestion. Plasmids were digested with *Eco*RI, RNase A-treated, and the DNA fragments separated through an agarose gel (Figure 3.4.).

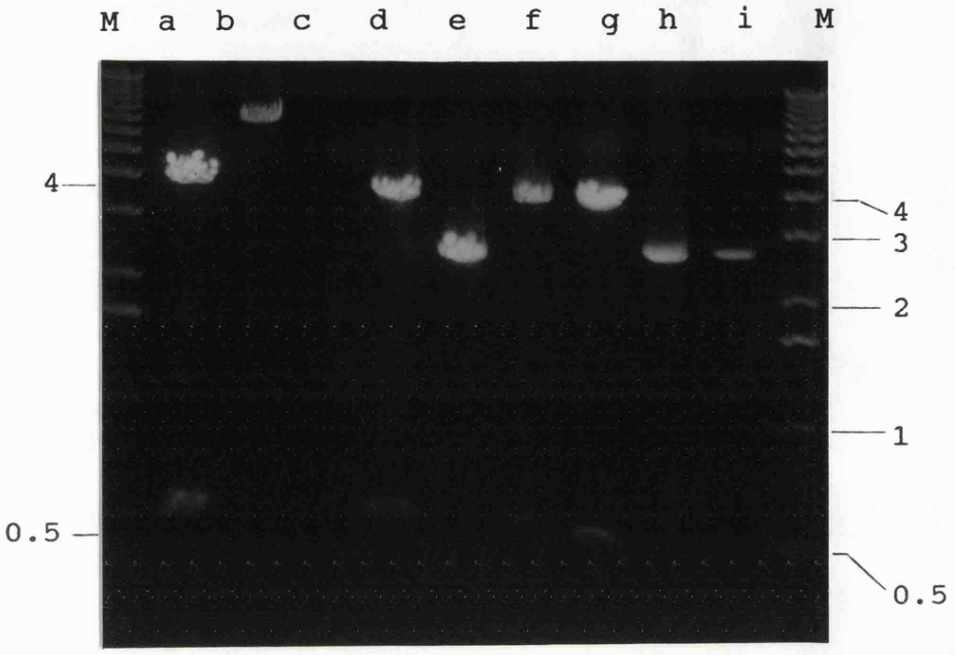


Figure 3.4. Agarose gel separation of EcoRI plasmid digests. Eight small scale plasmid preparations (lanes a to h) were derived from white colonies (on agar containing IPTG and X-gal) produced from the transformation of competent cells with the products of the ligation reaction between linearised pUC18 DNA and the 1.75kb fragment. These plasmids were treated with RNase-A prior to electrophoresis. CsCl-purified pUC18 preparation (lane i) was not treated with RNase-A. Molecular weights are in kb. Markers (M) are 1kb ladder (see Appendix 4).

Parental plasmid pUC18 (lane i) gave a single DNA fragment when digested with EcoRI, of approximately 2.7kb. Four out of the eight chosen plasmids contained fragments of approximately 3.9kb and 0.6kb (lanes a, d, f and g). The fragment sizes corresponded with those predicted for a gC fragment pUC18 recombinant plasmid. A map depicting EcoRI sites is shown in Figure 3.5. The four plasmids were designated pGCH1 to 4, respectively. The four other plasmid constructs (lanes b, c, e and h), two of which appeared to be vector DNA only, were discarded.

A purified preparation of pGCH1 was obtained following large-scale culture of bacteria containing the plasmid and chloramphenicol amplification. Extraction of plasmid DNA and caesium chloride (CsCl) centrifugation generated a purified preparation of pGCH1 at a concentration of 795μg/ml DNA. The plasmid was compared with pUC18 by restriction enzyme analysis. The agarose gel separation of the fragments resulting from digestion with EcoRI, NarI and XbaI is shown in Figure 3.6. Plasmid pGCH1 digested with EcoRI (lane a) gave fragments of 4.0kb and 0.5kb, with NarI (lane c) gave 3.1kb and 1.3kb and with XbaI (lane e), gave 4.0kb and 0.4kb. Digestion of pUC18 with each of these enzymes (lanes b, d and f, respectively) gave a single fragment of approximately 2.7kb. The digests performed here were not treated with RNase prior to electrophoresis and the agarose gel shows the plasmid preparations to be free from RNA contamination. The fragments obtained from these digests confirmed the structure of pGCH1 as depicted in Figure 3.5. This recombinant plasmid was used for further manipulations of the gC sequence.

3.1.3. Construction of pGCV Plasmids.

Plasmids designated pGCV were generated by the cloning of the HSV-1 gC gene into plasmid vector pSC11, under the control of the vaccinia virus P7.5 promoter contained within that plasmid. Plasmid pSC11 was prepared as for pUC18 (SmaI/BAP) and 80μl (approximately 0.25μg) of the 1.75kb fragment from KpnId was ligated with 0.5μl (approximately

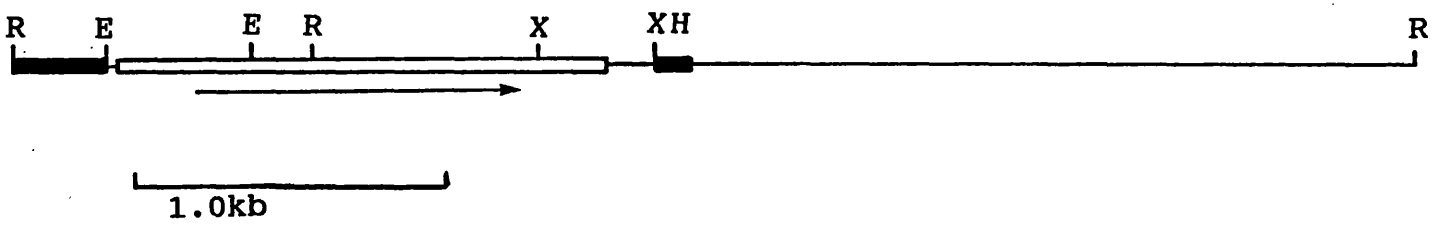


Figure 3.5. Linear representation of pGCH plasmids. DNA originating from parental pUC18 plasmid is represented as a single line. The position (open box) and transcriptional orientation of the HSV-1 gC gene (arrow below box), with respect to the plasmid polylinker region and the plasmid copy of the E. coli lacZ gene (closed box) are indicated. The insertion of the HSV-1 gC gene interrupts the lacZ gene. Restriction enzyme sites indicated are EcoRI (E), HindIII (H), NarI (R) and XbaI (X). Derived from information within Figures 3.1. and 3.4. and from pUC18 restriction maps.

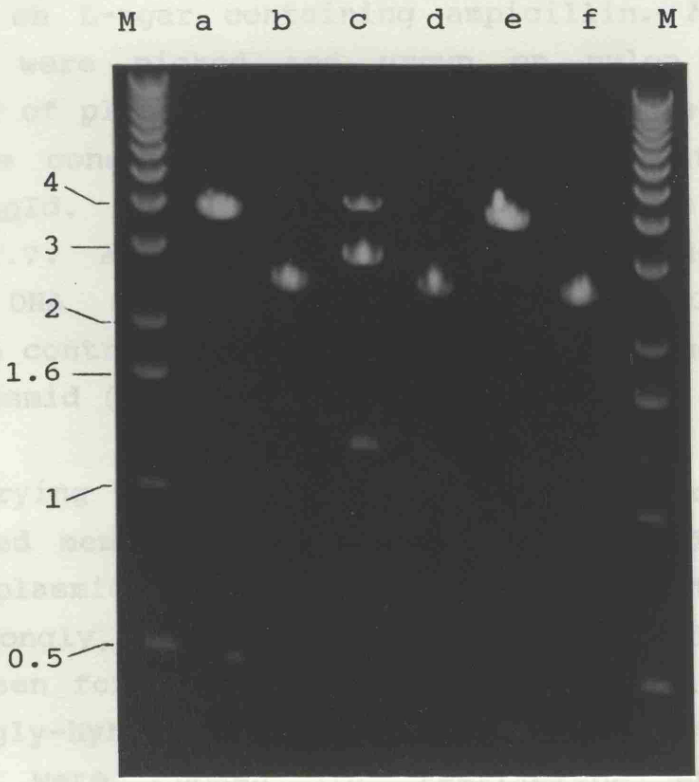


Figure 3.6. Agarose gel separation of restriction digests of pGCH1 and pUC18. The position and orientation of gC sequences within pGCH1 (lanes a, c and e) was confirmed by comparison with pUC18 (lanes b, d and f). Restriction enzymes were EcoRI (lanes a and b), NarI (lanes c and d) and XbaI (lanes e and f). Molecular weights are in kb. Markers (M) are 1kb ladder (see Appendix 4).

0.2μg) of the prepared plasmid vector. Competent E. coli JM101 cells were transformed with the ligation reaction and selected on L-agar containing ampicillin. A total of 192 colonies were picked and grown on nylon membranes for screening of plasmid DNA by radioactive probe hybridisation. The probe consisted of the 1.75kb fragment excised from pAT153/KpnId. The resulting autoradiograph is shown in Figure 3.7. A small membrane was included, containing control DNA samples (20ng each) of pSC11 and pUC18 (negative controls, top left and right, respectively) and of pGCH1 plasmid (positive control, bottom).

A varying degree of hybridisation was observed across the probed membranes, and some was observed with negative control plasmids. However, the positive control hybridised more strongly, and so only strongly-hybridising colonies were chosen for restriction enzyme analysis. Of a total of 37 strongly-hybridising positions on the test membranes, 11 colonies were chosen for restriction enzyme analysis corresponding to the autoradiograph positions indicated below.

Plate A: 1G, 3C, 7G, 10F, 10H
Plate B: 2H, 4B, 5A, 5G, 6B, 7E

Small plasmid preparations were derived from each of the 11 colonies and digested with restriction enzyme XbaI (Figure 3.8.). Restriction data for parental plasmid pSC11 indicated digestion with XbaI would produce two fragments of sizes 4.04bp and 3.85bp. In Figure 3.8. these two fragments were seen to migrate together as a brightly staining band, a doublet (lane 1), approximating to a size of 3.8-3.9kb, closer to the size of the smaller fragment. This suggests the fragment bands have run slightly faster than desired, due to overloading of the gel, which has lead to a poor separation. The orientation of the cloned 1.75kb gC fragment with respect to the vaccinia virus P7.5 promoter is crucial for transcription of the gene by the recombinant vaccinia virus. Restriction site data for pSC11 and for the HSV-1

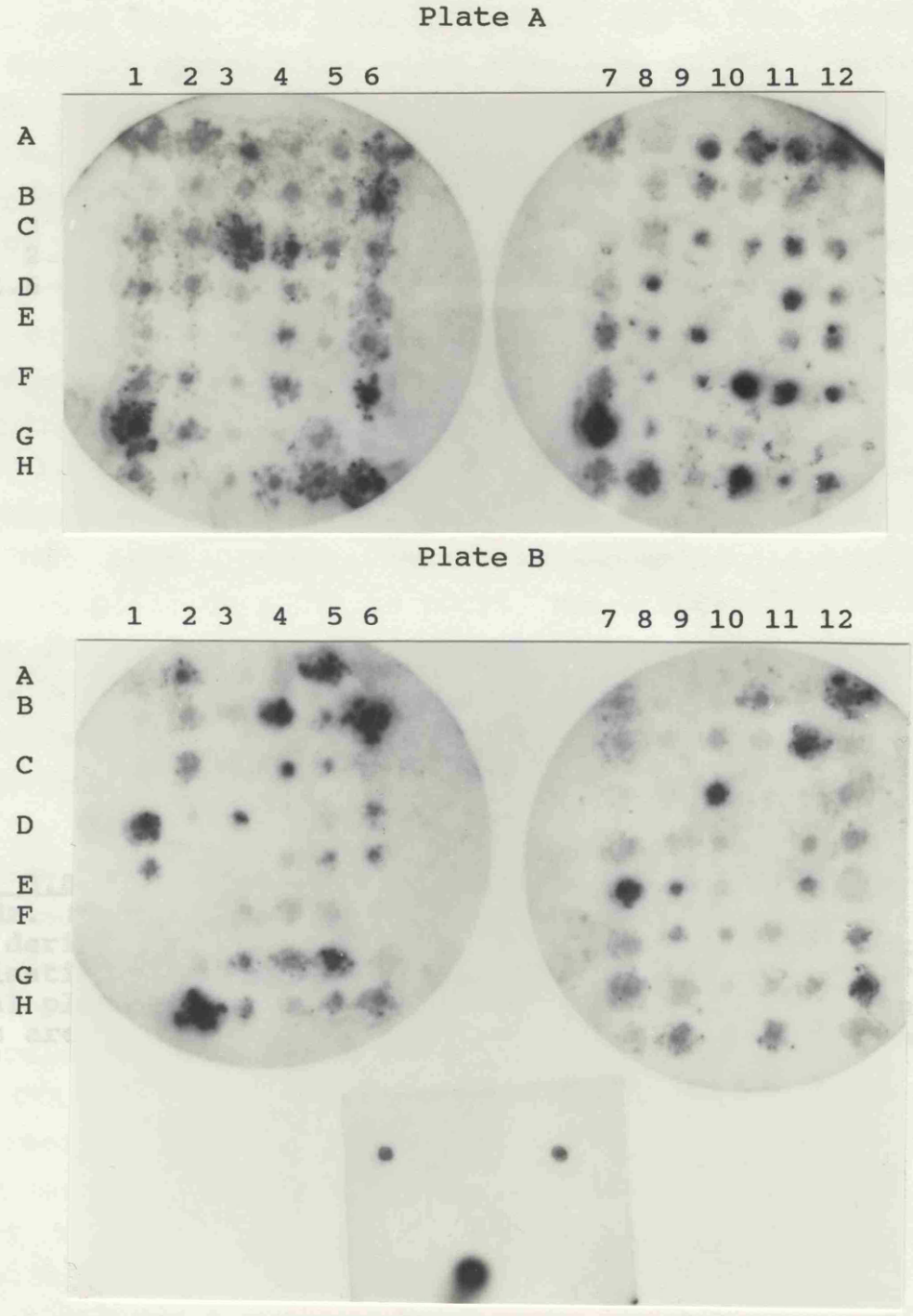


Figure 3.7. Autoradiograph of nylon membranes, carrying transformed colonies, probed with radiolabelled 1.75kb DNA fragment (containing the HSV-1 gC gene) derived from the NheI/SphI digest of pAT153/KpnId. Prior to transfer onto membranes, transformed bacterial colonies were grown in the wells of 2 plates, A and B, which contained 8 rows (A to H) and 12 columns (1 to 12). The control membrane contains 20ng of plasmids pUC18 (top left), pSC11 (top right) and pGCH1 (bottom).

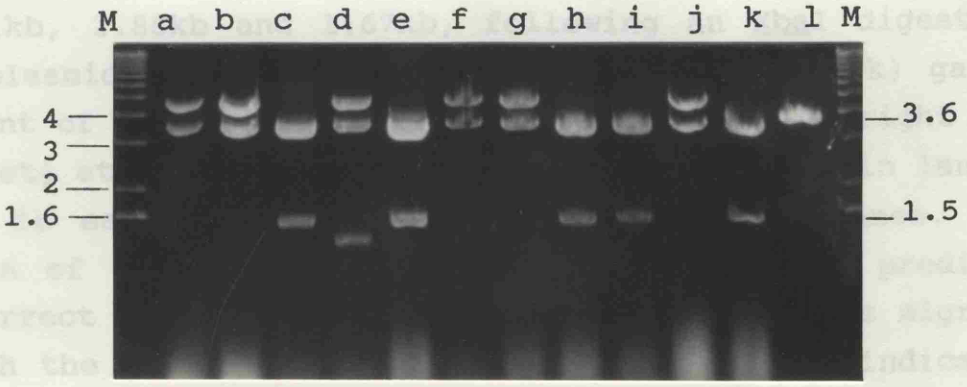


Figure 3.8. Agarose gel separation of XbaI digest of plasmids. Small scale plasmid preparations (lanes a to k) were derived from bacterial cultures demonstrating hybridisation to a gC-containing DNA probe (Figure 3.7.). Parental plasmid pSC11 was also included (lane l). Molecular weights are in kb. Markers (M) are 1kb ladder (see Appendix 4).

(KOS) gC gene predicted that cloning of the 1.75kb fragment in the desired orientation would give rise to DNA fragments of 4.1kb, 3.85kb and 1.67kb, following an XbaI digestion. Five plasmid preparations (lanes c, e, h, i and k) gave a fragment of approximately 1.6kb in addition to a bright band (doublet) at approximately 3.6kb. The doublet within lanes c and e is easier to distinguish than in other lanes. This pattern of fragment sizes corresponds with that predicted for correct insertion of the gC gene. The fragments migrated through the gel faster than predicted, which was indicative of the lanes being overloaded with DNA. These plasmid preparations corresponded to colonies A/7G, A/10H, B/5A, B/5G and B/7E, which were subsequently designated recombinant plasmids pGCV1 to pGCV5. The compositions of the plasmids in other lanes (a, b, d, f, g and j) were unknown, but none corresponded to the insertion of the 1.75kb in the alternative orientation to that desired. As a result, these plasmids were discarded.

Figure 3.9. depicts the desired orientation of gC within pGCV plasmids, as confirmed by the fragment sizes obtained from the above XbaI digest, ensuring the gC gene is transcribed in the correct orientation within the plasmid. A large-scale, CsCl-purified pGCV2 preparation (concentration 570μg DNA/ml) was compared to pSC11 by restriction endonuclease digests and the fragments separated by electrophoresis (Figure 3.10.). Plasmid pGCV2 gave rise to fragment sizes of approximately 4.5kb, 3.1kb, 1.4kb and 0.8kb with EcoRI (lane a); 8.0kb, 2.4kb with NarI (lane c) and 3.8-3.9kb and 1.7kb with XbaI (lane e). The sizes of the fragments obtained with each restriction enzyme confirm that pGCV2 is as depicted within Figure 3.9. The corresponding sizes of the fragments resulting from pSC11 digests were 4.5kb, 3.1kb (lane b); 8.0kb (lane d) and a 3.9kb doublet (lane f). RNAse A was not added to plasmid preparations prior to electrophoresis, but the gel shows no contaminating RNA within the plasmid preparations.

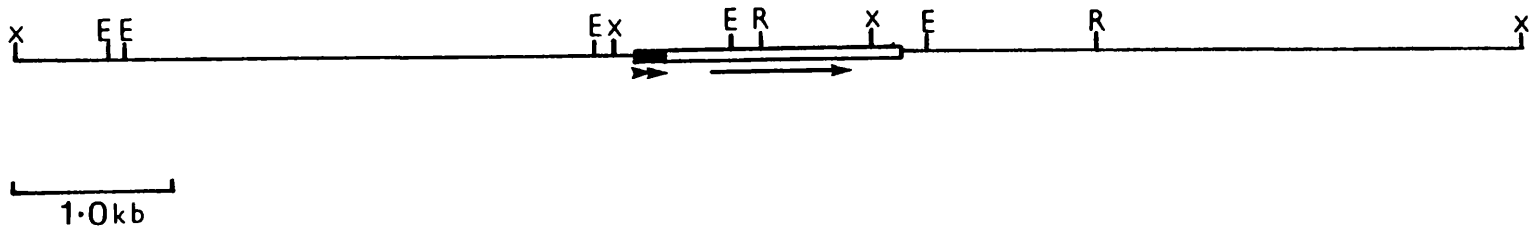


Figure 3.9. Linear representation of pGCV plasmids. DNA originating from the parental plasmid pSC11 is represented as a single line. The position of the cloned HSV-1 gC sequences (open box) and the transcriptional orientation (single arrow) are indicated with respect to the P7.5 promoter (closed box; double arrow). Restriction enzyme sites indicated are EcoRI (E), NarI (R) and XbaI (X). Derived from information within Figures 3.1. and 3.8. and from pSC11 restriction maps.

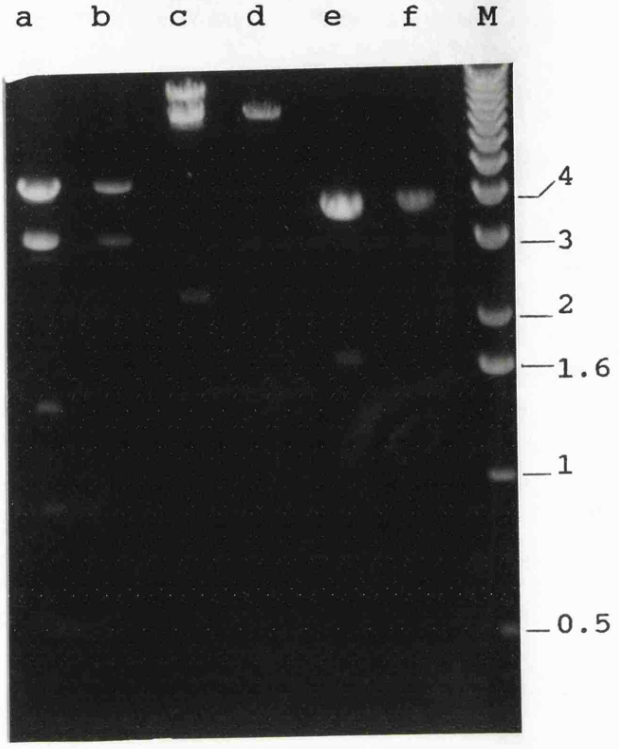


Figure 3.10. Agarose gel separation of restriction enzyme digests of pGCV2 and pSC11 plasmids. Purified preparations of pGCV2 (lanes a, c and e) and pSC11 (lanes b, d and f) were digested with enzymes EcoRI (lanes a and b), NarI (lanes c and d) and XbaI (lanes e and f). Molecular weight markers (M) are 1kb ladder (see Appendix 4).

3.2. GENERATION OF RECOMBINANT VC- AND VpS-VIRUSES.

Recombinant vaccinia viruses were generated within vaccinia-infected mammalian cells following co-transfection with plasmid DNA. Transfection with plasmid pGCV2 gave rise to recombinant viruses designated with the prefix VC. Transfection with pSC11 gave rise to recombinant viruses designated with the prefix VpS. Infected/transfected monolayers were harvested, virus was released by freeze-thawing into 1ml fresh medium and titrated on CV-1 cells. Virus stock generated using recombinant plasmid pGCV2 was found to have a titre of 1.04×10^7 pfu/ml. Virus generated using plasmid pSC11 had a titre of 1.25×10^6 pfu/ml. The titres do not represent recombinant titres, but the combination of parental and recombinant viruses. However, the titres assist in the calculation of the proportion of recombinants generated by transfections.

3.2.1. Plaque-Purification of Recombinant Viruses.

CV-1 cells were infected with virus from transfected stocks, serially diluted in PBS between 10^{-2} and 10^{-7}, and were overlaid with solid medium containing agarose and X-gal. The plasmids used for transfection contain a copy of the *E. coli lacZ* gene (under the control of vaccinia P11 promoter) within the vaccinia TK regions which allow homologous recombination. During the infectious cycle of recombinant viruses, the ß-galactosidase generated acts on the chromogenic substrate X-gal to give rise to a blue-coloured product. At the lowest dilution with both virus types, the overlay contained an overall blue colour, probably due to the presence of unincorporated plasmid DNA contaminating the infecting virus stock. Monolayers infected with pGCV2-tranfected virus stock diluted between 10^{-2} and 10^{-4} displayed small areas of strong blue colouration. Examination of such areas by low power microscopy indicated these areas contained one or more plaques. The regions of the monolayer corresponding to these plaques were picked into 200µl PBS and virus released by freeze-thawing. The proportion of recombinant (VC-) vaccinia viruses derived from the transfection procedure with pGCV2 which were

recombinant viruses was 0.192%. Monolayers infected with pSC11-transfected virus gave rise to small areas of strong blue colouration within dilutions of 10^{-2} and 10^{-3}. The proportion of recombinant (VpS-) plaques out of the total of viruses generated by pSC11-transfection was 0.08%.

Virus plaques picked from infected monolayers were used for three subsequent rounds of plaque purification. CV-1 monolayers were infected with recombinant virus diluted between 10^-1 and 10^-3 in PBS and overlaid with solid medium containing X-gal as above. Not all virus samples chosen from the first selection gave rise to blue plaques in the second screening (the presence of plasmid DNA in the low dilution of the initial screening passage can give false positives which do not contain recombinant viruses; M. Mackett, personal communication). Following three rounds of plaque purification, a single VpS- plaque (designated VpS141) and nine VC- plaques were chosen. The VC- plaques were designated as below

VC211	VC231	VC232
VC341	VC351	VC741
VC751	VC851	VC861

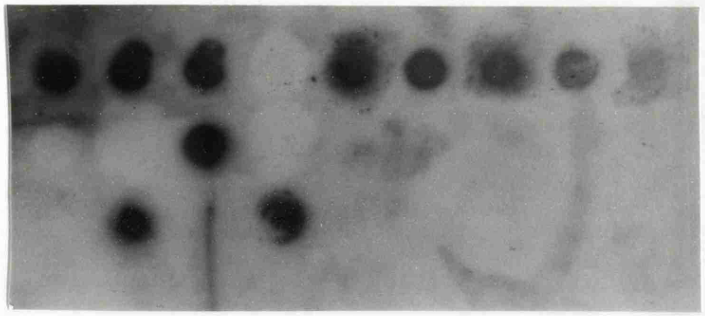
Recombinant viruses were characterised with respect to HSV-1 DNA content and gC protein synthesis within infected cells.

3.2.2. Detection of gC DNA in Recombinant Viruses.

Plaque-purified recombinant viruses were screened for the presence of HSV-1 gC sequences by Dot-Blot or Southern Blot DNA hybridisations.

3.2.2.1. Dot-Blot Assay.

Infected cells were applied to nylon membranes and probed *in situ* with a radiolabelled DNA probe. The probe consisted of the 1.75kb fragment obtained by the NheI/SphI digest of pAT153/KpnId. Figure 3.11. represents the resulting autoradiograph of this hybridisation, with each



1 2 3 4 5 6 7 8 9

10 11 12 13

14 15 16 17

Figure 3.11. Autoradiograph of infected cells probed with radiolabelled 1.75kb DNA fragment (containing the HSV-1 gC gene), derived from the NheI/SphI digest of pAT153/KpnId. Infected cell monolayers were harvested onto a nylon membrane. The lower diagram represents the numbering of each monolayer tested. Cells were infected with viruses VC211, VC231, VC232, VC341, VC351 VC741, VC751, VC851 AND VC861 (positions 1 to 9, respectively). Controls were cells infected with recombinant virus VpS141 (10), wild-type vaccinia (11), HSV-1 (12) or with PBS (13). Plasmid controls were also included, being 20ng each of pUC18, pGCH1, pSC11 and pGCV2 (positions 14 to 17, respectively).

point from 1 to 13 representing an infected monolayer. As expected, HSV-infected cells (12) hybridised to the gC probe, whilst cells infected with wild-type vaccinia, with VpS141 or mock infected cells (10, 11 and 13, respectively) did not hybridise to the probe. Plasmid controls (20ng each) were also included on the membrane, at positions 14 to 17. Negative controls pUC18 and pSC11 (14 and 16) showed no hybridisation, whilst positive controls pGCH1 and pGCV2 (15 and 17) hybridised well to the probe. Hybridisation of the probe to cells infected with recombinant viruses (points 1 to 9) occured at varying degrees of intensity. The differences in levels of hybridisation may have been due to differences in titre of infectious virus, as this was not determined for plaque-purified virus samples at this stage. Cells infected with viruses VC211, VC231, VC232 and VC351 (points 1, 2, 3 and 5) hybridised most strongly to the probe, whilst cells infected with VC741, VC751 and VC851 (6, 7 and 8) showed less intense hybridisation. Cells infected with VC861 (9) were very weakly hybridising and cells infected with virus VC341 (4) showed no hybridisation to the probe. This virus was discarded from further studies.

3.2.2.2. Southern Blot Analysis.

DNA was extracted from cells which had been infected with recombinant or wild-type vaccinia viruses, and was digested with restriction enzyme HindIII for 2 hours. Control DNA samples included 5ng each of BamHI-restricted pGCV2 or pSC11 plasmids. DNA samples were electrophoresed through an agarose gel and transferred onto a nylon membrane by Southern blotting. The membrane was probed with radioactively-labelled 1.75kb fragment DNA obtained, as before, from the NheI/SphI digest of pAT153/KpnId and the resulting autoradiograph is shown in Figure 3.12. The probe hybridised to a fragment of approximately 1.8kb generated by BamHI digestion of pGCV2 and very faintly to a fragment approximately 3.5kb in size (lane k). Faint hybridisation could be seen with a 3.5kb DNA fragment from pSC11 (lane l), but no strong hydridisations were seen with this plasmid. Recombinant viruses VC211, VC232, VC351, VC741 and VC751

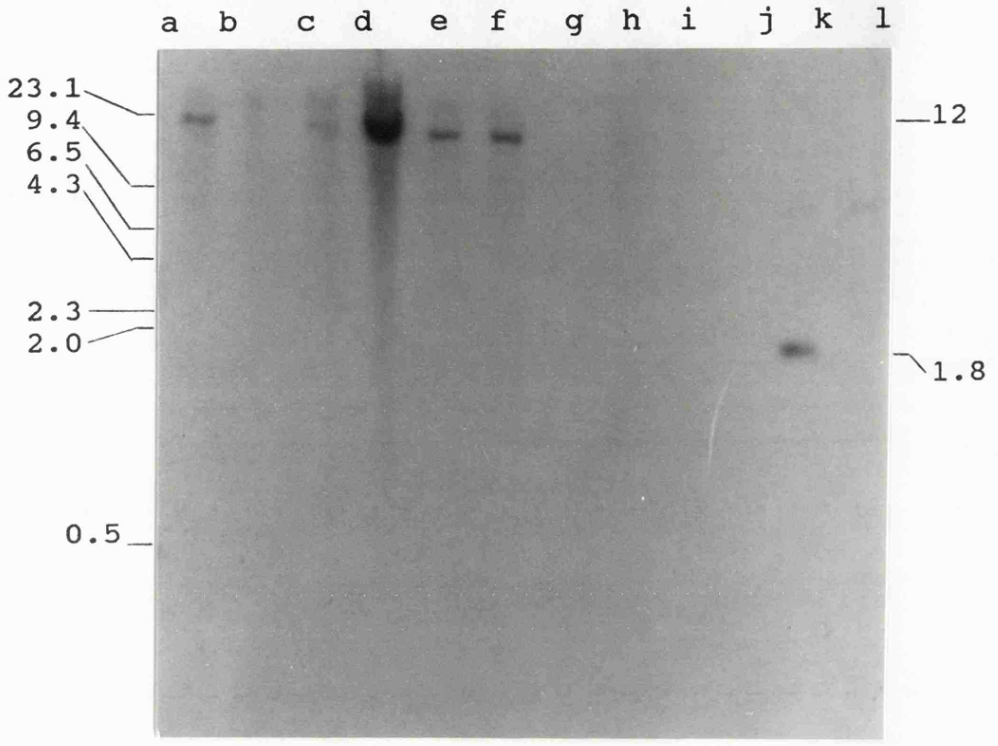


Figure 3.12. Autoradiograph of infected cellular DNA preparations. Cell monolayers were infected with viruses VC211, VC231, VC232, VC351, VC741, VC751, VC851 and VC861 (lanes a to h), with VpS141 (lane i) or with wild-type vaccinia (lane j). Plasmid controls were 5ng of BamHI-digested pGCV2 (lane k) or pSC11 (lane l). Following Southern transfer of DNA from agarose gel onto nylon membrane, the samples were screened for gC DNA with radiolabelled 1.75kb DNA fragment derived from NheI/SphI digest of pAT153/KpnId. Molecular weights are in kb. Markers were HindIII-digested lambda DNA (see Appendix 4).

show hybridisation to the probe within a distinct DNA fragment at approximately 12kb (lanes a, c, d, e and f). Vaccinia virus TK sequences (into which the cloned DNA is inserted) are contained within the genome fragment HindIII J (Weir, et al., 1982), which is approximately 5.0kb in length. The combined length of inserted DNA (HSV-1 gC and ß-galactosidase sequences, plus vaccinia promoter regions) is approximately 7kb. The virus genome fragment hybridising to the probe is therefore of the expected size, indicating recombination had most likely occured within the desired region of the virus genome. Hybridisation with VC231 and VC861 (lanes b and h) was poor, producing a faint smear, which may have been indicative of poor DNA preparations. VC851 (lane g) did not hybridise to the probe, nor did VpS141 or wild-type vaccinia (lanes i and j, respectively). Recombinant viruses VC211, VC351, VC741 and VC751 (lanes a, d, e and f) were chosen for further characterisation.

3.2.3. Expression of HSV-1 gC by Recombinant Viruses.

The detection of HSV-1 gC expressed by recombinant (VC) viruses during the infectious cycle was attempted by indirect immunofluorescence (IIF) techniques. CV-1 cells were grown on glass coverslips and infected with HSV-1, recombinant or wild-type vaccinia virus or mock-infected with PBS. The infected monolayers were washed and fixed at 24 hours post infection (pi). The vaccinia virus P7.5 promoter has been shown to reach peak activity between 12 and 24 hours pi (Mackett, et al., 1984). At this time, a positive fluorescence was observed with cells infected by HSV-1, but infection of cells with either recombinant or wildtype vaccinia viruses gave no fluorescence (no photographs were taken, as all samples except HSV-1 were negative). It was concluded that the recombinant vaccinia viruses contained HSV-1 gC DNA sequences, but were unable to synthesise gC protein from these sequences within infected cells.

CHAPTER 4

RESULTS PART 2

VACCINIA VIRUS EXPRESSION OF HSV-1 gC

4.1. SEQUENCE VERIFICATION OF CLONED DNA.

The failure of the recombinant vaccinia viruses generated in Chapter 3. to express HSV-1 gC during infection of susceptible cells could be due to an abberation of the coding sequence for the glycoprotein, occurring as a consequence of cloning manipulations. In order to examine this, the gC coding region was excised from the recombinant plasmid pGCH1, and cloned into M13mp19 bacteriophage vector, which allowed DNA sequence analysis of the gene.

4.1.1. Generation of pLEC Plasmids.

Insertion of the HSV-1 gC coding sequences into the replicative form (ds) DNA of the bacteriophage M13mp19 generated plasmids designated pLEC. Plasmids pGCH1 and pUC18 were digested with restriction enzymes BamHI and KpnI and the resulting DNA fragments were separated by electrophoresis (Figure 4.1.). The agarose gel shows a fragment of approximately 1.7kb, released from pGCH1 (lane a; arrow) which is not present in the parental pUC18 plasmid (lane b). This was the 1.75kb (gC) fragment and was subsequently excised from pGCH1 (10μg) with BamHI and KpnI. M13mp19 vector (2μg) was prepared by restriction digest with BamHI and KpnI (sample run in lane c) and isopropanol extraction to attempt to remove the small fragment of DNA excised by the digestion (not visible on the gel). A ligation reaction was performed, with a 40μl volume (approximately 0.5μg) of the 1.75kb fragment DNA obtained from LMP agarose and approximately 0.2μg of vector DNA, in a 50μl volume. M13-competent E. coli JM101 cells were transformed with ligated DNA or with undigested M13mp19 vector, as described in Materials and Methods. The top agar contained IPTG and X-gal, as a means of selecting recombinant plaques over wild-type plaques. Cells transformed with M13mp19 bacteriophage, which contains a copy of the E.coli lacZ gene, gave rise to blue-coloured plaques within the bacterial lawn as the gene product ß-galactosidase converted the chromogenic X-gal substrate to a coloured product. The ligation reaction gave rise to a mixture of blue and colourless plaques within the bacterial

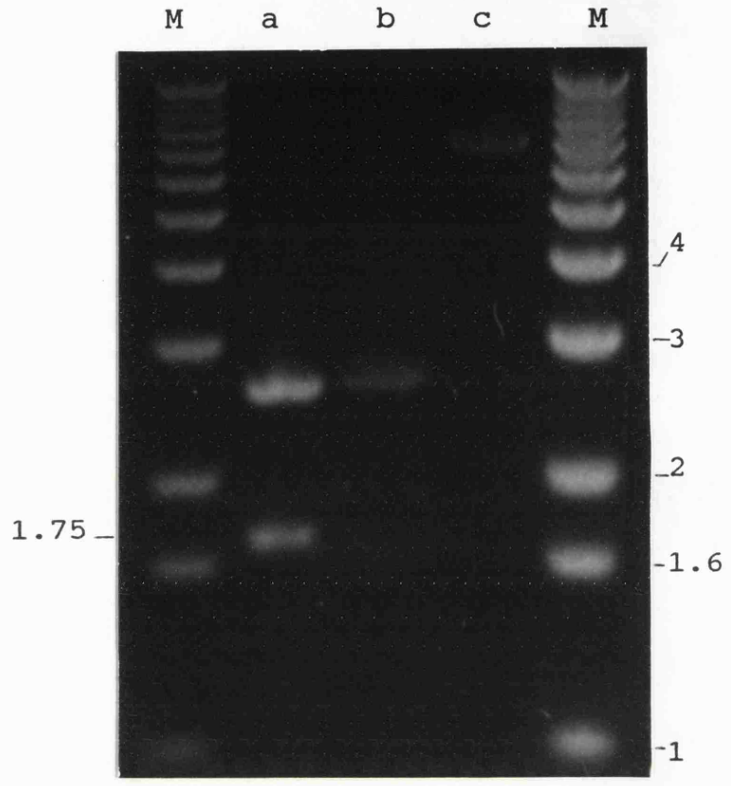


Figure 4.1. Agarose gel separation of digested plasmids. The recombinant plasmid pGHC1 was digested with BamHI and KpnI (lane a), and yielded the 1.75kb fragment (arrow) containing the HSV-1 gC gene. Plasmid pUC18 (lane b) and bacteriophage M13mp19 (lane c) were also restricted with the same enzymes. Molecular weights are in kb. Markers (M) were 1kb ladder (Appendix 4).

lawn. The generation of recombinant bacteriophages causes inactivation of the lacZ gene, due to the insertion of the 1.75kb DNA fragment into the gene. The presence of blue plaques indicated parental bacteriophage had recircularised, possibly due to the presence of stuffer fragment DNA which had not been completely removed by the isopropanol extraction.

The ratio of clear plaques with respect to blue (wild-type) plaques was 57.2%, a high ratio due to the presence of cohesive ends on DNA fragments encouraging efficient ligation. As both vector and insert were digested with 2 different restriction endonucleases, each DNA fragment contained non-identical termini. Ligation of vector and insert should therefore occur in one orientation only, and Figure 4.2. illustrates the structure of the recombinant bacteriophage DNA expected from such a ligation. Bacteriophage derived from five colourless plaques and one blue (wild-type) plaque were used to infect E. coli JM101 cultures for small-scale preparations of replicative form bacteriophage DNA. The preparations were digested with BglI enzyme and the fragments separated by electrophoresis (Figure 4.3.). The parental M13mp19 vector (lane f) produced a single fragment at approximately 7.5kb, which corresponded well to the size of linearised M13mp19, at 7.25kb. All five recombinant samples (lanes a to e) gave rise to two DNA fragments, one within the region of 7kb and one of approximately 1.4kb. The larger fragment observed within each recombinant bacteriophage digest migrated slightly more rapidly than expected through the agarose gel. Figure 4.2. suggested sizes of 7.65kb and 1.4kb, from a BglI digest of the recombinant bacteriophage DNA. The rapid migration of the large fragment may be due to an excess of sample within each well (the large fragments appear as curved bands, indicative of overloading), or due to the electrophoresis being carried out at too high a voltage. However, the five recombinant DNA samples were deemed to be representative of

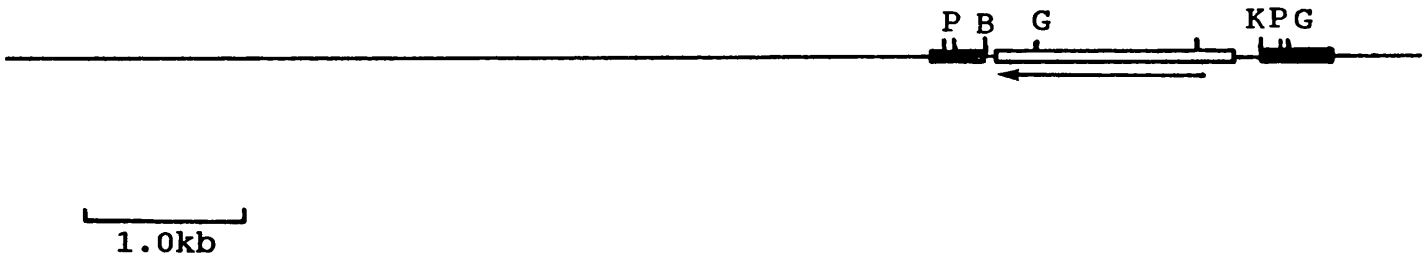


Figure 4.2. Linear representation of pLEC plasmids. DNA originating from the parental plasmid M13mp19 is represented as a single line. The position of the cloned HSV-1 gC sequences (open box) and the transcriptional orientation (single arrow) are indicated. The cloned sequences interrupt the functioning of the bacteriophage copy of the lacZ gene (closed box). The position of several restriction sites are indicated, being BamHI (B), BglI (G), KpnI (K) and PvuII (P). Derived from information within Figure 4.1. from M13mp19 restriction maps.

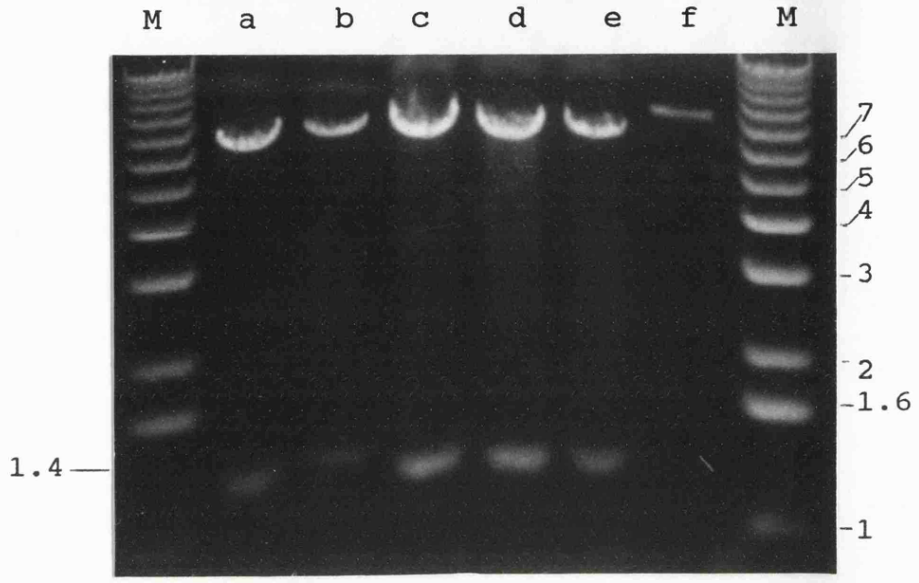


Figure 4.3. Agarose gel separation of BglI digest of bacteriophage RF (double-stranded) DNA preparations. Small-scale DNA preparations were derived from colourless (recombinant) plaques picked from a bacterial lawn transformed with the products of ligation between the 1.75kb BamHI/KpnI fragment of pGCH1 (containing the HSV-1 gC gene) and BamHI/KpnI digested M13mp19 DNA (lanes a to e). Parental bacteriophage DNA was derived from blue plaques picked from a bacterial lawn transformed with undigested M13mp19 DNA (lane f). Molecular weights are in kb. Markers (M) are 1kb ladder.

the fragment sizes expected from Figure 4.2. The recombinant bacteriophages were designated pLEC1 to pLEC5 and were used as templates for sequencing reactions.

4.1.2. Sequence Verification of Cloned gC Gene.

The sequence of the cloned 1.75kb DNA fragment was determined using the pLEC recombinant plasmids. Figure 4.4. shows the complete sequence of the 1753bp cloned fragment, including the entire HSV-1 (17) gC coding sequence from position +1 (ATG) to +1536 (TAA). The complete sequence of the HSV-1 (strain 17) long unique region (McGeoch, et al., 1988) had subsequently become available through the Genbank database. The sequence of the gC gene determined here experimentally was compared to the published sequence for HSV-1 (17) gC gene. The sequence determined within this study showed two differences from the database published sequence. Nucleotide 170 was an A, compared to the published G residue, whilst nucleotides 1146-1148 were GCA (as for KOS sequence), rather than the published CAT for strain 17. These changes correspond to amino acid changes of serine to lysine (residue 57) and asparagine to histidine (residue 383) with respect to the database HSV-1 (17) gC protein. The data confirmed that no abberations had occured within the gC gene during the cloning manipulations used.

4.2. MANIPULATIONS OF NON-CODING SEQUENCES.

The nucleotide sequence of the cloned 1.75kb fragment revealed 34 bases upstream of the gC gene initiation codon, as predicited by KOS sequence data and the position of the NheI site. A communication with Dr. Richard Eberle (Oklahoma, USA) indicated that removal of these upstream nucleotides may release the block to gC expression by recombinant vaccinia viruses. The upstream 34bp were removed and replaced with a specific linker, which contained recognition sites for two additional restriction enzymes:- PvuII and PflMI (see Appendix 3). Digestion with these restriction enzymes would then allow cloning of the gC gene into pSC11 plasmids for the generation of recombinant vaccinia viruses. Restriction with PvuII would generate a

-1
CTAGCCGATCCCTCGCGAGGGGGAGGCGTCGGGC

1 60
ATGGCCCCGGGGCGGGTGGGCCTTGCCGTGGTCCTGTGGAGCCTGTTGTGGCTCGGGGCG

61 120
GGGGTGTCCGGGGGCTCGGAAACTGCCTCCACCGGGCCCACGATCACCGCGGGAGCGGTG

121 * 180
ACGAACGCGAGCGAGGCCCCCACATCGGGGTCCCCCGGGTCAGCCGCCAACCCGGAGGTC

181 240
ACCCCCACATCGACCCCAAACCCCAACAATGTCACACAAAACAAAACCACCCCCACCGAG

241 300
CCGGCCAGCCCCCCAACAACCCCCAAGCCCACCTCCACGCCCAAAAGCCCCCCCACGTCC

301 360
ACCCCCGACCCCAAACCCAAGAACAACACCACCCCCGCCAAGTCGGGCCGCCCCACTAAA

361 420
CCCCCCGGGCCCGTGTGGTGCGACCGCCGCGACCCATTGGCCCGGTACGGCTCGCGGGTG

421 480
CAGATCCGATGCCGGTTTCGGAATTCCACCCGCATGGAGTTCCGCCTCCAGATATGGCGT

481 540
TACTCCATGGGTCCGTCCCCCCCAATCGCTCCGGCTCCCGACCTAGAGGAGGTCCTGACG

541 600
AACATCACCGCCCCACCCGGGGGACTCCTGGTGTACGACAGCGCCCCCAACCTAACGGAC

601 660
CCCCACGTGCTCTGGGCGGAGGGGGCCGGCCCGGGCGCCGACCCTCCGTTGTATTCTGTC

661 720
ACCGGGCCGCTGCCGACCCAGCGGCTGATTATCGGCGAGGTGACGCCCGCGACCCAGGGA

721 780
ATGTATTACTTGGCCTGGGGCCGGATGGACAGCCCGCACGAGTACGGGACGTGGGTGCGC

781 840
GTCCGCATGTTCCGCCCCCCGTCTCTGACCCTCCAGCCCCACGCGGTGATGGAGGGTCAG

841 900
CCGTTCAAGGCGACGTGCACGGCCGCCGCCTACTACCCGCGTAACCCCGTGGAGTTTGTC

901 960
TGGTTCGAGGACGACCACCAGGTGTTTAACCCGGGCCAGATCGACACGCAGACGCACGAG

961 1020
CACCCCGACGGGTTCACCACAGTCTCTACCGTGACCTCCGAGGCTGTCGGCGGCCAGGTC

1021 1080
CCCCCGCGGACCTTCACCTGCCAGATGACGTGGCATCGCGACTCCGTGACGTTCTCGCGA

1081 1140
CGCAATGCCACCGGGCTGGCCCTGGTGCTGCCGCGGCCAACCATCACCATGGAATTTGGG

1141 *** 1200
GTCCGGCATGTGGTCTGCACGGCCGGCTGCGTCCCCGAGGGCGTGACGTTTGCCTGGTTC

1201 1260
CTGGGGGACGACCCCTCACCGGCGGCTAAGTCGGCCGTTACGGCCCAGGAGTCGTGCGAC

1261 1320
CACCCCGGGCTGGCTACGGTCCGGTCCACCCTGCCCATTTCGTACGACTACAGCGAGTAC

1321 1380
ATCTGTCGGTTGACCGGATATCCGGCCGGGATTCCCGTTCTAGAACACCACGGCAGTCAC

1381 1440
CAGCCCCCACCCAGGGACCCCACCGAGCGGCAGGTGATCGAGGCGATCGAGTGGGTGGGG

1441 1500
ATTGGAATCGGGGTTCTCGCGGCGGGGGTCCTGGTCGTAACGGCAATCGTGTACGTCGTC

1501 1560
CGCACATCACAGTCGCGGCAGCGTCATCGGCGG**TAA**CGCAAGACCCCCCCGTTACCTTTT

1561 1620
TAATATCTATATAGTTTGGTCCCCCCTCTATCCGCCCACCGCTGGGCGCTATAAAGCCGC

1621 1680
CACCCTCTCTTCCCTCAGGTCATCCTTGGTCGATCCCGAACGACACACGGCGTGGAGCAA

1681 1719
AACGCCTCCCCCTGAGCCGCTTTCCTACCAACACAACGG

Figure 4.4. Complete sequence of the cloned 1.75kb fragment. The DNA fragment spans the region of the HSV-1 genome surrounding the gC coding sequence, from the upstream NheI site (position -34) to the downstream SphI site (position 1719). The entire HSV-1 (17) gC gene is contained within the fragment, beginning at position 1 (ATG; bold type) and terminating at position 1536 (TAA ;bold). The position of nucleotide differences between this and the published HSV-1 (strain 17) gC sequence are marked by asterisks.

blunt-ended fragment with only 3 nucleotides upstream of the initiation codon. Restriction with PflMI would yield a fragment with a 3′ overhang of 4 nucleotides prior to the initiation codon. Treatment of such a fragment with Klenow enzyme or T4 DNA polmerase would remove the overhanginig nucleotides to generate blunted termini at precisely the initiation codon.

4.2.1. Design of Oligonucleotide Primers.

The replacement of upstream non-coding bases by a specific linker was achieved in a two-step mutagenesis reaction involving the annealing of oligonucleotide primers to a single-stranded template. Single-stranded, recombinant bacteriophage DNA was passaged through a bacterial strain deficient in dut and ung functions, which meant replication of the template within these cells allowed the incorporation of uracil residues into the template (in place of thymidine at random sites), and then failed to recognize and repair these errors. Secondary strand DNA was generated from an annealed primer by polymerase and ligase reactions, and was then selected over the original template by passage through a competent bacterial strain, which recognized the errors within the original template and degraded it (Kunkel, et al., 1987). The oligonucleotide primers were designed such that at least 10 nucleotides would anneal to either side of the template region to be mutated. The melting temperature (Tm) of the two annealing regions of each primer were designed to be as close as possible, determined by the equation:-

$$Tm\ (^{o}C) = [4x(G+C)] + [2x(A+T)]$$

4.2.1.1. Primer P-MUT1.

Removal of the 34 non-coding nucleotides upstream of the ATG codon was achieved by mutagenesis of template DNA (passaged through E. coli strain RZ1032) with a 30-nucleotide primer designated P-MUT1 (Appendix 3). The 5′ sequence of this primer annealed to 16 nucleotides of vector DNA prior to the region to be removed, the last base of the

vector KpnI restriction site being the last annealing base. The 3′ portion (14 nucleotides) of the primer annealed to the gC coding sequence, beginning with the first nucleotide of the gene. The Tm values of the 5′ and 3′ annealing regions of the primer were calculated as follows, using the above equation.

5′ region ATTCGAGCTCGGTACC

$$Tm = [4x(9)] + [2x(7)]$$
$$= 36 + 14$$
$$= 50^{o}C$$

3′ REGION ATGGCCCCGGGGCG

$$Tm = [4x(11)] + [2x(2)]$$
$$= 44 + 4$$
$$+ 48^{o}C$$

4.2.1.2. Primer P-MUT2.

The second primer P-MUT2, was designed to insert 5 nucleotides between the (now adjacent) vector KpnI restriction site and the ATG codon. The sequence of P-MUT2 (Appendix 3) allowed 10 nucleotides to anneal to both vector and gC sequences, with the 5 extra bases to be inserted into the new template coming between these two termini. The Tm of the 5′ and 3′ annealing regions were calculated as follows.

5′ region GTCTGGTACC

$$Tm = [4x(6)] + [2x(4)]$$
$$= 24 + 8$$
$$= 32^{o}C$$

3′ region ATGGCCCCGG

$$Tm = [4x(8)] + [2x(2)]$$
$$= 32 + 4$$
$$= 36^{o}C$$

Following the mutagenesis with primer P-MUT1, newly-synthesised DNA was used to transform competent E. coli JM 101 cells. The composition of the templates in the region of the initiation codon was examined rapidly by sequence analysis using only the ddATP nucleotide (A-tracking). Several templates showed differences in the position of adenosine residues, with respect to the original template. These templates were thought to have lost the non-coding 34 bases between the vector KpnI site and the gC ATG codon. One template, designated pLED1, was sequenced within this region, and compared to the original template, pLEC1 (Figure 4.5.), to verify this. The pLED1 bacteriophage template was subjected to the second stage of mutagenesis, with P-MUT2. Sequence analysis (A-tracking) again indicated differences in the position of residues within several templates generated from the mutagenesis reaction. One such template, designated pLEM1, was sequenced and compared with pLEC1 and pLED1 (Figure 4.5.), and again, the desired mutation was seen to have taken place. The loss of nucleotides between the vector KpnI site and the gC initation codon in template pLED1 compared with pLEC1, and the addition of 5 nucleotides into this region within pLEM1 are illustrated. Due to the high content of G and C residues within the region, there are areas of compression (asterisk), where it is difficult to distinguish individual bands. Template pLEM1 was used to re-sequence the complete gC gene, and such compressions were overcome, giving the complete sequence of the gene and indicating that no sequence alterations had been created within the gene other than those designed above. A large-scale, CsCl preparation of plasmid pLEM1 was generated (395μg/ml) and used for recloning of gC sequences.

4.3. CONSTRUCTION OF pMCV PLASMIDS.

Following the manipulations described, the HSV-1 (17) gC gene was again cloned into the plasmid pSC11, to generate vectors (designated pMCV plasmids) for the purpose of transfecting the gene into the vaccinia virus genome. Bacteriophage pLEM1 (ds) DNA was digested with restriction enzyme PvuII, which produced a blunt-ended, 1.85kb fragment

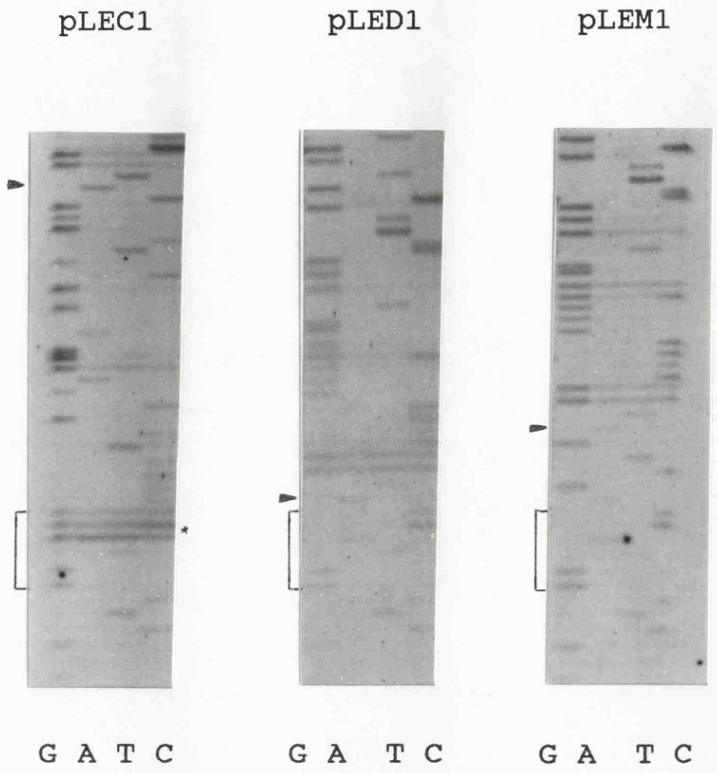


Figure 4.5. Comparison of nucleotide sequences upstream of the HSV-1 gC initiation codon, prior to, and following mutagenesis reactions with primers P-MUT1 and P-MUT2. Single-stranded DNA preparations of pLEC1, pLED1 and pLEM1 were sequenced using Sequenase Version 2.0. Tracks read G-A-T-C from left to right. Boxed region indicates vector KpnI recognition sequence. Arrow indicates A residue of gC initiation codon ATG. Asterisk indicates areas of compression, due to GC-rich regions of template.

containing the HSV-1 gC gene. Digestion with this enzyme therefore removed the need for Klenow treatment of DNA fragments, as the blunted ends allow direct cloning into the pSC11 SmaI site. The enzyme PvuII generates a fragment which contains only 3 nucleotides upstream of the gC initiation codon.

The 1.85kb fragment was excised from LMP agarose and 80µl (approximately 0.25µg) were ligated into prepared pSC11 DNA (SmaI/BAP; approximately 0.2µg). Competent cells were transformed with the ligation mix and grown on agar plates containing ampicillin. A total of 288 colonies were picked from the agar plates to screen for the presence of gC DNA in the plasmids which had transformed the bacteria. Colonies were grown on nylon membranes (supported on agar plates) and were probed with radiolabelled DNA containing the gC gene (the 1.85kb fragment used in the above ligation reaction). The resulting autoradiograph is shown in Figure 4.6. A control membrane was also included, containing 20ng samples of pGCH1 (top left), pLEM1 (top right), pUC18 (centre), pSC11 (bottom left) and M13mp19 (bottom right). Background hybridisation was observed with the negative controls, probably due to the presence of vector DNA regions within the fragment used for the probe. Positive controls were more strongly hybridising than negative controls and six colonies hybridised more strongly to the probe than others. These six colonies were chosen for restriction enzyme analysis and corresponded to plate positions of

Plate A : C7, H2
Plate B : B8, C8
Plate C : E1, F1

Figure 4.7. shows an agarose gel separation of DNA fragments obtained by BamHI digestion of these 6 plasmids and of pSC11. Vector plasmid pSC11 (lane g) gave rise to DNA fragments of approximately 4.4kb, 3.0kb and 0.4kb. An additional DNA fragment of approximately 1.8kb was observed with three of the above plasmids (lanes c, d and f).

Plate A

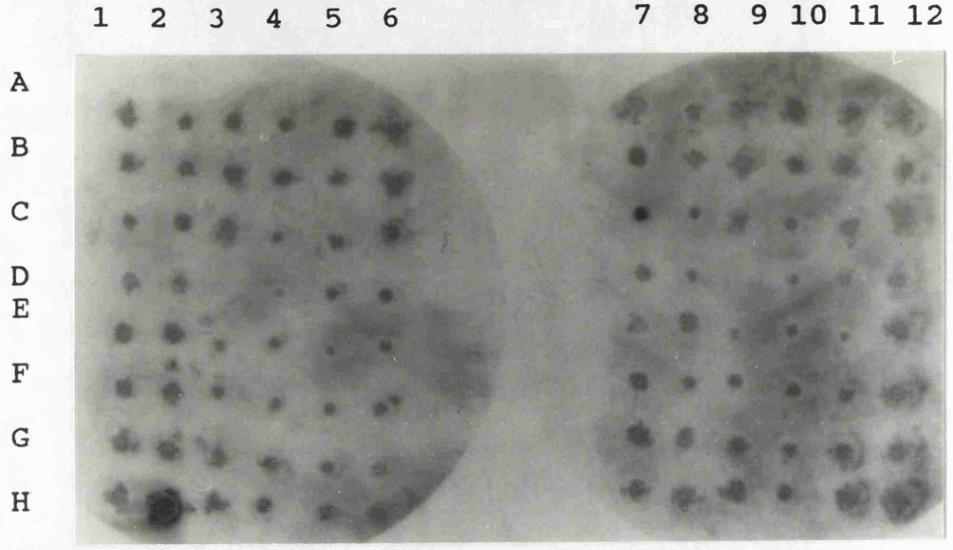
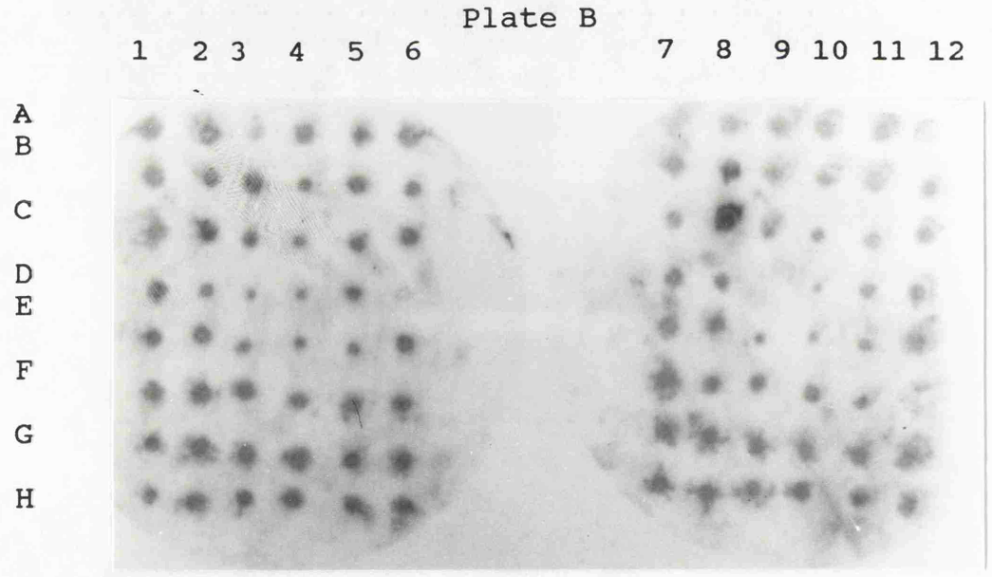


Plate B



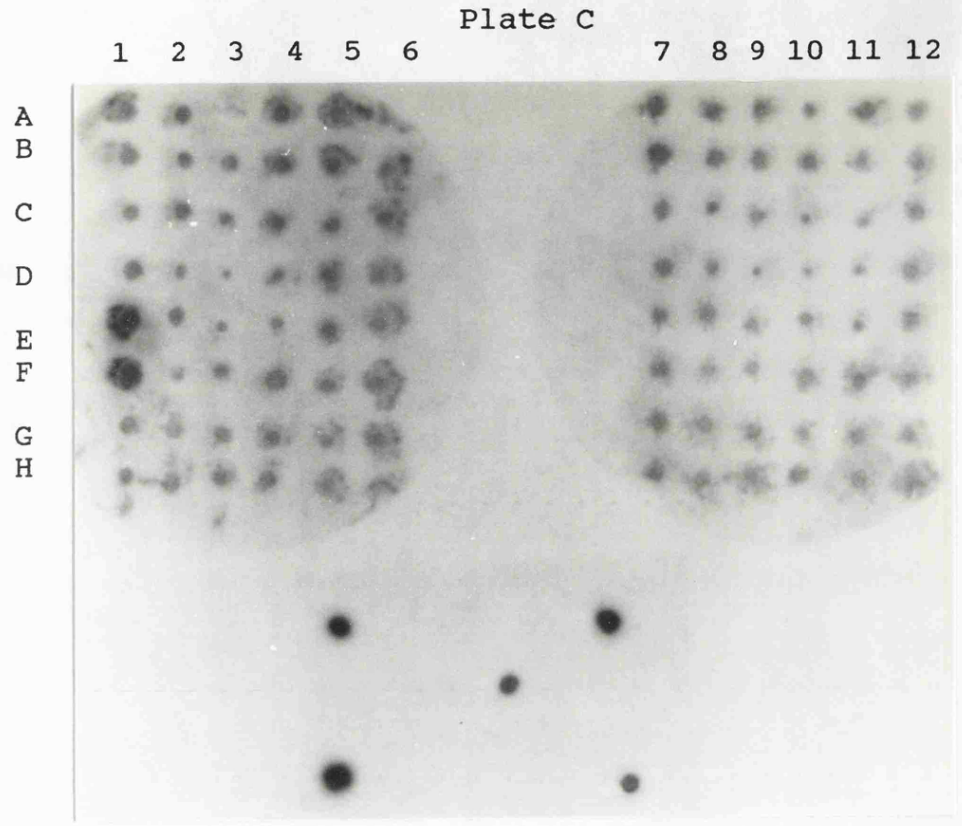


Figure 4.6. Autoradiograph of colonies transformed with ligation reaction between the 1.85kb PvuII (gC-containing) DNA fragment from pLEM1 and SmaI-digested pSC11 plasmid. Colonies were grown on nylon membranes and probed with radiolabelled 1.75kb fragment, containing the HSV-1 gC gene, excised from pAT153/KpnID with NheI and SphI. Three 96-well plates (A, B and C) are represented, each of 8 rows (A to H) and 12 columns (1 to 12). Square control membrane consists of 20ng samples of pGCH1 (top left), pGCV2 (top right), pSC11 (centre), pLEM1 (bottom left) and M13mp19 (bottom right).

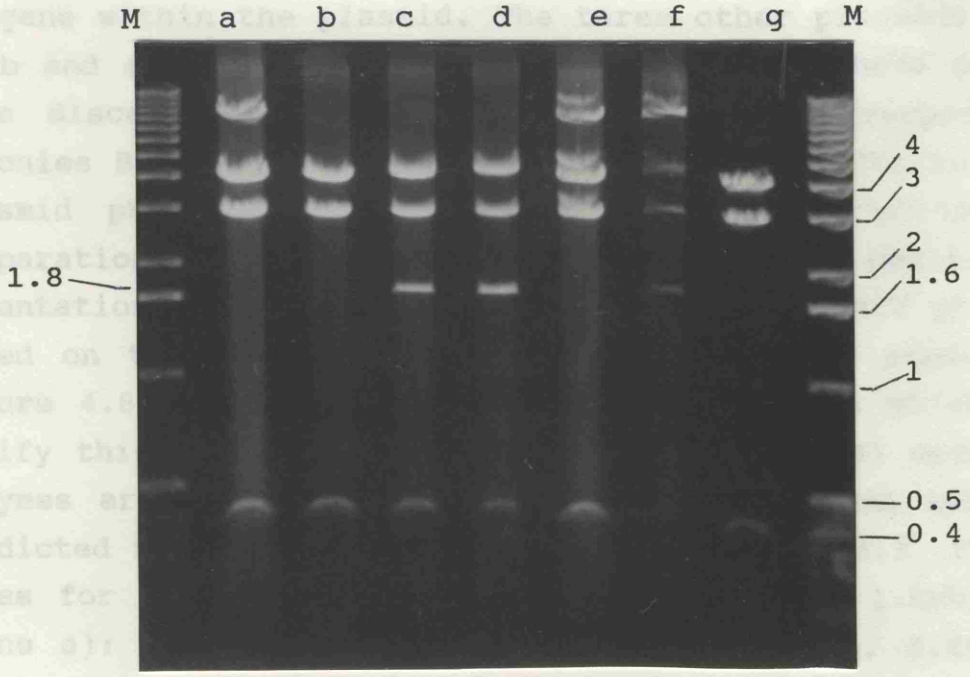


Figure 4.7. Agarose gel separation of BamHI plasmid digests. Small scale preparations were derived from colonies (Figure 4.6.) which hybridised to a radiolabelled probe containing HSV-1 gC sequences (lanes a to f). Parental plasmid pSC11 was also digested with xBaI (lane g). Molecular weights are in kb. Markers (M) were 1kb ladder (Appendix 4).

Restriction enzyme data for pSC11 and gC sequeneces indicated the fragment sizes represent correctly orientated gC gene within the plasmid. The three other plasmids (lanes a, b and e) indicated parental DNA only and these plasmids were discarded. The recombinant plasmids corresponded to colonies B8, C8 and F1, and were designated pMCV1 to pMCV3. Plasmid pMCV1 was used for a large-scale, CsCl-purified preparation (DNA concentration 1.5mg/ml). The position and orientation of the HSV-1 gC gene within the pMCV plasmids, based on the results of the BamHI digest, is depicted in Figure 4.8., including other restriction sites which would verify this. Restriction enzyme digests of pMCV1 with these enzymes are shown in Figure 4.9. and correspond well with predicted fragment sizes illustrated in Figure 4.8. Fragment sizes for pMCV1 with BamHI were 4.3kb, 3.0kb, 1.8kb, 0.4kb (lane a); with EcoRI were 4.0kb, 3.0kb, 1.5kb, 0.85kb and 0.4kb (lane c); and with XbaI were 3.8-4.0kb, and 1.73kb (lane e). The corresponding fragment sizes for pSC11 with the same enzymes were 4.3kb, 3.0kb, 0.4kb (lane b); 4.3kb, 3.0kb, 0.4kb (lane d) and 3.9-4.0kb, (lane f).

4.4. GENERATION OF RECOMBINANT VMC-VIRUSES.

The generation of recombinant vaccinia viruses containing the HSV-1 gC gene was carried out as described previously. Viruses generated by transfection with plasmid pMCV1 were designated the prefix VMC. The virus harvested from pMCV1-transfected monolayers was titred at 9.875×10^6 pfu/ml and the proportion of recombinant viruses within this virus stock (determined by blue plaques) was 0.11%. Three rounds of plaque purification using X-gal to select for ß-galactosidase function gave rise to 12 recombinant plaques, designated as below.

VMC241	VMC242	VMC243	VMC244
VMC245	VMC246	VMC247	VMC248
VMC249	VMC301	VMC302	VMC303

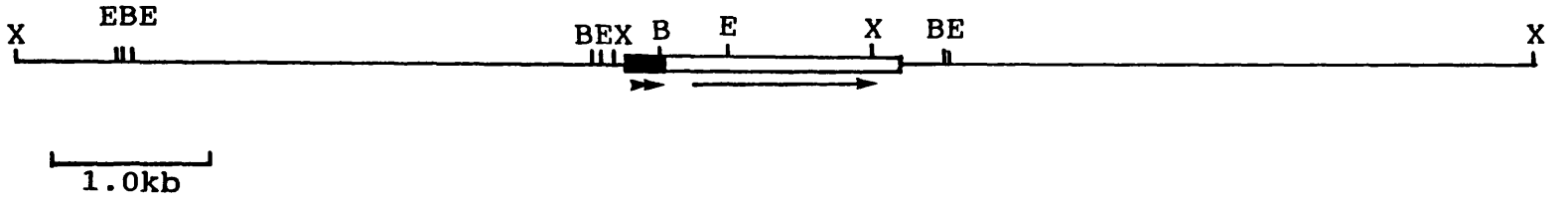


Figure 4.8. Linear representation of pMCV plasmids. The position of the cloned HSV-1 gC sequences (open box) and the transcriptional orientation (single arrow) are indicated with respect to the P7.5 promoter (closed box; double arrow). Restriction enzyme sites indicated are BamHI (B), EcoRI (E) and XbaI (X). Derived from information within Figures 3.1. and 4.7. and from pSC11 restriction maps.

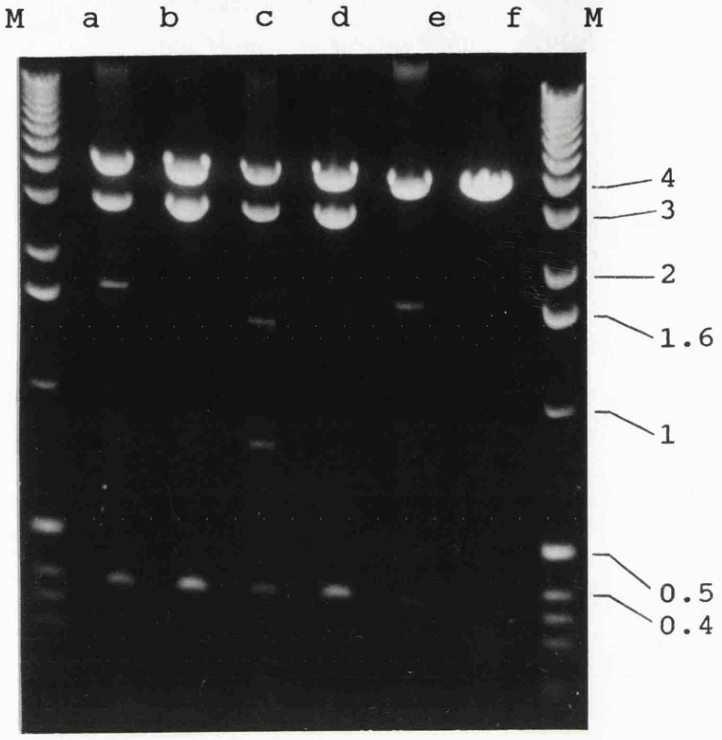


Figure 4.9. Agarose gel separation of restriction digests of plasmids pMCV1 and pSC11. Purified preparations of pMCV1 (lanes a, c and e) and pSC11 (lanes b, d and f) were digested with BamHI (lanes a and b), EcoRI (lanes c and d) or with XbaI (lanes e and f). Molecular weight markers were 1kb (Appendix 4).

4.4.1. Detection of HSV-1 gC DNA in Recombinant Viruses.

DNA was extracted from cells infected with recombinant (VMC- or VpS141) or wild-type vaccinia viruses and digested with HindIII for 2 hours. Plasmids pMCV1 and pSC11 were digested with BamHI as controls (5ng each). DNA samples were separated by agarose gel electrophoresis, transferred onto nylon membrane and probed as described previously. In an attempt to eliminate background levels of hybridisation observed in earlier probing experiments, a smaller DNA fragment was used for generation of the probe. The fragment consisted of gC sequences within a 0.9kb fragment, from an EcoRI/EcoRV digest of pGCH1 (see Figure 3.1.). The smaller size of this fragment meant an improved separation of DNA samples within an agarose gel, and hence a lower probability of containing contaminating vector DNA fragments.

Figure 4.10. shows hybridisation of this probe to the 1.8kb fragment generated from a BamHI digest of pMCV1 (lane o), but no hybridisation is observed with BamHI-digested pSC11 (lane p). DNA preparations from cells infected with all 12 recombinant VMC-viruses (lanes a to l) showed hybridisation to the probe, within a fragment of approximately 12kb in size. Cells infected with with VpS141 (lane m) or with wild-type vaccinia (lane n) showed no distinct hybridisation to the probe, other than a faint smear with no distinct features. As before, the size of the DNA fragment within recombinant-infected cells to which the probe hybridised indicated the HSV-1 gC gene had probably recombined into the virus thymidine kinase gene (HindIII J fragment) as desired. Viruses VMC241 and VMC303 (lanes a and l) were chosen for analysis of HSV-1 gC expression within infected cells.

4.4.2. Expression of gC by Recombinant Viruses.

Cells were infected with plaque-purified recombinant viruses, wild-type vaccinia, with HSV-1 or with PBS (mock-infected). After 24 hours incubation, the monolayers were fixed onto glass coverslips with acetone, and glycoprotein expression was detected by indirect immunofluorescence as

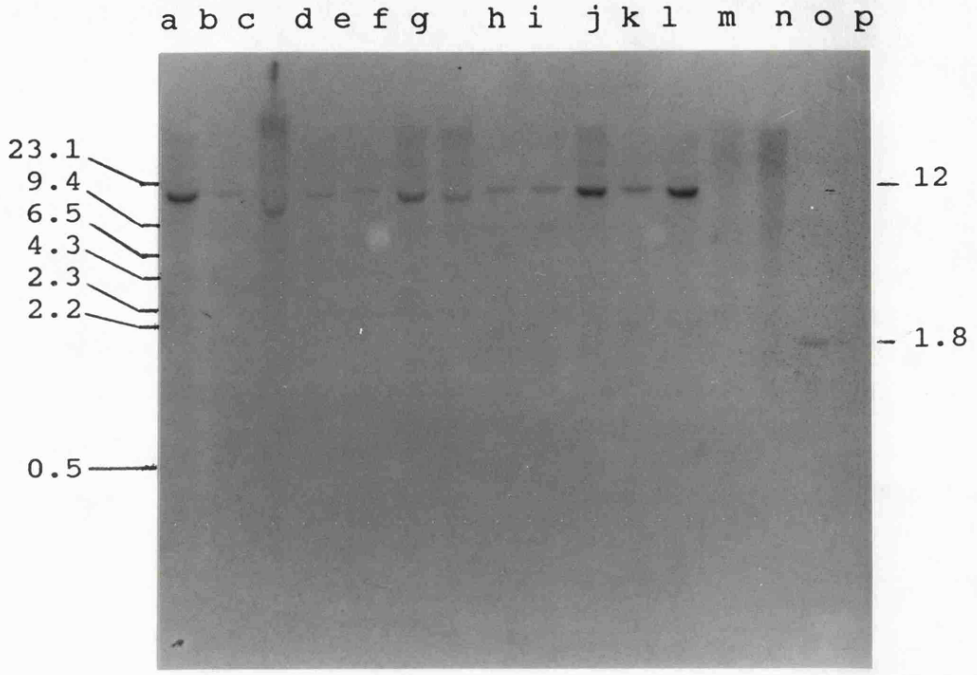


Figure 4.10. Autoradiograph of infected cellular DNA preparations probed with radiolabelled gC DNA sequences. Cells were infected with recombinant viruses VMC241 to VMC249 and VMC301 to VMC303 (lanes a to l). Control cells were infected with VpS141 (lane m) or wild-type vaccinia (lane n). Plasmid controls were 5ng each of BamHI-digested pMCV1 (lane o) or pSC11 (lane p). DNA samples were transferred onto a nylon membrane and probed with radiolabelled 0.9kb DNA fragment (containing gC sequences) derived from an EcoRI/EcoRV digest of plasmid pGCH1. Molecular weights are in kb. Markers were HindIII digested lambda DNA (Appendix 4).

decribed in Materials and Methods. Treated monolayers were viewed under UV-illumination and photographed using ASA 400 high-speed film (Figure 4.11.). Cells infected with HSV-1 (a) developed a rounded appearance, frequently clumped together and demonstrated bright fluorescence as a positive control. The fixative used (acetone) permeabilised cells and allowed antibodies to penetrate the plasma membrane. As a result, cytoplasmic and nuclear membrane staining was observed. Cells infected with VMC241 and VMC303 (b and c, respectively) retained their shape and did not clump together. The infected cells demonstrated immunofluorescence on the nuclear membrane and within the cytoplasm. HSV-infected cells demonstrated a more granular and brighter flourescence than VMC-infected cells. This may be accounted for by the presence of glycoproteins gE and gI, which form a receptor for the Fc region of IgG molecules within HSV-1-infected cells (see Introduction 1.6.4.). This was not present within recombinant vaccinia virus-infected cells. Cells infected with VpS141 (d), with wild-type vaccinia (e) or mock-infected cells (f) did not show fluorescence.

4.5. IMMUNISING PROPERTIES OF VMC241.

Large-scale, purified stocks of viruses VMC241, VpS141, vaccinia (Lister) and HSV-1 (17) were prepared and titrated. Virus titres were as follows

VMC241 : 4.875×10^7 pfu/ml
VpC141 : 3.125×10^7 pfu/ml
Vaccinia : 1.375×10^9 pfu/ml
HSV-1 (17) : 1.750×10^8 pfu/ml

Groups of 30 BALB/c mice were inoculated by scarification at the base of the tail, with purified virus stocks or with PBS (mock-infection, 20μl volume). Virus inocula were one of the following

HSV-1 (17) : 1×10^5 pfu/mouse

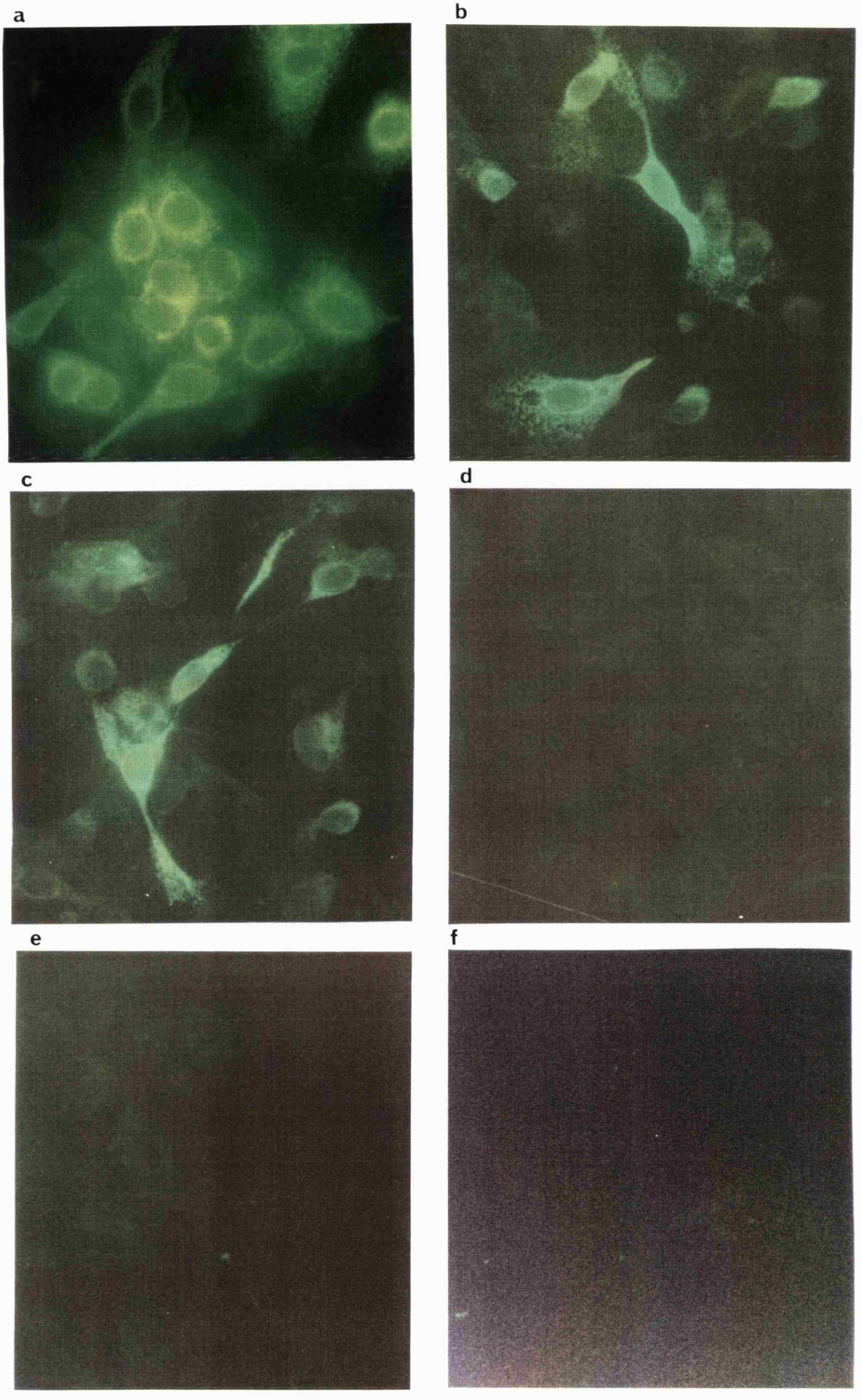

a
b
c
d
e
f

Figure 4.11. UV-immunofluorescence of infected cell monolayers. Cell monolayers (on glass coverslips) were infected with HSV-1 (a), VMC241 (b), VMC303 (c), VpS141 (d), wild-type vaccinia (e) or with PBS (f). Monolayers were fixed with acetone at 24 hours post infection and were probed with for expression of HSV-1 gC using anti-gC monoclonal antibody, followed by fluorescein-congugated anti-mouse IgG. Photographs were taken under UV-illumination at x40 magnification.

VMC241 : 1.5 x10^6 pfu/mouse

VpS141 : 1.5 x10^6 pfu/mouse

Vaccinia : 1.5 x10^6 pfu/mouse

Mice were boosted at 21 days with identical doses for all inocula except HSV-1, which were not given booster immunisations.

4.5.1. Effects of Immunisations on Mice.

All mice which received HSV-1 immunisation displayed a roughened coat from 3-5 days following virus administration. In most cases, no other signs of infection were observed, but some mice suffered to a greater degree and developed more obviously roughened fur and a slightly hunched stance. All but 2 mice recovered to apparent full health within 21 days of inoculation. The 2 mice in question developed puffed eyes, an indifference to handling and died within the 21 days. Mice receiving all other immunisations (VMC241, VpS141, Vaccinia Lister or PBS) showed no signs of ill health throughout the 28 days prior to challenge.

4.5.2. Immunoreactivity of Immunised Mouse Sera.

Five mice from each group were sacrificed on days 7, 14, 21 and 28 and bled by cardiac puncture. Sera from each group of mice were pooled and used to determine HSV-neutralising antibody titres stimulated by each inoculation. Figure 4.12. indicates the neutralision titres for each group of pooled sera (not heat inactivated) over the course of the experiment. The neutralisation titre was taken as that dilution of the serum which caused a 50% plaque reduction of HSV-1 (17) within a cell monolayer. For mice immunised with HSV-1, the mean titre rose very rapidly to 1/64 at just 7 days post inoculation, peaking at 14 days at greater than 1/256 (the limits of the test). This level was maintained to 21 days, then began to decline to a level of 64 at 28 days. Mice immunised with VMC241 developed a mean neutralisation titre of 1/16, detected at 7 days which remained at this level to 21 days. Following the booster immunisation at 21 days, the titre rose to 1/32 by day 28. Mice immunised with

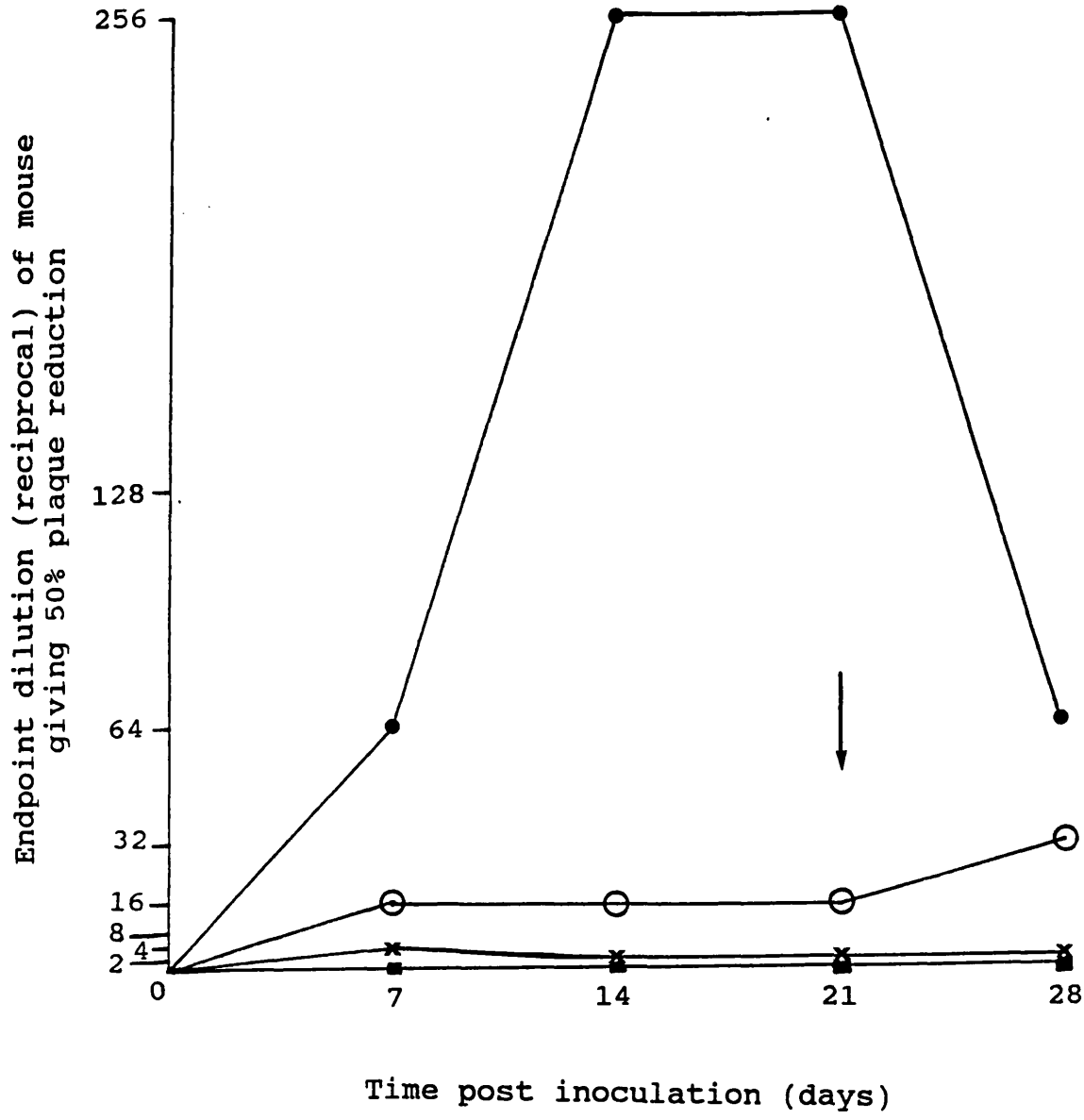


Figure 4.12. Neutralising antibody titres stimulated in mice against HSV-1 in immunised mice following immunisation. BALB/c mice were immunised at day 0 with HSV-1 (●), VMC241 (○), VpS141 (×), vaccinia (■) or with PBS (□). At days 7, 14, 21 and 28 post inoculation, sera from 5 mice were harvested by cardiac puncture, pooled and tested for neutralising activity against HSV-1 in a plaque-reduction assay. Neutralisation titres were calculated as the highest dilution at which sera could induce a 50% reduction in plaque numbers. Titres are expressed as the reciprocal of the endpoint dilutions. The arrow at day 21 indicates the time of a booster dose, give to all mice except those that had received HSV-1 immunisation at day 0.

recombinant VpS141 developed a mean neutralising antibody titre of 1/4 after 7 days, which dropped to 1/2 by day 14 and did not rise again even following booster. Immunisation with wild-type vaccinia or with PBS caused no detectable neutralising antibody development. Sera inactivated by heating to 56^{o}C showed no HSV-neutralisation, irrespective of the inoculation used. This would suggest the antibodies developed within immunised mice act in a complement-dependent manner, as heat inactivation destroys the complement proteins within the sera.

4.5.3. Protection from Lethal HSV-1 Challenge.

Following the immunisation of mice with the above preparations, mice were challenged at day 28 with a lethal dose ($1x10^{6}$ pfu/mouse; personal communication A. Minson, Cambridge) of HSV-1 by scarification as before. Mice were observed for 14 days following challenge and survival levels of each group determined (Table 4.1.). All mice which had received HSV-1 immunisations (total 8) remained free from signs of infection following lethal challenge. Mice which received VMC241 immunisations (total 10) displayed varying degrees of illness. They developed roughened fur, but whilst 5 mice showed no other signs and recovered by day 14, 5 mice developed a more severe infection and died. Similar signs (very poor coat, hunched stance, puffed eyes, indifference to handling) were also observed to preceed death in 9 out of 10 mice immunised with either wild-type vaccinia virus or PBS, and 8 out of 10 mice immunised with VpS141. Thus whilst VMC241 immunisations provided a 50% level of protection from lethal challenge, VpS141 gave only 20% protection and vaccinia and PBS were associated with 90% mortality.

Using Fisher's exact test, the levels of protection resulting from VMC241 immunisation is marginally significant (0.07). Using contingency tables, the sample size required to find a significant difference between VMC241 and the vaccinia virus control (significance level of <0.05; >0.01)

TABLE 4.1.

SURVIVAL OF IMMUNISED MICE TOWARDS LETHAL HSV-1 CHALLENGE.

Immunising agent	No. mice surviving No. mice infected	Percent survival	P-value [a]
HSV-1 (17)	8/8	100	0.00021
VMC241	5/10	50	0.070[b]
VpS141	2/10	20	NS
Vaccinia	1/10	10	NS
PBS	1/10	10	--

a. Calculated using Fisher's exact test, versus vaccinia control.
b. Marginally significant
NS. Not significant

would be between 25 and 30 mice. This assumes the figures within Table 4.1. represent an accurate estimate of the relative proprtions of positive and negative values.

CHAPTER 5

DISCUSSION

5.1. CLONING OF HSV-1 gC SEQUENCES.

The aim of this project was to clone the DNA sequence encoding the glycoprotein gC of HSV-1 strain 17 into the genome of a vaccinia virus, and obtain expression of the herpes glycoprotein during infections with the recombinant virus. Due to the non-infectious nature of the vaccinia virus genome, cloning was performed via an intermediary plasmid, pSC11, engineered specifically for this purpose. The plasmid contains vaccinia TK sequences, which permit homologous recombination with the viral genome (in infected cells), and a copy of the E. coli ß-galactosidase (lacZ) gene which provides a simple screening mechanism for recombinant viruses (Chakrabarti, et al., 1985). Both the lacZ gene and HSV-1 gC coding sequences are positioned such that their transcription is controlled by vaccinia virus promoters contained within the plasmid. This is essential for expression within recombinant vaccinia virus-infected cells, as the virus encodes it's own RNA polymerase which only recognizes vaccinia virus transcription signals.

Restriction enzyme maps of the HSV-1 genome indicated the glycoprotein C gene was contained entirely within the KpnId fragment, which was available within the plasmid vector pAT153 (Davison and Wilkie, 1983). DNA sequence data for HSV-1 (KOS) suggested the complete gC gene could be cloned within a 1.75kb fragment obtained from a dual digest of KpnId using restriction enzymes NheI and SphI (Figures 3.1. and 3.2.). Digestion with these enzymes gave rise to the expected fragment (Figure 3.3.), which was subsequently used for cloning of the gC coding sequence. The gene was cloned into pSC11 and also into pUC18. The purpose of using the second plasmid was to allow easier manipulations of the cloned DNA, as pUC18 replicates to a high copy number within bacterial cells (the plasmid can be amplified with chloramphenicol) and contains a multiple cloning site for easy excision of cloned DNA. For both plasmids, cloning was performed in the vector SmaI site, which generates blunt-ended fragments. The cohesive termini of the DNA fragment containing the gC gene generated by the enzymes NheI and

SphI were converted to blunt termini for ligation into the SmaI site using Klenow enzyme. Plasmids produced on insertion of gC into pUC18 were termed pGCH plasmids, whilst cloning of gC sequences into pSC11 gave rise to pGCV plasmids.

The orientation of the gC gene within pGCH plasmids was not important, although it was determined with respect to the enzyme sites within the multiple cloning region and is illustrated within Figure 3.5. The orientation of the cloned sequences within pGCV, however, was vital, as the gene was to be transcribed from a vaccinia virus promoter (the P7.5 promoter) contained within the pSC11 plasmid. The foreign gene is therefore required to be downstream of and in the same orientation as the vaccinia promoter. Verification that the cloned gC gene was in the correct orientation was by restriction digest and is illustrated in Figure 3.9. The recombinant plasmid pGCV2 was chosen for homologous recombination of the gC gene into the genome of vaccinia virus strain Lister.

Homologous recombination occurred between the vaccinia TK sequences contained within the plasmid and the viral genome within an infected cell monolayer. The transfected virus stocks were screened for inserted DNA by metabolism of X-gal by recombinant viruses, which now contained the lacZ gene (controlled by vaccinia P11 promoter regions) from the pSC11 plasmid. Blue colouration could be seen with the unaided eye, and low-power microscopy confirmed the presence of virus plaques within the cell monolayers at these points. Recombinant viruses were plaque purified and examined by hybridisation to radiolabelled gC DNA from the Nhe/Sph fragment. A dot-blot assay (Figure 3.11.), using cells infected with recombinant viruses indicated that 8 of the 9 plaque-purified virus stocks contained gC sequences, and hybridised to the probe. The 8 viruses were used to infect cells and DNA was extracted and analysed by Southern blot. Restriction of the cellular DNA preparations with HindIII, Southern blotting and subsequent probing revealed a gC-

containing DNA fragment of approximately 12kb in each recombinant virus (Figure 3.12.). The vaccinia TK gene, into which the cloned sequences were inserted, is contained within the HindIII J fragment of the virus genome (Weir, et al., 1982), which is approximately 5.0kb in size. Insertion of pSC11 sequences containing a 1.75kb Nhe/Sph fragment increased the size of the HindIII J fragment by approximately 7kb. The Southern blot therefore indicated the cloned gC sequences had entered the correct region of the virus genome.

The recombinant viruses were examined for their ability to synthesise HSV-1 gC during their replicative cycle within infected cells. Infected monolayers were fixed and probed by indirect immunofluorescence (IIF). Whilst HSV-1-infected cells showed positive fluorescence, no expression of HSV gC was detected in recombinant vaccinia virus-infected cells.

5.2. MANIPULATIONS OF CLONED SEQUENCES.

A communication with Dr. Richard Eberle, Oklahoma, USA, suggested that the failure of the recombinant viruses to express the HSV glycoprotein C may be due to the presence of non-coding sequences, situated upstream of the ATG initiation codon, within the 1.75kb fragment used for cloning of the gene. Challberg and Englund (1979) demonstrated that secondary structures within DNA templates dramatically reduce the efficacy of replication of such regions by the vaccinia virus DNA polymerase. It may be that the RNA polymerase enzymes of vaccinia are similarly susceptible to secondary structures. Certainly the gC gene of HSV-1, and the regions surrounding the gene are known to be rich in nucleotides G and C. As a result, these DNA regions may readily form fairly stable secondary structures which could account for the lack of gC expression from a recombinant vaccinia virus. In contrast to the gC gene, vaccinia promoters are AT-rich (Venkatesen, et al., 1981; Bertholet, et al., 1985; Mars and Beaud, 1987), which suggests these proposals may be relevant. Considering these

arguments, the non-coding sequences upstream of the gC gene were removed, and the gene re-cloned into the vaccinia genome.

Removal of non-coding sequences was performed via a 2-stage mutagenesis protocol. The HSV-1 sequences were excised from pGCH1 and cloned into the bacteriophage vector M13mp19 (Figures 4.1. and 4.2.). The gC sequence was determined and was found to be unaltered by the cloning strategies employed (Figure 4.4.). The sequence derived compared well with the published HSV-1 (17) gC sequence (obtained through the Genbank database), varying at only 2 amino acid positions. The upstream sequences prior to the coding region were then subject to two mutagenesis reactions which involved replacing the 34 nucleotides upstream of the ATG codon with a specific 5-nucleotide linker sequence (Figure 4.5.). The linker contained sites for two restriction enzymes, PflMI and PvuII, which would allow subsequent cloning of the gene at precise points upstream of the initation codon. The PvuII site was chosen for cloning into pSC11 as it generated blunted termini (removing the need for Klenow treatment to remove cohesive ends), and also as the M13mp19 vector contains convenient PvuII sites that allow excision with a single restriction enzyme digest. Excision of the gC gene with PvuII generated a 1.85kb fragment, containing the gC gene with only 3 non-coding nucleotides prior to the ATG codon.

Following the same procedures as before, the gC gene was cloned into pSC11, it's orientation with respect to the P7.5 vaccinia promoter verified (Figures 4.7. and 4.8.), and the recombinant plasmid (pMCV1) was used to recombine the gene into vaccinia virus genomes. Recombinant viruses were plaque-purified three times on the basis of ß-galactosidase function, and infected cells were used to prepare DNA samples for Southern blotting. HindIII-digested cellular DNA preparations probed with a radiolabelled gC DNA probe again demonstrated hybridisation to a fragment of approximately

12kb (Figure 4.10.). This suggested that as before, the HindIII J region of the virus genome had taken up the cloned sequences in their entirety.

5.3. EXPRESSION OF HSV-1 gC.

The recombinant viruses generated with manipulated gC sequences were examined for gC expression using IIF as before and were found to synthesise the glycoprotein within infected cells. As with HSV-infected cells the glycoprotein was found on the nuclear membrane and within the cytoplasm (Figure 4.11.). The ability of the monoclonal antibody to recognise gC expressed from a recombinant vaccinia virus, and the distribution of the glycoprotein within the cell (which correlates well with the distribution of fluorescence within HSV-infected cells) suggest the authenticity of the glycoprotein has been maintained within the recombinant vaccinia virus. Verification of the molecular weight of the expressed glycoprotein, either using Western blotting or immunoprecipitation would help to further confirm it's authenticity.

The ability of these recombinant viruses to express gC (whereas the previously constructed viruses failed to express the glycoprotein) may confirm the suggestion that the upstream sequences form secondary structures which inhibit vaccinia transcription activity. Certainly in this instance, removal of the upstream sequences released the block to gC expression from recombinant vaccinia viruses within infected cells. The expression of HSV-1 gC from recombinant vaccinia viruses has been notoriously difficult and a gC-expressing vaccinia has been elusive until recently despite the presence of numerous vaccinia viruses expressing other known HSV glycoproteins. The first successful gC expression in a vaccinia virus was reported by Weir and colleagues (1989a). The nucleotide sequences this group used to transfer the gC gene into the virus genome contained only 9 nucleotides upstream of the initiation codon. More recently, other successful vaccinia-gC constructs have been made (Allen et al., 1990). These included recombinants which

contained an additional 125bp of the gC promoter region between the vaccinia virus promoter and the coding sequence. This had the effect of decreasing the levels of gC expression, compared to recombinants containing the gene situated in closer proximity to the vaccinia promoter. These two reports would appear to confirm the suggestion by Dr. Eberle, that non-coding sequences upstream of the gC initiation codon have detrimental effects on expression of the glycoprotein within recombinant vaccinia viruses. Whether the block is indeed at the level of transcription and due to secondary structures, as the report of Challberg and Englund (1979) suggests, is yet to be investigated. It may be countered that the entire HSV-1 gC gene is rich in CG pairs, and yet the vaccinia RNA polymerase appears able to transcribe the sequence, as indicated by expression of the protein within the recombinant viruses described.

5.4. IMMUNOGENICITY OF CLONED gC.

The recombinant virus VMC241 was tested for it's immunogenic capacity within BALB/c mice. Mice were inoculated via the base of the tail with HSV, vaccinia recombinants VMC241 or VpS141, wild-type vaccinia or with a placebo of PBS. Following immunisation, mice which had received HSV showed signs of infection, (a roughened coat and, in some animals, a hunched stance). All but 2 mice recovered from their illness, and these 2 mice died. No such signs were observed in mice receiving other immunisations and no other mice died.

The immunisation of mice with HSV-1 (17) or with VMC241 both resulted in the production of anti-HSV neutralising antibodies (Figure 4.12.). The levels of antibody produced by the gC-expressing recombinant were lower than produced following HSV immunisations. This was to be expected, as the immune response to HSV involves a variety of viral antigens in addition to gC, which are not represented within VMC241. Mice immunised with recombinant VpS141 or wild-type vaccinia did not develop levels of HSV-neutralising antibodies above those developed by mice mock-immunised with PBS. At 14 days

post immunisation, the titres of neutralising antibody produced following VMC241 immunisation reached a plateau, and booster immunisations were given at 21 days to all mice except those which had received HSV as an immunisation. The levels of antibody attained within HSV-immunised mice were deemed to be sufficiently high at this stage (greater than 1/256) to provide protection and this was examined in the next stage of the experiment. The ability of VMC241 to generate anti-HSV neutralising antibodies suggested the virus was stable within the mice, and was capable of producing an authentic HSV-1 antigen (gC) within the animal, which was not produced by either VpS141 or wild-type vaccinia viruses.

Immunised mice were challenged with a normally lethal dose of HSV-1 (17), 28 days after the initial immunisation (7 days after the booster dose given to mice receiving recombinant and wild-type vaccinia viruses or placebo). All mice previously immunised with HSV remained free from any observed ill effects during the course of the challenge (14 days) and all eight mice survived. The majority of mice immunised with PBS, VpS141 or vaccinia wild-type showed severe signs of infection, including a roughened coat, puffed eyes, a hunched stance and lack of response to handling. In addition some developed lesions at the site of HSV infection. Of mice immunised with PBS or wild-type vaccinia, only 1 of the 10 mice survived challenge with mild signs of illness to day 14, and only 2 out of 10 mice immunised with VpS141 survived (Table 4.1.). Vaccination of mice with VMC241 appeared to protect against HSV challenge. The mice within this group displayed roughened coats, but only 5 of the 10 mice succumbed to severe illness and death. The 5 surviving mice appeared to recover well over the 14 days and their coats became much less roughened by day 14.

The degree of protection afforded by VMC241 was lower than observed with HSV immunisation, but was greater than that obtained with recombinant or wild-type vaccinia viruses which did not express the HSV glycoprotein C. As with the

levels of neutralising antibody developed, this was perhaps to be expected, as the recombinant virus expresses only one of the HSV antigens involved in the induction of immunity. The levels of protection obtained by the VMC241 recombinant virus within this project were also lower than the levels reported for the gC-expressing recombinant vaccinia virus used by Weir and colleagues (1989a). The virus used in that instance provided 90% protection against a lethal HSV challenge. However, Allen and colleagues (1990) reported variable levels of protection against HSV with gC-expressing recombinant viruses, based on clearance of the virus from the ear pinna. Although the recombinant virus VMC241 did not protect mice from lethal HSV-1 challenge as efficiently as previous exposure to HSV itself, the recombinant immunisation caused no observable illness in the mice. In contrast, HSV immunisation gave rise to signs of infection and even caused the death of two mice. The recombinant vaccinia virus would seem to be a less harmful immunogen than a sub-lethal dose of HSV. The results presented here agree with those of Weir, et al. (1989a) and Allen et al. (1990). The glycoprotein gC of HSV is capable of eliciting a protective immune response in mice. In addition, the recombinant vaccinia virus vector is capable of replicating within the mouse (with few adverse effects) and of presenting an immunogenic protein to the immune system of mice with protective results.

5.5. RECOMBINANT VACCINIA VIRUSES INDUCING HSV IMMUNITY.

Since the demonstration that vaccinia viruses were able to accept foreign genes into their genomes and express proteins from those cloned genes, the range of proteins studied has continued to grow at a great rate. Proteins from many viruses have been expressed in tissue culture, and in animals infected with recombinant vaccinia viruses. Several recombinant viruses have induced immune responses to the foreign proteins expressed and to the pathogen those proteins represent (see Introduction 1.9.). All the known HSV-1 glycoproteins have been expressed in vaccinia viruses (see Table 1.2.), and all but gH have induced immune

responses to HSV in infected animals (Cantin, et al., 1987; Sullivan and Smith, 1987, 1988; Weir, et al., 1987; Allen, et al., 1990; Blacklaws, et al., 1990).

T-cell responses are of great importance during virus infections and this has been shown to be particularly so for HSV infections (Introduction 1.7.3.). A vector system which stimulates these responses is therefore advantageous. Vaccinia virus infections are known to elicit both humoral and cellular immune responses in animals and in man (Koszinowski and Thomssen, 1975; Perrin, et al., 1977; Morishima, et al., 1978; Novembre, et al., 1985) and recombinant vaccinia viruses stimulate T-cell responses to the foreign proteins they express (Bennink, et al., 1984, 1986; Yewdell, et al., 1986). The HSV-1 glycoproteins gB, gC and gD, expressed by recombinant vaccinia viruses, have induced anti-HSV T-lymphocytes within mice (Zarling, et al., 1986b; Martin, et al., 1987; Martin and Rouse, 1987; McLaughlin-Taylor, et al., 1988; Weir, et al., 1989a; Allen, et al., 1990).

The responses induced by recombinant vaccinia viruses expressing HSV glycoproteins have given significant protection against HSV infections (Paoletti, et al., 1984; Cantin, et al., 1987; Weir, et al., 1989a; Allen, et al., 1990). In addition to protection against primary infections, there have been reports of recombinant vaccinia viruses expressing HSV glycoproteins gB or gD which have prevented the establishment of HSV latency and reduced the incidence of recrudescence in animals (Cremer, et al., 1985; Wachsman, et al., 1987; Blacklaws, et al., 1990). These reports underline the success of vaccinia virus as a eukaryotic expression vector for immunogenic proteins. The ability of these recombinants to protect animals suggests that they have potential for use as vaccines in man. However, there is a significant difference between herpesvirus infections in animal models and man and data obtained cannot be applied to humans. The outlook for a recombinant vaccinia virus vaccine for HSV (or for any other pathogen) is not without problems.

In a review of the subject, Kaplan (1989) points out several features of vaccinia virus which cast doubt on it's safety as a vaccine.

Several complications may arise following vaccinia virus infections, some of which are life-threatening. One such complication is generalised vaccinia, a rare occurrence in normal individuals, but more common in immunocompromised patients. In such cases, lesions fail to heal and further lesions may develop, there is a systemic infection and death often follows within approximately 6 months. Redfield and colleagues (1987) documented such a case in a patient identified as HIV-positive. Although the patient responded to treatment and recovered, he later died as a result of AIDS, which lead to the (unproven) suggestion that the vaccinia infection converted the patient from the asymptomatic stage of HIV infection into the AIDS stage of the infection. Other complications following vaccinia infections may include eczema, post-vaccinal encephalitis and carcinoma development.

The pathogenicity of the many strains of vaccinia virus varies widely in man, from the relatively highly pathogenic strains such as Copenhagen and Bern, through to the less pathogenic Equador and Lister strains (Kaplan, 1989). Several different strains have been used both for vaccination programmes against smallpox and for the construction of recombinant vaccinia viruses, making correlation of data difficult to assess in order to make a suitable choice for a recombinant vaccinia vaccine. The pathogenicity of vaccinia virus may be attenuated. Buller et al. (1985) and Taylor, et al. (1991) correlated the lower pathogenicity of recombinant vaccinia viruses, with respect to wild-type, with the loss of TK function. In both cases, however, infectious virus was recovered from the brains of mice, demonstrating the reduction in virulence does not necessarily prevent CNS invasion. Inactivation of other regions of the vaccinia genome also result in decreased pathogenicity of the virus, including the viral

haemagglutinin gene or the HindIII C region (Flexner, et al., 1987) and the virus growth factor (VGF) gene (Buller, et al., 1988). The expression of interleukin-2 (IL-2) by a recombinant virus also reduced the severity of vaccinia infection with respect to wild-type in mice (Flexner, et al., 1987).

There has been some debate on the subject of vaccine "take", in subjects which have previously encountered vaccinia. Immunisation should provide protection, thereby preventing replication of the same agent during a second encounter. Some of the adult population has been previously vaccinated with vaccinia virus, and the immunity induced by this inoculation must be overcome if a recombinant vaccinia vaccine expressing proteins from another pathogen is to be successful in these individuals. However, it would appear that both humoral and cellular immunity may be readily stimulated in adult volunteers receiving vaccinia virus 15-18 years after their first vaccination (Moller-Larsen, et al., 1978a+b). The speed with which these responses developed suggested immunological memory to the virus. Etlinger and colleagues (1991) were able to overcome the problems of prior exposure to vaccinia virus by additional challenges with recombinant viruses. Documented cases of accidental infection of human subjects with recombinant vaccinia viruses have resulted in signs of vaccinia infection (local swelling, restriction of movement at the site of infection, pock lesion or rash, and lymph node swelling) despite previous vaccination with wild-type virus (Jones, et al., 1987; Openshaw, et al., 1991). In both individuals, specific immune responses against both vaccinia and the foreign protein expressed were detected. These reports demonstrate not only the feasibility of vaccinia recombinants to induce immunity within pre-vaccinated individuals, but also that such recombinants are capable of stimulating immunity to foreign proteins within humans.

Poxviruses have very wide host ranges and are known to cross-infect different species (Kaplan, 1989). As demonstrated by the production of recombinant vaccinia viruses, and by the work of Gershon and colleagues (1989), poxviruses are also capable of recombination in the laboratory and within the wild. Therefore a potential hazard could arise from the spontaneous recombination between an engineered vaccinia virus vaccine and a naturally occurring poxvirus, resident in a non-target host accidentally infected with the vaccine. This could lead to the formation of a new virus, the pathogenicity of which may differ from (and conceivably be greater than) either parent virus.

5.6. FUTURE STUDIES.

Despite the problems outlined above, there remains the potential for recombinant vaccinia viruses to be used as vaccines. The continuation of this line of research, including the recombinant virus VMC241 constructed here, will undoubtedly contribute valuable information to the search for a successful human herpesvirus vaccine. The widespread benefits which will ultimately arise from such a vaccine (the removal of financial burdens on health services, reductions in patient suffering and distress, elimination of the need for antivirals which can cause selective mutations, etc.) justify the efforts required to overcome the problems encountered. A successful herpesvirus vaccine may also play a role in the prevention of cancers linked with herpesvirus infections. A recombinant vaccinia virus expressing the EBV glycoprotein gp340 has already been demonstrated to protect cottontop tamarins from EBV-induced malignant lymphomas (Morgan, et al., 1988). It would be of interest to examine whether recombinant viruses expressing HSV proteins could exert similar effects on the development of cervical carcinomas. It would be important to determine the ability of the VMC-construct to generate T-cell responses within vaccinated animals, considering the importance of such responses during HSV infections. In vitro proliferation and cytotoxic tests would give information about the T-cells induced. A series of recombinant viruses

could be constructed, expressing various regions of the gC protein, generated by deletion of the coding sequences within plasmid vectors prior to recombination. Such viruses could yield information as to the position of antibody and T-cell epitopes, and to the presence of any cross-reactive epitopes on the glycoprotein. These recombinant viruses may also be useful to investigate the role of gC in protecting the virus from complement-mediated immunity, linking the function with specific regions. This may in turn lead to some mechanism to overcome immune escape by the virus, perhaps by stimulating antibody to bind to the regions involved.

The recombinant virus VMC241 is probably most valuable for the opportunity it presents to study the glycoprotein gC itself. For some time the generation of such a recombinant virus eluded workers, possibly because of the curious nature of the glycoprotein. It appears that the regions surrounding the gC gene are unusual, as they prevent vaccinia virus expression of the protein, whilst the regions surrounding other HSV glycoproteins appear to present no such problems for the vector. It would be of value to establish if the block to expression were at the level of transcription or translation, and this may be verified by the study of RNA species synthesised during infection of cells by VgC- and VMC-recombinant viruses. The proposed functions of gC within virus attachment and fusion may be better understood through use of the virus by presenting the glycoprotein separate from other HSV proteins. The rate of entry of gC-negative HSV-1 strains into cells infected with recombinant (VMC) or wild-type vaccinia viruses may help to confirm or refute these roles for the glycoprotein. The suggestion by Friedman, et al. (1989) that gC is toxic to cells may also be investigated with this construct. The state of cell lines following infections with gC-expressing recombinants may give some indication as to the levels of gC which appear to be toxic. The effects of different levels of expression, and of variations in the time of expression within the infectious cycle, on toxicity and immunogenicity can be

studied. Recombinant vaccinia viruses containing the gC gene under the control of vaccinia promoter regions other than P7.5 may be constructed for such a purpose. It may be that expression of the glycoprotein from alternative promoters can improve expression levels and raise the degree of protection against HSV, and these constructs would also yield information on this. Recently, expression of the HIV gp160 was obtained at higher levels when under the influence of the P11 late promoter than when controlled by the P7.5 promoter (Klaniecki, et al., 1991). The choice of vaccinia promoter was shown to influence the immune responses against foreign proteins (Coupar, et al., 1986). This report demonstrated an early expression of protein was essential for the induction of CTL responses in mice against the protein.

The construction of a multiple vector, containing one or more HSV proteins in addition to gC would be an interesting advance. The immune response to any pathogen is developed towards many antigens, and the used of several HSV proteins together is likely to increase the levels of immunity. In addition, the recombinant virus may be used to vaccinate against both serotypes of HSV if, for instance, the gD protein was included. For early immune responses to HSV, the use of an immediate-early protein could be invaluable, as cellular immunity towards such proteins has been demonstrated for CMV (Reddehase, et al., 1986; Volkmer, et al., 1987; Koszinowsi, et al., 1988; Alp, et al., 1991) and for HSV (Martin, et al., 1988).

5.7. SUMMARY.

The DNA sequences encoding the glycoprotein gC of HSV-1 have been inserted into the genome of a vaccinia virus by homologous recombination. The resulting vaccinia virus expresses the HSV-1 gC during infection of cells and of mice, such that anti-HSV neutralising antibodies are developed by recombinant-immunised animals. These animals also demonstrated some protection against a lethal HSV

challenge, which was not observed in mice immunised with either wild-type vaccinia virus or with a recombinant vaccinia virus which did not possess the gC gene. In addition to further studies examining the immunity induced by vaccination with this construct, the recombinant virus can be viewed as a valuable tool for the study of gC, an unusual HSV glycoprotein which has yet to be fully characterised.

APPENDICES

APPENDIX 1 : BUFFERS AND SOLUTIONS

OLIGOLABELLING BUFFER.

Solution O.
1.25M Tris-HCl pH 8.0
0.125M $MgCl_2$
Stored at 4°C

Solution A.
1ml Solution O
18µl ß-mercaptoethanol
5µl 50mM dCTP, dGTP, dTTP in TE
Stored -20°C

Solution B.
2M HEPES, brought to pH 6.6 with 4M NaOH
Stored at 4°C.

Solution C.
90 OD units of hexaribonucleotides in TE
Stored at -20°C.

Oligolabeling Buffer (OLB).
Mix the three solutions in the ratio

A : B : C
50 : 125 : 75

Stored at -20°C.

20 x SSC.

3M NaCl
0.3M Na citrate

50 x DENHARDT'S SOLUTION.

1% BSA
1% Ficoll
1% polyvinyl pyrrolidine
Made up in 6 x SSC.

PREHYBRIDISATION SOLUTION.

50% formamide
5 x SSC
1 x Denhardt's solution

APPENDIX 2 : POLYACRYLAMIDE SEQUENCING GELS

40% Acrylamide Stock Solution.
38g acrylamide
2g N,N'-methylenebisacrylamide (bis-acrylamide)
Made up to 100ml with dH_2O, deionised with 10g Amberlite resin, 30 minutes and filtered.

10x TBE buffer.
108.99g Tris base
55.65g Boric acid
9.31g EDTA
Made up to 1 litre with dH_2O.

Sequencing gel mix.
75ml 40% acrylamide stock solution
230g urea
50ml 10x TBE buffer
Made up to 500ml with dH_2O, stored 4^oC.

6% Sequencing gel.
100ml Sequencing gel mix
600µl 10% ammonium persulphate (APS)
60µl N,N,N',N'-tetramethylethylenediamine (TEMED)

APPENDIX 3 : OLIGONUCLEOTIDE PRIMERS.

SEQUENCING PRIMERS.

The following set of primers were used to determine the sequence of the cloned HSV-1 (17) gC gene. Primers were synthesised at the Department of Genetics, University of Leicester. HSV-1 (KOS) gC gene sequence was used as a reference for the design of these primers (Frink, et al., 1983) and each primer anneals 200 bases downstream of the the previous primer.

P-HSV2 : 5′-C-G-G-G-T-C-A-G-C-C-G-C-C-A-G-3′
P-HSV3 : 5′-G-G-C-C-G-C-C-C-C-A-C-T-A-A-A-3′
P-HSV4 : 5′-C-A-C-C-G-C-C-C-C-A-C-C-C-G-G-3′
P-HSV5 : 5′-T-G-G-A-C-A-G-C-C-C-G-C-A-C-G-3′
P-HSV6 : 5′-A-C-G-C-A-G-A-C-G-C-A-C-G-A-G-3′
P-HSV7 : 5′-G-C-A-T-G-T-G-G-T-C-T-G-C-A-C-3′
P-HSV8 : 5′-C-C-G-G-G-A-T-T-C-C-C-G-T-T-C-3′
P-HSV9 : 5′-C-C-C-C-G-T-T-A-C-C-T-T-T-T-T-3′

MUTAGENESIS PRIMERS.

The following primers were used to remove upstream noncoding sequences from the cloned HSV-1 (17) gC gene and replace the sequence with a short run of bases to generate restriction endonuclease sites at the ATG start codon of the gene, for further cloning. Primers were synthesised by Dr. J. Kite, Department of Genetics, University of Leicester.

P-MUT1

5′ 3′
A-T-T-C-G-A-G-C-T-C-G-G-T-A-C-C-A-T-G-G-C-C-C-C-G-G-G-G-C-G

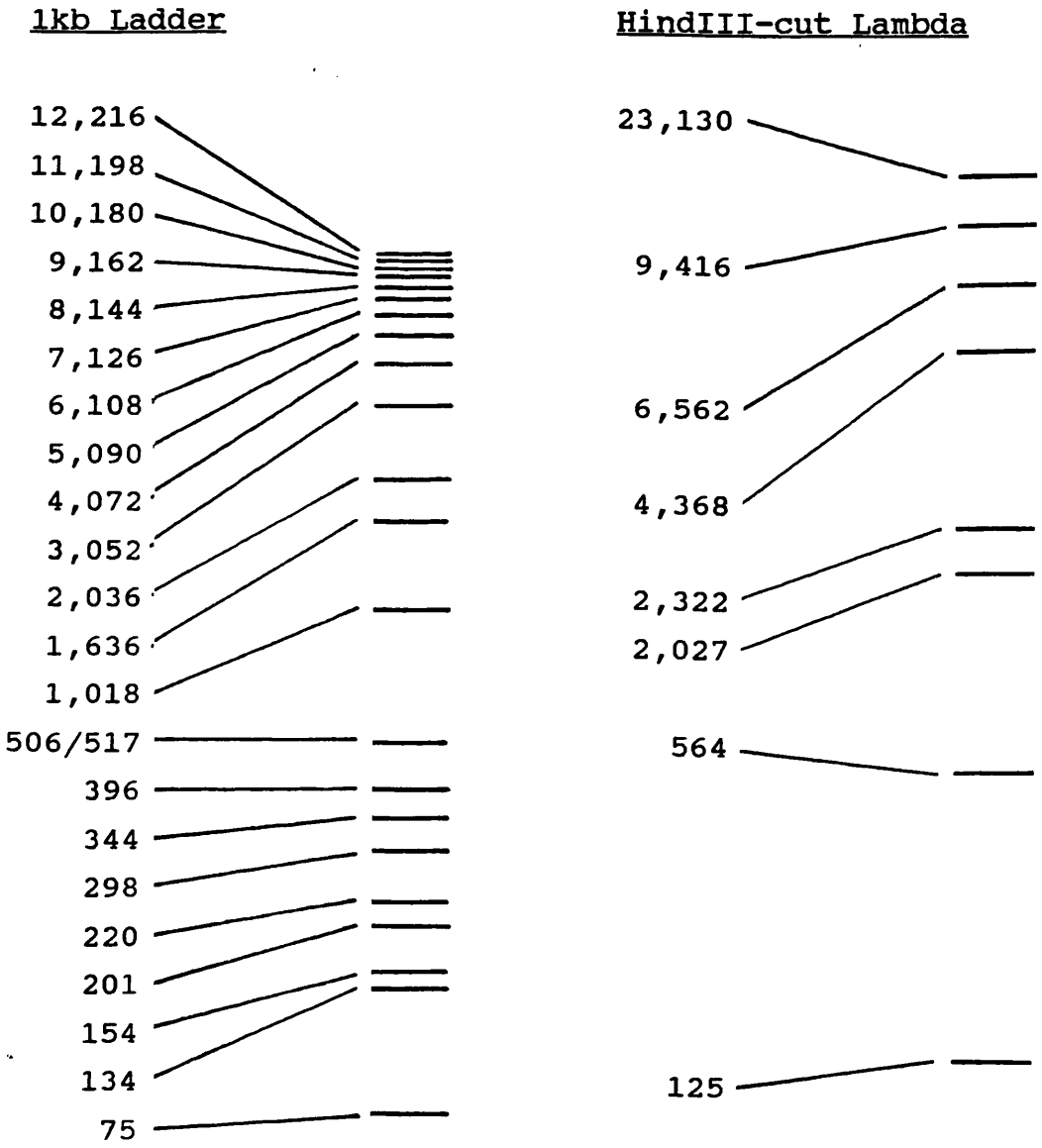
P-MUT2

5′ 3′
G-T-C-T-G-G-T-A-C-C-A-G-C-T-G-A-T-G-G-C-C-C-C-G-G

Emboldened text refers to PvuII recognition sequence.
Underlined text refers to PflMI recognition sequence.

APPENDIX 4 : DNA MOLECULAR WEIGHT MARKERS.

Molecular weight markers used for agarose gel separations of restriction digests were standard 1kb ladder, supplied by BRL. Markers used for Southern blot analyses was lanbda DNA digested with HindIII. The sizes of fragments obtained with each set of markers, and the expected pattern visible within agarose gels are indicated below. All sizes are in basepairs.



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