SEQUENCE ANALYSIS OF SELECTED REGIONS OF THE EQUINE HERPESVIRUS 4 GENOME

by

Marcello Pasquale Riggio, B.Sc.(Hons).

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, University of Glasgow.

Department of Veterinary Pathology,
University of Glasgow,
December 1990.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To my family

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I am most grateful to my supervisor, Professor David E. Onions, for his endless advice, guidance and support during the course of these studies.

Many thanks are due to Professor W.F.H. Jarrett for the use of the facilities in his department, the staff of the department for providing such a friendly and stimulating atmosphere in which to work, Mr. John Fuller for skilled artwork and Mr. Alan May for skilled photographic work. I would also like to thank Dr. Ann Cullinane for providing the EHV-4 genomic library and Dr. Anne Stokes for carrying out the immunological studies. Many thanks are also due to the Horserace Betting Levy Board and the Equine Virology Research Foundation for their generous financial support.

Finally, a big thank you to my parents and my wife, Aileen, for their never-ending support and encouragement and to my daughter, Gina-Marie, for the great fun and joy she brought to me during the preparation of this manuscript.

P R E F A C E

The work described in this thesis was carried out in the Department of Veterinary Pathology from April 1986 to April 1989, under the able supervision of Professor David E. Onions, BVSc, PhD, MRCVS.

These studies represent original work carried out by the author, except for the immunological studies which were carried out by Dr. Anne Stokes using material provided by the author, and have not been submitted in any form to any other university. Where use has been made of material provided by others, due acknowledgement has been made in the text.

December 1990.

Marcello Pasquale Riggio

SUMMARY

The gene encoding glycoprotein gB of EHV-4 strain 1942 was located within a BamHI genomic library by a combination of dot blot and Southern blot hybridisation using probes derived from the HSV-1 gB DNA sequence, and its DNA sequence determined. The major portion of the gene mapped to a 2.9kb fragment at the left-hand end of BamHI-C with the transcriptional control domains and start of the coding sequences of the gene located at the right-hand end of BamHI-M. The unprocessed EHV-4 gB protein was predicted to be 975 amino acids (110kDa) in size and composed of an unusually long amino-terminal signal sequence, a hydrophilic surface domain containing 11 potential N-linked glycosylation sites and 10 cysteine residues, a hydrophobic transmembrane domain thought to span the membrane three times and a highly charged carboxyl-terminal cytoplasmic anchor domain. A potential internal proteolytic cleavage site was identified near the centre of the protein. The EHV-4 gB protein demonstrated high levels of conservation of primary amino acid sequence with other herpesvirus gB-like proteins. Furthermore, the conservation of all cysteine residues and some N-linked glycosylation sites in the surface domain between the gB-like proteins suggested possible conservation of secondary and tertiary structures. The gB-like proteins may have a similar, conserved function across the herpesviruses.

With a view to evaluating EHV-4 gB as a potential candidate for development as a subunit vaccine, segments from the hydrophilic surface domain were expressed as fusion proteins using prokaryotic vector systems and fusion proteins

successfully purified. Three peptides from the EHV-4 gB amino acid sequence were also synthesised. Fusion proteins and peptides were examined for their ability to induce strong immune responses in hamsters. No peptide could stimulate the production of virus neutralising antibodies or confer protective immunity in hamsters. Similarly, fusion proteins could not stimulate the production of virus neutralising antibodies in hamsters although immunisation with one fusion protein protected one out of three animals from a lethal EHV-1 challenge. Furthermore, immunisation of hamsters with all three peptides induced lymphocytes to proliferate in response to whole virus antigen in vitro. These results suggested that EHV-4 gB may have a more important role in cell-mediated immunity than in humoral immunity.

The DNA sequence of the EHV-4 genome between 0.067 and 0.122 map units was determined. The EHV-4 genome was demonstrated to have an identical gene arrangement with the HSV-1 (I_L) and VZV genomes in this region and DNA sequence for genes analogous to HSV-1 UL51 to UL47 and VZV genes 7 to 11 was obtained. One of these EHV-4 genes is that for the unique glycoprotein gp10, although its gene product does not possess features characteristic of herpesvirus envelope glycoproteins. gp10 is a glycosylated non-envelope protein which probably resides in the virus tegument as does the analogous but unglycosylated protein specified by HSV-1 UL47. Additional DNA sequence data for BamHI-Q demonstrated that this genomic fragment encodes part of the major DNA binding protein of EHV-4.

Suitable approaches for the development of recombinant herpesvirus vaccines and the genetic relatedness between herpesvirus genomes are discussed.

A B B R E V I A T I O N S

HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
VZV	varicella-zoster virus
HCMV	human cytomegalovirus
EBV	Epstein-Barr virus
HHV-6	human herpesvirus 6
BHV-1	bovine herpesvirus 1
PRV	pseudorabies virus
EHV-1	equine herpesvirus 1 (equine herpesvirus 1, subtype 1)
EHV-2	equine herpesvirus 2 (equine cytomegalovirus)
EHV-3	equine herpesvirus 3
EHV-4	equine herpesvirus 4 (equine herpesvirus 1, subtype 2)
MDV	Marek's disease virus
HVT	herpesvirus of turkeys
CCV	channel catfish virus
HVS	herpesvirus saimiri
H. aotus-1	herpesvirus aotus type 1
H. aotus-2	herpesvirus aotus type 2
H. aotus-3	herpesvirus aotus type 3
H. ateles	herpesvirus ateles
BHV-4	bovine herpesvirus 4 (bovine cytomegalovirus)
AHV-1	alcelaphine herpesvirus 1
HV papio	herpesvirus papio
HV pan	herpesvirus pan
BHV-2	bovine herpesvirus 2 (bovine mammillitis virus)

FHV-1	feline herpesvirus 1
kb	kilobases
bp	base pairs
M	molar
IgG	immunoglobulin G
VSV	vesicular stomatitis virus
RER	rough endoplasmic reticulum
SRP	signal recognition protein
TK	thymidine kinase
UV	ultraviolet
kDa	kilodaltons
m.u.	map units
ORF	open reading frame
p.i.	post-infection
CTL	cytotoxic T lymphocyte
ADCC	antibody-dependent cellular cytotoxicity
CDL	complement-dependent antibody lysis
IE	immediate-early
ICP	infected-cell polypeptide
RK	rabbit kidney
PAA	phosphonoacetic acid
DIP	defective interfering particle
Amp^R	ampicillin resistant
Tet^R	tetracycline resistant
E.coli	Escherichia coli
dH_2O	distilled water
OD	optical density
λ	bacteriophage lambda
mar	monoclonal antibody-resistant

IPTG	isopropyl-β-D-thiogalactopyranoside
GST	glutathione-S-transferase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
dUTPase	deoxyuridine-5'-triphosphate nucleotidohydrolase
α-TIF	alpha-trans-inducing factor
BUdR	5-bromodeoxyuridine
ACV	acyclovir (9-[2-hydroxyethoxymethyl]guanine)
HPMPA	(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine

CHAPTER 1

General Introduction

THE FAMILY HERPESVIRIDAE

General Description

The herpesviruses are infectious agents which are widely distributed throughout the animal kingdom and infect many species including birds, fish, reptiles and mammals such as monkeys, apes and man. Over 80 different herpesviruses are known (Roizman, 1982) and more are continuing to be identified. They are genetically complex and possess large, linear, double-stranded DNA genomes with base pair compositions of 32 to 75% G+C containing of the order of 60 to 100 genes, and show considerable variation with respect to host range and biological properties. To date, six distinct human herpesviruses have been identified and characterised, namely herpes simplex virus types 1 (HSV-1) and 2 (HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and human herpesvirus-6 (HHV-6). Another human herpesvirus, HHV-7, has very recently been identified.

HSV-1, which is regarded as the prototype of the Herpesviridae, is the most extensively studied herpesvirus. HSV-1 and its closely related counterpart, HSV-2, are the causative agents of similar kinds of clinical syndromes. These include minor skin infections of the face (cold sores, blisters) and genital organs, more fatal complications in the exposed newborn and immunocompromised host, viral encephalitis and possible involvement in urogenital cancers (Spear and Roizman, 1980, and references therein). The common childhood ailment of chicken pox is a result of primary infection with VZV and infected individuals can suffer from painful shingles in later

life as a result of virus reactivation. Infection with HCMV is often subclinical, but can lead to mortality in foetuses and neonates and can cause serious problems in immunocompromised individuals such as those undergoing surgery or suffering from acquired immune deficiency syndrome (Stagno et al., 1983; Meyers, 1985). EBV immortalises human B lymphocytes and is the causative agent of infectious mononucleosis. It has also been associated with Burkitt's lymphoma and nasopharyngeal carcinoma (Epstein et al., 1964; Klein, 1979). HHV-6 has been isolated from patients with acquired immune deficiency syndrome and lymphoproliferative disease (Salahuddin et al., 1986; Tedder et al., 1987) and is capable of infecting B and T lymphocytic cell lines in addition to cells of neuronal origin (Ablashi et al., 1987).

Infection of animals with herpesviruses can cause serious diseases which often lead to large economic losses. Bovine herpesvirus 1 (BHV-1) gives rise to numerous clinical conditions in cattle such as rhinotracheitis, vulvovaginitis, conjuctivitis, abortion and fatal systemic infection in newborn calves (Ludwig, 1983). Pseudorabies virus (PRV) causes Aujeszky's disease in swine which can be lethal in young pigs (Gustafson, 1986). Equine herpesviruses 1 (EHV-1) and 4 (EHV-4) (previously known as EHV-1 subtypes 1 and 2, respectively) cause a number of diseases in the horse. Whilst both viruses are associated with severe respiratory illness, EHV-1 can also cause abortion, neonatal foal death and neurological disease (Campbell and Studdert, 1983; O'Callaghan et al., 1983; Allen and Bryans, 1986). Herpesviruses infecting birds include Marek's disease virus (MDV) (Churchill and Biggs, 1967) and herpesvirus of

turkeys (HVT) (Kawamura et al., 1969). MDV causes cytolytic infection of B and T cells and induces T cell lymphomas in chickens, whereas HVT is non-oncogenic, only mildly infectious and is not associated with any apparent disease (Payne, 1982). An example of a herpesvirus of fish is channel catfish virus (CCV) (Wolf and Darlington, 1971), whilst green iguana virus is one herpesvirus which infects reptiles (Zeigel and Clark, 1972). Herpesvirus saimiri (HVS) of the squirrel monkey (Melendez et al., 1968) is a well known herpesvirus that infects non-human primates.

The variable biological properties of the herpesviruses are such that infection can lead to fatal systemic illnes, localised infection or even no apparent symptoms in the host. However, one distinct property common to all herpesviruses is the ability to establish a latent infection. The state of latency is established after primary infection, in which the virus lies dormant in the host. Reactivation of latent virus leads to renewed episodes of disease and may occur on any number of occasions during the lifetime of the host, or not at all. Attempts to control herpesvirus infection by vaccination have been hindered by the latent nature of these viruses.

Structure of the Herpesvirion

All members of the Herpesviridae show a remarkably high degree of conservation of virion structure. The virion consists of four distinct structural elements: the core, capsid, tegument and envelope (for reviews see Wildy et al., 1960; Roizman and Furlong, 1974). A schematic representation of a herpesvirus virion is shown in Figure 1.1. The electron-opaque core contains

F I G U R E 1.1

Structure of the Herpesvirus Virion

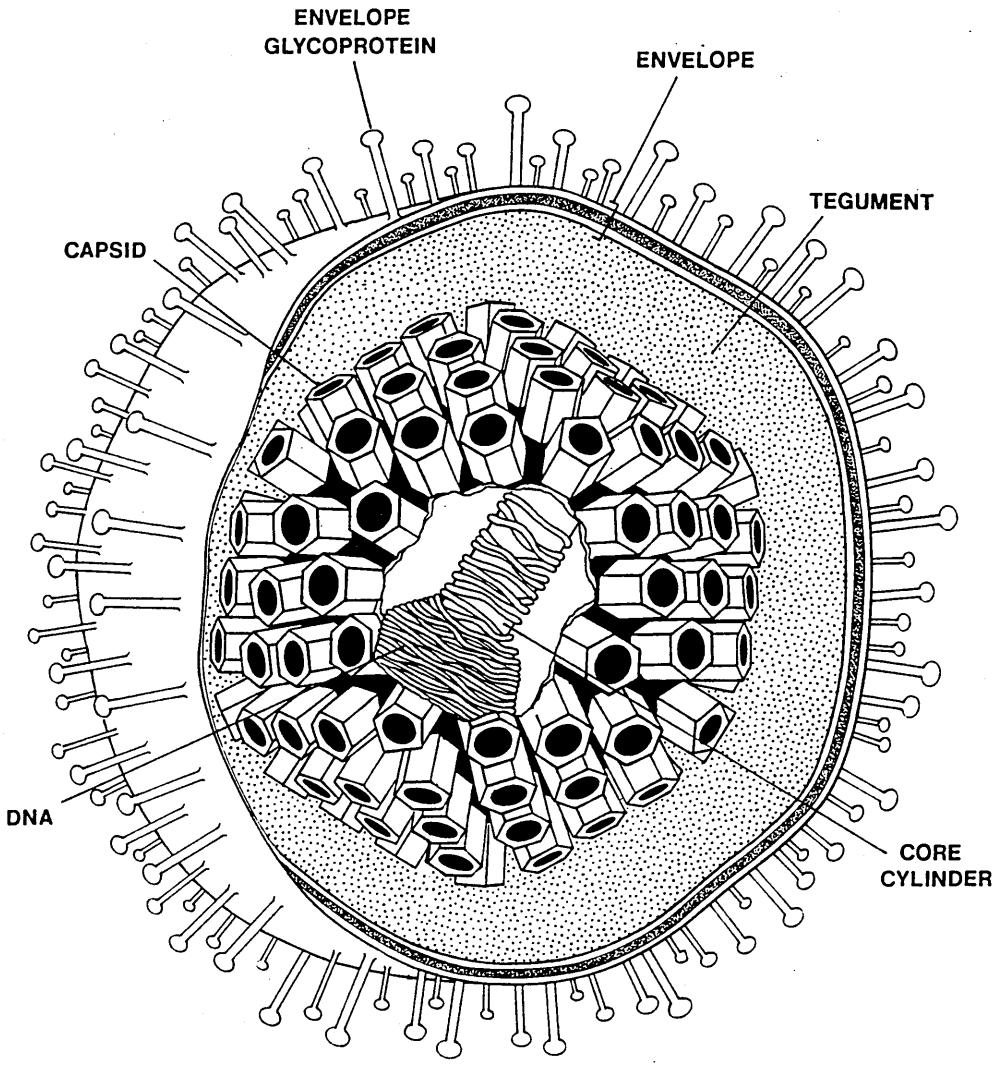


Figure 1.1 Schematic representation of the herpesvirus virion showing the main structural components (see text for details).

DNA wrapped around a fibrillar spool (core cylinder) (Furlong et al., 1972) and the ends of the fibres are attached to the underside of the capsid shell. The core is enclosed by the capsid, which has a diameter of 100 to 110nm and contains 12 pentameric and 150 hexameric capsomeres arranged in the form of an icosadeltahedron on the surface - these two features of capsid structure are especially typical of the herpesviruses. The hexameric capsomeres are approximately 12.5nm long and contain a hole 4nm in diameter which runs through the long axis of the structure. The tegument surrounds the capsid and contains globular material which is often asymmetrically distributed and variable in amount from virion to virion, even in the same cell. The outer envelope, which immediately surrounds the tegument, is a membrane composed of a lipid bilayer and contains a number of immunologically important glycoproteins which protrude as spikes from the outer surface. The overall diameter and volume of the virion depends on the diameter of the capsid and the volume of the tegument, which can be variable. In addition, discontinuities in the envelope may be present which tend to increase the overall diameter of the virion.

Classification

Newly isolated herpesviruses have readily been identified as such on the basis of morphology since their virions share structural features as described in the previous section. However, with the increasing number of herpesviruses that have been identified over the last 50 years, the need for a suitable subclassification system has become of paramount importance. Recent studies have demonstrated that they have undergone much

divergence during the course of their evolution in terms of antigenic and biological properties and the size, composition and arrangement of their double-stranded DNA genomes. Although the antigenic properties of virions can be a useful system for identifying closely related viruses, this approach cannot provide a firm foundation for subclassification. Due to their complexity, the herpesviruses are difficult to subclassify unequivocally. Despite such limitations, attempts have been made to divide them into subfamilies on the basis of biological properties and, to a lesser extent, genome structure.

Biological Properties as a Subclassification System

The Herpesviridae have been quite successfully divided into three subfamilies on the basis of biological properties, namely alpha-, beta- and gammaherpesviruses (Honess and Watson, 1977; Roizman et al., 1981; Roizman, 1982; Honess, 1984). The proposal of these subfamilies was based largely upon host range, characteristics of latent infection, length of replication cycle and cytopathology. Features of the Alphaherpesvirinae are that they can replicate in a wide variety of cells in vivo and in vitro and have a relatively short replication cycle (<18hr). They spread rapidly in tissue culture with mass destruction of infected cells and carrier cultures of susceptible cells harbouring non-defective genomes are difficult to establish. Common clinical manifestations include mild primary infection of the epithelial cells of the skin, genitalia, eyes and respiratory tract, giving rise to vesicular lesions, and they can also cause severe generalised infection in neonates and immunocompromised adults. They persist in a latent form

frequently, but not exclusively, in the neurones of ganglia serving the primary infected area (Wildy et al., 1982; Wildy, 1985). The Alphaherpesvirinae comprise two subgroups, α_1 and α_2, the members of which more closely resemble HSV and VZV, respectively. Members of the α_2 subgroup differ from those of the α_1 subgroup in that they replicate more slowly, have a narrow host cell range and usually remain cell-associated (Wildy, 1985). The vast majority of alphaherpesviruses belong to the α_1 subgroup and include HSV-1, HSV-2, PRV, BHV-1 and EHV-1, whilst the only members of the α_2 subgroup are VZV, Liverpool vervet monkey virus, patas monkey herpesvirus and simian varicella virus (Honess, 1984). The Betaherpesvirinae, or cytomegaloviruses, have a very narrow host range in vitro and in vivo (species-specific) and generally replicate best in host fibroblast cells in vitro. They have a relatively long replication cycle, infected cells may become enlarged (cytomegalia) and carrier cultures of infected cells are readily established. Infection in adults is usually subclinical but severe generalised disease can result in neonates or immunocompromised adults of their natural host. They persist in a latent form in the salivary gland epithelium, lymphoreticular cells, kidneys and other tissues. Members of this subfamily include HCMV, equine cytomegalovirus (EHV-2) and herpesvirus aotus types 1 and 3 (H. aotus-1 and -3). The Gammaherpesvirinae have a very narrow host cell range in vivo and are usually restricted to the same family or order as the host they naturally infect, and their replication cycle is variable in length. All members of this subfamily replicate in lymphoblastoid cells and some can cause lytic infection of

epithelial and fibroblast cells. They immortalise either B lymphocytes (γ_1 subgroup) or T lymphocytes (γ_2 subgroup) and some can cause lymphoproliferative disease in the host eg. HVS and MDV. Latent virus can usually be detected in lymphoid tissue of the host. Members of the γ_1 subgroup comprise EBV and related viruses of Old World monkeys and apes, whilst the γ_2 subgroup includes HVS and viruses of New World monkeys and lower vertebrates (Honess, 1984).

Genome Structure as a Subclassification System

The three main features of genome structure which can be considered when attempting to group herpesviruses are base composition (G+C content), genome size and the arrangement of reiterated sequences within the genome (Roizman, 1982). Grouping on the basis of G+C content is a rather arbitrary approach. However, the base composition of their genomes does show some correlation with the biological subgroupings described in the previous section. For instance, the 12 herpesviruses with a G+C content greater than 60% all belong to the alphaherpesvirus subfamily, the betaherpesviruses have a G+C content of 50 to 60% whilst the γ_2 herpesviruses typically have a G+C content of less than 50% (Honess, 1984). In addition, the γ_2 herpesviruses are characterised by possessing genomes of low G+C content in their coding sequences with a high G+C content in their repeat sequences. Despite the large variations in genome size (120 to 240 kilobases [kb]), the only information that can be gleaned from this is that the betaherpesviruses possess relatively large genomes. The only useful method for grouping on the basis of genome structure is by analysis of reiterated

sequences within the genome, which must consist of at least 100 base pairs (bp) (Roizman, 1982). Using this approach, five distinct groups, A to E, have been proposed. The genome structure of each group is shown diagrammatically in Figure 1.2.

i) Group A

Genomes in this group comprise a unique long sequence (U_L) which is bounded by a single reiteration of one set of sequences in the same orientation at both termini. The sole known member of the group is CCV (Chousterman et al., 1979).

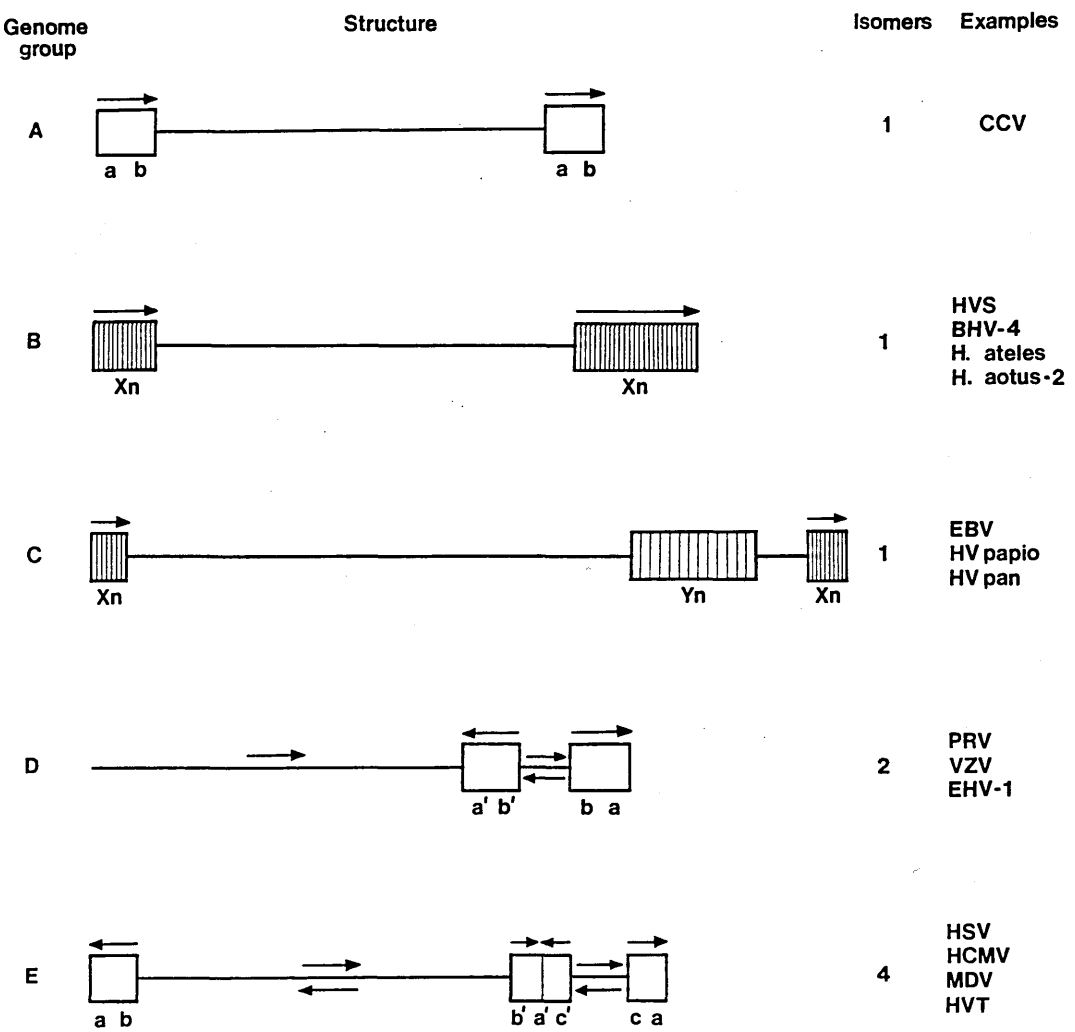
ii) Group B

Group B genomes are also terminally redundant, comprising a U_L sequence bounded by multiple reiterations of one set of sequences in the same orientation at both termini. Members of this group include the viruses of non-human primates HVS (Bornkamm et al., 1976), herpesvirus ateles (H. ateles) (Fleckenstein et al., 1978) and herpesvirus aotus type 2 (H. aotus-2) (Fuchs et al., 1985), and the viruses of non-primates bovine cytomegalovirus (BHV-4) (Ehlers et al., 1985), herpesvirus sylvilagus (Medveczky et al., 1989) and alcelaphine herpesvirus 1 (AHV-1) (Bridgen et al., 1989). Their genomes consist of an A+T-rich U_L sequence flanked by G+C-rich direct terminal repeats. The genomes of HVS and H. ateles are about 160kb and the direct terminal repeats, which account for about one-third of the genome, comprise a variable number of identical tandemly repeated sequences of 1444bp and 1600bp, respectively (Bornkamm et al., 1976; Fleckenstein et al., 1978; Fleckenstein and Desrosiers, 1982). The genome of H. aotus-2 is about 151kb and its direct terminal repeats comprise two repeat units of 2.3kb and 2.7kb which are homologous over at least 1.3kb of

Figure 1.2 Schematic diagram showing the arrangement of DNA sequences in the herpesvirus genomes of groups A, B, C, D and E. Unique sequences are represented by horizontal lines and their possible orientations are indicated by arrows. Single repeat sequences are shown as unfilled boxes and their orientation is indicated by arrows. The letters a, b, c and their complements a′, b′, c′ are also used to indicate the orientation of these repeat sequences. Multiple tandem repeat sequences (Xn, Yn) are shown as vertical lines. The use of Xn and Yn does not imply homology between the tandem repeat sequences of different herpesviruses. The number of isomers for each genome type and examples of herpesviruses that possess such genome structures are also indicated.

F I G U R E 1.2

Structure of Herpesvirus Genomes



their sequences, are tandemly arranged in a random order and represent 41kb of sequence (Fuchs et al., 1985). The genome of BHV-4 is about 144kb and contains polyrepetitive DNA segments composed of about 15 monomeric units of either 1.95kb, 2.35kb or 2.7kb which are present in a tandem orientation at both genomic termini and account for 20 to 25% of the genome (Ehlers et al., 1985). Herpesvirus sylvilagus has a genome comprising a U_L sequence of 120kb and direct terminal repeats containing tandemly arranged repeat units of 553bp which are variable in number (Medveczky et al., 1989). The genome of AHV-1 (isolate WC-11) possesses a 130kb U_L sequence and a further 30kb of tandemly arranged direct repeats. The major repeat unit is 950bp and is interspersed with a smaller number of related sequences of different lengths (Bridgen et al., 1989). Another AHV-1 isolate, C500, differs from isolate WC-11 in that only one type of repeat unit of 1050bp is present (Bridgen et al., 1989).

iii) Group C

Genomes in this group contain multiple reiterations of one set of sequences in the same orientation at both termini in addition to a variable number of internal tandem reiterations of different sequences. The best studied member of this group is the B lymphotropic virus of man, EBV, which possesses a genome of about 172kb in length. The direct terminal repeats are about 0.5kb and there are 4 to 8 copies at each terminus. The genome is divided into long (U_L) and short (U_S) unique regions by up to 12 internal direct sequence repeats of 3kb (Given and Kieff, 1979). The complete DNA sequence of the B95-8 strain of EBV has revealed the presence of many other repeat sequences distributed throughout the genome (Baer et al., 1984). The other members of

this group are the primate B lymphotropic viruses herpesvirus papio (HV papio) (Heller et al., 1981) and herpesvirus pan (HV pan) (Heller et al., 1982), whose genomes are similar in size and organisation to that of EBV.

iv) Group D

Examples of viruses possessing a group D genome are PRV, EHV-1, equine herpesvirus 3 (EHV-3), EHV-4, VZV and BHV-1. Their genomes comprise two covalently linked segments, L (long) and S (short). L is a unique sequence (U_L) and S consists of a unique sequence (U_S) bounded by inverted repeats, namely an internal repeat (IR_S) and a terminal repeat (TR_S). The L segment is in a fixed orientation but the S component can invert relative to L, thus giving rise to two isomeric forms of the genome.

Such a genome structure was deduced by electron microscopic studies of self-annealed single strands of the DNA of PRV (Stevely, 1977; Ben-Porat et al., 1979), EHV-1 (Ruyechan et al., 1982), EHV-3 (Atherton et al., 1982), EHV-4 (Cullinane et al., 1988) and VZV (Ecker and Hyman, 1982). Single strands of DNA reannealed to form a single-stranded loop (the U_S region) at one end of a double-stranded region (the reannealed repeats), with a long segment of single-stranded DNA (the U_L region) emerging from the other end of the double-stranded region. The existence of two isomeric forms of the genome was shown by cleavage of genomic DNA with a restriction endonuclease that cleaves only within U_L and U_S and not within the repeat sequences. Such analysis yielded four 0.5 molar (M) fragments (relative to the molarity of intact DNA) and revealed the presence of three terminal fragments of which two are present in 0.5M concentrations and are located at the ends of the S region,

whilst the other is present at a concentration of 1M. This indicated that the S segment can invert to give two terminal fragments, whilst the L segment is fixed and yields only one terminal fragment (Dumas et al., 1981; Henry et al., 1981; Whalley et al., 1981; Atherton et al., 1982). Variant strains of PRV have been reported whose genomes contain a sequence at the left end of the L component that has been translocated to the right end of the L component next to the inverted repeat, IR_S. Such strains have thus acquired a genome in which both the L and S components can invert relative to each other, giving rise to four isomeric forms (see Group E, below). This was first demonstrated with the Norden vaccine strain of PRV (Lomniczi et al., 1984a), and the nucleotide sequence of regions of the genome of three strains with an invertible L component that contain the translocated segment has been determined (Lu et al., 1989). It was concluded that the size of the translocated fragments is fairly similar (1.3kb to 1.4kb) and the translocation of such sequences is accompanied by the deletion of sequences from the right end of the L component which ranged from 0.8kb to 2.3kb. Similarly, the genome of VZV has been shown to contain an inverted repeat sequence of 88.5bp from the left end of the L segment which is also present at the L-S junction, and about 5% of virion DNA contains an invertible component (Davison, 1984). However, since the reiterated sequence is less than the 100bp minimum required for classification purposes (Roizman, 1982), the VZV genome remains classified as a group D member.

v) Group E

Group E genomes are exemplified by the extensively

characterised members of the Herpesviridae, HSV-1 and HSV-2. HSV DNA exists as two covalently linked components, L and S. L consists of a unique long sequence (U_L) bounded by inverted repeats (IR_L, TR_L) and S comprises a unique short sequence (U_S) bounded by inverted repeats (IR_S, TR_S). The L and S components can both invert relative to each other and DNA extracted from wild-type virions consists of a mixture of four equimolar isomers differing in the relative orientations of L and S, designated prototype (P), inversion of S (I_S), inversion of L (I_L) and inversion of both S and L (I_{SL}) components (Hayward et al., 1975; Roizman, 1979).

The elucidation of the genome structure was accomplished by electron microscopic studies of self-annealed single strands of HSV DNA (Sheldrick and Berthelot, 1975). Upon reannealing, single strands formed two single-stranded loops of unequal size (corresponding to U_L and U_S) bridged by a double-stranded region (the reannealed inverted repeats), resulting in a barbell structure. The reiterated sequences bracketing the L component (IR_L, TR_L) each account for about 6% of the total DNA. Similarly, reiterated sequences bracketing the S component (IR_S, TR_S) each represent 4.3% of the total DNA. Such conclusions were arrived at by measurement of the double-stranded regions formed by self-annealing of single strands and by partial denaturation studies of intact molecules (Wadsworth et al., 1975). That the L and S components can invert relative to each other was demonstrated by the use of restriction endonucleases that cleave only within U_L and U_S and not within the inverted repeats. Cleavage of the P isomer of HSV-1 DNA in this way (Roizman, 1979) produces three sets of fragments. The first set comprises

four terminal fragments, each present in 0.5M concentrations relative to the molarity of intact DNA. The second set consists of four fragments spanning the L-S junction which are each present at a 0.25M concentration, and the third set contains 1M fragments which are situated between the terminal and junction fragments. The generation of three sets of fragments in this way is consistent with the assortment of the L and S components at random relative to each other (Hayward et al., 1975).

Bovine mammillitis virus (BHV-2) was the first non-human herpesvirus shown to have a similar genome arrangement to HSV (Buchman and Roizman, 1978a,b). Other members of this group include HCMV (Weststrate et al., 1980), MDV and its closely related counterpart HVT (Cebrian et al., 1982), and the primate herpesviruses H. aotus-1 and -3 (Ebeling et al., 1983).

Limitations of Subclassification Methods

Although subclassification on the basis of biological properties is considered as the dominant criterion for the grouping of herpesviruses (Roizman, 1982) and the vast majority have been assigned to three such subgroups, there exist numerous herpesviruses which have not been assigned to any such subgroup eg. black stork herpesvirus (Kaleta et al., 1980). In a similar vein, the majority of herpesviruses have not been assigned to any of the groups A to E on the basis of genome structure eg. guinea pig cytomegalovirus (Nayak, 1971). Of particular significance is the fact that subclassification of some herpesviruses by biological properties has been directly contradicted by analysis of their genome structure. For example, whilst BHV-4 was originally classified as a betaherpesvirus

since it had a comparable pathobiology with the cytomegaloviruses of other species (Storz et al., 1984), analysis of its genome structure revealed that it has a similar arrangement to those of the γ_2 herpesviruses HVS, H. ateles and H. aotus-2 (Ehlers et al., 1985). MDV and the closely related HVT were originally classified as γ_2 herpesviruses mainly due to their tropism for lymphocytes (Roizman, 1982), but electron microscopic studies revealed that they possess a similar genome structure to HSV (Cebrian et al., 1982). Furthermore, the mapping and sequencing of numerous genes from these two viruses shows that they are more homologous to the alphaherpesviruses HSV and VZV than to the gammaherpesvirus EBV, and their genomes appear to be colinear with that of VZV at least within U_L (Buckmaster et al., 1988). Thus, as the DNA sequence and genome structure of more herpesviruses comes to light, more importance may be placed on genome structure and gene conservation as a suitable criterion for the subclassification of herpesviruses.

HERPESVIRUS GLYCOPROTEINS

General Description

The membrane glycoproteins play major roles in the early stages of viral infection and are important in determining the biological and pathogenic properties of the virus (for review see Spear, 1984). They are exposed on the surfaces of the virion envelope and membranes of infected cells and are targets for both humoral and cellular immune responses. They are involved in the recognition, adsorption and penetration of virus into susceptible host cells, mediate the cell-to-cell spread of infection by cell fusion and play a role in the envelopment and release of progeny virus from infected cells. Furthermore, glycoproteins are associated with the expression of receptors for the C3b component of complement and the Fc region of immunoglobulin G (IgG) by the virus-infected cells.

The following sections describe the basic structure of glycoproteins, review the advances made in the identification of the glycoproteins of HSV and describe their roles in the infectious process. Finally, glycoproteins specified by other herpesviruses and their relation to those of HSV is reviewed.

Glycoprotein Structure

The advent of recombinant DNA technology has brought about major advances in the determination of glycoprotein structure. Much information on the domains of virus glycoproteins has been obtained from the study of a number of non-herpesvirus membrane-bound glycoproteins, such as the G protein of vesicular stomatitis virus (VSV) (Schlesinger and Schlesinger, 1987).

Subsequent studies of the glycoproteins of HSV led to the conclusion that they are similar in structure and adopt an orientation in membranes similar to the VSV G protein.

Viral glycoproteins consist of four distinct domains: the signal sequence, surface, transmembrane and cytoplasmic anchor domains. The signal sequence, which is located at the amino-terminus of the molecule, plays a central role in the process of protein export as defined by the signal hypothesis (Blobel and Dobberstein, 1975). Most exported proteins, which include membrane-bound glycoproteins and secreted proteins, are synthesised by membrane-bound ribosomes on the rough endoplasmic reticulum (RER) and the signal sequence serves to attach the nascent peptide to the membranes of the RER. The signal sequence then initiates export of the growing protein chain across the membrane of the RER. Upon emergence of the nascent peptide from the ribosome, the polypeptide-ribosome complex interacts with a cytosolic nucleoprotein called the signal recognition protein (SRP), leading to a rapid decrease or even total arrest of translation (Walter and Blobel, 1981). After this structure is presented to the surface of the RER it associates with the SRP-receptor or 'docking protein' (Gilmore et al., 1982; Meyer et al., 1982), resulting in the displacement of the SRP from the polypeptide chain and thus facilitating the subsequent translocation of the protein across the membrane of the RER. During translocation the signal sequence interacts with at least two more membrane components, the signal sequence receptor (Wiedmann et al., 1987) and the signal peptidase (von Heijne, 1984). The signal peptidase cleaves the signal sequence at a specific site consisting of three amino acids. The mature

protein is then incorporated into the plasma membrane of the cell, whilst secreted proteins are released into the surrounding medium. Thus, the signal sequence serves to export the protein across the membranes of the RER and to insert the protein into the plasma membrane of the cell. The largest domain is the surface domain which is oriented such that it is exposed on the outer surface of the plasma membrane. This domain is hydrophilic and contains antigenic sites against which virus neutralising antibodies are directed. Additionally, sites at which both N-linked and O-linked oligosaccharides are added reside in this domain. Potential N-linked glycosylation sites are defined by the consensus sequence Asn-X-Ser/Thr (Hubbard and Ivatt, 1981) where X is any amino acid except proline, with oligosaccharides being added to the Asn residue of this motif. The hydrophobic transmembrane domain is located towards the carboxyl-terminus and spans the lipid bilayer, being oriented such that the small hydrophilic cytoplasmic domain at the carboxyl-terminus projects into the cytoplasm. The cytoplasmic domain is highly charged and anchors the glycoprotein into the membrane.

Glycoproteins Specified by HSV

The existence of four major, antigenically distinct glycoprotein species in the envelope of HSV-1 and the plasma membrane of HSV-1-infected cells has been known for some time (Spear, 1976; Baucke and Spear, 1979; Norrild, 1980). These species were designated gB, gC, gD and gE. More recently, the use of monoclonal antibody techniques and DNA sequence analysis has allowed the characterisation of a further three minor species, namely gG, gH and gI. Thus HSV-1 encodes at least seven

glycoproteins, and homologues of all these glycoproteins have been detected in HSV-2. The determination of the DNA sequence of HSV-1 has identified the genes which specify the glycoproteins mentioned above (McGeoch et al., 1985, 1988a). This analysis has identified another, as yet uncharacterised, potential glycoprotein which is encoded by gene US5 (McGeoch et al., 1985). The vast majority of work regarding HSV glycoprotein function has been carried out on HSV-1. The following description of individual glycoprotein function is therefore biased towards HSV-1, with occasional reference being made to their HSV-2 counterparts.

Functions of HSV Glycoproteins

Of the seven characterised glycoproteins of HSV, the structural genes for three of them are located within the U_L component of the genome (gB, gC and gH), whilst the remaining four species are encoded by the U_S region (gD, gE, gG and gI).

i) Glycoprotein B (gB)

gB is one of the most extensively characterised glycoproteins of HSV and the DNA sequence of the gene encoding gB has been determined for both HSV-1 (Bzik et al., 1984a; Pellett et al., 1985b) and HSV-2 (Bzik et al., 1986; Stuve et al., 1987). gB was the first glycoprotein shown to be required for virus infectivity (Sarmiento et al., 1979). That gB is indeed essential for viral entry and the production of infectious virus was shown by the study of the gB temperature-sensitive mutants tsB5 (Manservigi et al., 1977) and tsJ12 (Little et al., 1981). Such mutants replicate normally at 34°C but at 39°C virus produced is non-infectious due to inhibition

of virus penetration, and these mutants are unable to fully glycosylate the major gB precursor to the mature form (Manservigi et al., 1977; Sarmiento et al., 1979; Little et al., 1981). Since these non-infectious mutant viruses can bind to cells and treatment of cell-adsorbed mutant virus with the membrane-fusing agent polyethylene glycol greatly enhances virus infectivity (Sarmiento et al., 1979; Little et al., 1981), the defect in these mutants is in the membrane fusing activity required for viral penetration. Therefore, gB has a role in such a membrane-fusing activity required for viral entry.

The role of gB in cell fusion was studied using the tsB5 mutant virus which, in addition to being temperature-sensitive, is also syncytial (Manservigi et al., 1977). This ability to promote fusion of infected cell membranes is due to a syncytial mutation in the gB gene as mapped by DeLuca et al. (1982). tsB5 enters cells more rapidly at 30°C than does the wild-type KOS strain. The mutation responsible for this fast-entry phenotype also maps within the gB gene and is separate from the syncytial and temperature-sensitive mutations (DeLuca et al., 1982). DNA sequencing subsequently revealed that these phenotypes arise due to point mutations in the gB gene of tsB5, with the substitutions valine-to-alanine at residue 552 and arginine-to-histidine at residue 857 of the gB protein relative to KOS accounting for the fast-entry and syncytial phenotypes, respectively (Bzik et al., 1984b). This places the syncytial mutation in the cytoplasmic domain of the protein. In addition, the temperature-sensitive lesions for two mutants defective in accumulation of gB and viral growth, tsJ12 and tsJ20, are also the result of point mutations in the gB sequence, resulting in

an arginine-to-cysteine substitution at residue 376 for tsJ12 and a valine-to-isoleucine substitution at residue 272 for tsJ20 (Bzik et al., 1984a).

It has been shown by the use of gB-defective mutants which fail to synthesise gB as a transmembrane protein that gB is not essential for viral assembly and nucleocapsid envelopment (Cai et al., 1987).

ii) Glycoprotein C (gC)

Together with gB and gD, gC is a prominent and highly immunogenic antigen on the surface of virions and HSV-1-infected cells (Glorioso et al., 1983, 1984). The gC gene of HSV-1 (gC-1) has been mapped and sequenced (Frink et al., 1983) and its HSV-2 counterpart, gC-2, has also been mapped (Zezulak and Spear, 1984) and sequenced (Dowbenko and Lasky, 1984). A number of discrete functions have been assigned to gC. It is not essential for virus growth in tissue culture since a number of viral mutants that fail to produce gC-1 have emerged (Holland et al., 1983a, 1984b), and an HSV-2 mutant that fails to synthesise gC-2 has also been reported (Zezulak and Spear, 1984). An interesting observation regarding such mutants is that they are also associated with a syncytial phenotype and it has previously been suggested that the inability to express gC may be directly responsible for the syncytial phenotype (Manservigi et al., 1977). However, this was shown not to be the case for the strain HSV-1 (MP), when insertion of the gC-1 gene into the locus specifying the thymidine kinase (TK) gene resulted in the successful expression of gC-1 without altering the syncytial phenotype (Lee et al., 1982b). It thus seems likely that there exists some, as yet, undefined relationship between the gC-

negative and syncytial phenotypes. The fact that such mutant strains exhibit a syncytial plaque morphology suggests that gC may inhibit cell fusion, and it seems likely that gC influences virus infectivity and virulence by inhibiting gB-promoted cell fusion between host cell and virion membrane (Epstein et al., 1984) and between infected cells (Manservigi et al., 1977).

Endothelial cells infected with HSV-1 develop receptors for the C3b component of complement and it has been demonstrated that gC-1 does indeed form part of, or act as, such a receptor (Friedman et al., 1984). This study showed that a gC-negative mutant could not express C3b receptors and monoclonal antibodies against gC-1 were found to block C3b-binding activity. The mutant which failed to express gC-1, and therefore could not express C3b receptors, was shown to be viable, thus suggesting that receptor activity is not essential for HSV-1 replication in cell culture. It has recently been reported that the C3b receptor is expressed on cells transfected with the gC-1 and gC-2 genes (Seidel-Dugan et al., 1988) and that purified gC-1 and gC-2 can both bind complement component C3b (Eisenberg et al., 1987). It has been been demonstrated by the use of monoclonal antibodies directed against cell-derived gC-1 that the central segment of gC-1 is not involved in C3b binding (Huemer et al., 1989). All three monoclonal antibodies recognised sites in the central third of the molecule (which is hydrophobic and non-glycosylated) although none of them affected C3b binding to gC-1, which is a glycosylation-dependent process. Marlin et al. (1985) identified two major antigenic sites in gC-1 by using monoclonal antibodies specific for gC-1. Site I is thought to be located in the carboxyl-terminal half of the molecule, whilst

site II probably resides in the amino-terminal half which contains a clustering of sites for N-linked glycosylation. Monoclonal antibodies against sites I and II directly block C3b binding by gC-1 (Friedman et al., 1986). These independent studies suggested that only the amino- and carboxyl-terminal segments of gC-1 are involved in the binding of the C3b component of complement.

iii) Glycoprotein D (gD)

The gD genes of HSV-1 and HSV-2 have been successfully mapped and were the first HSV glycoprotein genes to be sequenced (Lee et al., 1982a; Watson et al., 1982; Watson, 1983). The role of HSV-1 gD in the infectious process has been widely studied. The first function assigned to gD was that of promoting the fusion of infected cells, as was shown by the ability of anti-gD monoclonal antibodies to inhibit HSV-1-induced cell fusion (Noble et al., 1983). A role in virus attachment to cells was demonstrated by the use of monoclonal antibodies specific for gD which inhibited adsorption (Fuller and Spear, 1985). Such a role was also suggested by Johnson et al. (1984) who demonstrated that liposomes containing gD were able to bind cells. However, gD is not essential for adsorption as mutant viruses lacking gD can still adsorb to cells (Ligas and Johnson, 1988). However, gD is essential for viral penetration of cells and therefore indispensable for virus infectivity, since mutant viruses lacking gD are unable to penetrate cells (Ligas and Johnson, 1988). gD is a very abundant and immunogenic component of the HSV virion, being a primary target for virus neutralising antibodies (Cohen et al., 1984; Para et al., 1985). Such neutralising antibodies are thought to

inhibit virus penetration of the cell surface membrane (Highlander et al., 1987). Further evidence that gD plays an important role in the viral penetration of infected cells was provided by experiments which showed that some anti-gD monoclonal antibodies block HSV-1 infection by inhibiting fusion of the virus and cell at the cell surface, although adsorption remained unaffected (Fuller and Spear, 1987). Cell lines that express gD have been shown to inhibit the penetration of HSV-1 but not virus adsorption to cells (Campadelli-Fiume et al., 1988). Similar conclusions by Johnson and Ligas (1988) led to the proposal of a model by which gD promotes viral penetration. They showed that pretreatment of cells with ultraviolet (UV)-inactivated virions containing gD rendered them resistant to infection with HSV-1 and HSV-2, whilst pretreatment with UV-inactivated virions lacking gD resulted in successful infection, and the prior addition of infectious HSV-1 to UV-inactivated virions containing gD also resulted in the successful entry and replication of infectious virus. On the basis of these results, it was proposed that virions containing gD bind a set of cell surface receptors which are limited in number, thus promoting viral penetration (Johnson and Ligas, 1988). Since virions lacking gD were unable to bind these receptors, it is apparent that gD is an essential receptor-binding polypeptide.

iv) Glycoproteins E (gE) and I (gI)

In addition to expressing receptors for the C3b component of complement, virions and cells infected with HSV-1 and HSV-2 also express receptors which have affinity for the Fc domain of IgG. gE is present on the surface of virions (Para et al., 1980) and of infected cells (Baucke and Spear, 1979). gE has been

implicated as being a component of the Fc receptor as solubilised HSV-1 gE (gE-1) and HSV-2 gE (gE-2) have Fc-binding activity (Baucke and Spear, 1979; Para et al., 1982b). Furthermore, anti-gE-1 antiserum can neutralise virus infectivity in the presence of complement and the $F(ab')_2$ fragments of this antiserum partly block the Fc-binding activity of HSV-1-infected cells (Para et al., 1982a). Support for gE as the Fc receptor was provided by analysis of mutant viruses devoid of gE, which were unable to express Fc receptors (Neidhardt et al., 1987). The gene for HSV-1 gE has been mapped to the U_S component of the genome (Lee et al., 1982a; Para et al., 1982b) and sequenced (McGeoch et al., 1985). Para et al. (1982b) identified the antigenically related gE glycoprotein of HSV-2 in infected cell extracts and its gene has been partially sequenced (McGeoch et al., 1987).

More recently, a minor glycoprotein distinct from gE was detected. Longnecker et al. (1987) identified a monoclonal antibody specific for the product of HSV-1 gene US7 (McGeoch et al., 1985) which was then used to characterise the gene product, a glycoprotein designated gI. In a similar vein, a novel glycoprotein (g70) which binds human or rabbit IgG in conjunction with gE was identified, and it was proposed that g70 forms a complex with gE which binds IgG and that gE alone cannot bind IgG (Johnson and Feenstra, 1987). Further studies with a monoclonal antibody specific for g70 revealed that this glycoprotein is encoded by gene US7 and is therefore identical to gI (Johnson et al., 1988a). This monoclonal antibody could precipitate gI and a fraction of gE from infected cell extracts and rabbit IgG could precipitate such a complex from cells

harbouring a plasmid containing a HSV-1 fragment encompassing the genes for gE and gI (Johnson et al., 1988a). Rabbit IgG could not bind cells infected with mutant viruses which do not express gE or gI whereas cells coinfected with mutant viruses, one of which failed to express gE and one of which failed to express gI, were able to bind IgG to a similar level as wild-type virus (Johnson et al., 1988a). These studies demonstrated that gE and gI form a complex which binds to the Fc domain of IgG and that neither gE or gI alone can bind IgG. Both gE (Longnecker and Roizman, 1986) and gI (Longnecker et al., 1987) are deletable genes that are not essential for viral growth in tissue culture. Neidhardt et al. (1987) also showed that mutant viruses lacking gE are viable, being able to replicate to a level only slightly lower than wild-type virus. It has recently been reported that gE may have a role in the induction of infected cell fusion, since it was shown that a monoclonal antibody against gE inhibits syncytium formation in human cells by HSV-1 and a mutant devoid of gE failed to induce syncytium formation in tissue culture (Chatterjee et al., 1989).

v) Glycoprotein G (gG)

This minor glycoprotein was originally identified in HSV-2 by Marsden et al. (1984) who named it g92K and mapped its gene to the U_S component in a genomic region distinct from gD and gE. Ruyechan et al. (1979) mapped a 124 kilodalton (kDa) glycoprotein to a genomic region between 0.65 and 0.70 map units (m.u.) and named it gC, but it was subsequently found that this mapping was incorrect and the 124kDa protein was remapped to the U_S component and renamed gG-2 (Roizman et al., 1984). The map locations of the 92kDa and 124kDa proteins suggested that they

are the same species and the size anomaly was attributed to the use of different gel systems for resolving proteins. Although it was originally thought that HSV-1 did not encode a gG-2 homologue, the sequence of the U_S component of the HSV-1 genome revealed that gG-1 was potentially encoded by gene US4 (McGeoch et al., 1985). Subsequently, the HSV-2 gG-2 sequence was determined and it was demonstrated that HSV-2 US4 actually encodes gG-2 by the use of antiserum raised against a dodecapeptide representing amino acids near the carboxyl-terminus of the HSV-2 US4 gene product (McGeoch et al., 1987).

However, very little is known about the precise functions of gG in the infectious process. Anti-gG-1 antibody can neutralise virus infectivity in the presence of complement (Sullivan and Smith, 1987) and gG-1 is the target of monoclonal antibodies (Ackermann et al., 1986). The gene for gG (US4) is dispensable for growth in tissue culture (Longnecker et al., 1987; Weber et al., 1987) and the US4 gene may be involved in viral pathogenesis in the central nervous system of mice (Weber et al., 1987).

vi) Glycoprotein H (gH)

This minor glycoprotein species was first identified in HSV-1-infected cells and purified virions as a 110kDa protein which was recognised by monoclonal antibody 52S (Showalter et al., 1981). A neutralising monoclonal antibody (LP11) was shown to precipitate a glycoprotein similar to that precipitated by 52S (Buckmaster et al., 1984). The gene for this glycoprotein was mapped by the use of intertypic recombinants (Buckmaster et al., 1984) and by marker transfer studies (Gompels and Minson, 1986) to the left of the gene coding for TK between map

coordinates 0.27 and 0.31. The 110kDa protein was named gH-1 (Buckmaster et al., 1984) and the DNA sequence of its gene determined (Gompels and Minson, 1986; McGeoch and Davison, 1986).

A number of important roles have been assigned to gH. It can elicit the production of complement-mediated neutralising antibodies (Showalter et al., 1981; Buckmaster et al., 1984). Unlike most other neutralising antibodies, those specific for gH can inhibit plaque formation and thus the transfer of virus from infected to uninfected cells by syncytial or non-syncytial HSV-1 strains (Buckmaster et al., 1984; Gompels and Minson, 1986). It was thus inferred that gH has a role in virus release from infected cells or in the formation of intercellular junctions between cells and, consequently, the cell-to-cell spread of virus. A temperature-sensitive mutant of HSV-1, tsQ26, has been isolated (Chu et al., 1979) and the mutation mapped to a specific genomic fragment shown to reside within the coding sequence of gH (McGeoch and Davison, 1986). This mutant has an amino acid substitution in gH (Desai et al., 1988). Investigation of the phenotype of this mutant by Desai et al. (1988) revealed that at the non-permissive temperature the virus could enter cells and replicate to yield near normal levels of infectious virus, but intracellular virions contained an immature form of gH and no gH was expressed on the surface of infected cells. Virus particles excreted by these cells were devoid of gH and not infectious, whereas virus particles excreted at the permissive temperature were infectious and contained gH. The temperature-sensitive phenotype occurred due to the loss of gH from virions during transport to the cell

surface and resulted in a loss of infectivity. Thus gH was shown to be essential for virus infectivity. Furthermore, it was recently reported that three neutralising monoclonal antibodies specific for gH were able to block viral penetration without inhibiting adsorption of virus to cells, which supports the essential role of gH, like both gB and gD, in viral penetration of infected cells (Fuller et al., 1989).

Glycoproteins Specified by PRV

Five envelope glycoproteins (gI, gII, gIII, gIV and gV), in addition to the glycoprotein gX which is not incorporated into virions, have been defined for PRV (Hampl et al., 1984). The genes for six glycoproteins have been mapped and sequenced. The first glycoprotein gene to be sequenced was that for gX, a secreted glycoprotein, which was mapped to the U_S component of the genome and was shown to be antigenically distinct from, and not a proteolytic cleavage product of, any of the envelope glycoproteins (Rea et al., 1985). The gene for gp50, a minor species in the envelope, has been mapped to U_S (Wathen and Wathen, 1984). This glycoprotein is unusual in that it is not N-glycosylated (Petrovskis et al., 1986a) and determination of the DNA sequence of its gene indicated that it shows some homology to HSV-1 gD (Petrovskis et al., 1986a). It is not known whether gp50 corresponds to the gIV or the gV species defined above. The gIII gene has been mapped to the U_L component (Robbins et al., 1984; Wathen and Wathen, 1986) and sequenced (Robbins et al., 1986a). gIII is a major glycoprotein species and is the homologue of HSV-1 gC. The gene for gI was mapped by hybridisation-selection, in vitro translation and

immunoprecipitation techniques to the region 0.86 to 0.89 m.u. within U_S (Mettenleiter et al., 1985a). The gene encoding the glycoprotein complex gII was mapped to U_L (Mettenleiter et al., 1986) and subsequent sequencing analysis revealed that gII shows strong homology to HSV-1 gB (Robbins et al., 1987). gII is the species corresponding to that identified by Lukacs et al. (1985) as a disulphide-linked dimer. Petrovskis et al. (1986c) used the λgt11 system described by Young and Davis (1983a,b) to isolate two genes encoding glycoproteins. Mapping studies revealed that one of these genes was identical to the previously mapped glycoprotein gene for gI whilst the other was a previously unidentified species, named gp63. The gI and gp63 species mapped adjacent to each other within U_S and the DNA sequence of these genes revealed that gI shares homologous sequences with HSV-1 gE and gp63 is the homologue of HSV-1 gI. All PRV glycoproteins identified to date have homologues in HSV, with the exception of gX which is secreted into the medium of infected cells and appears to be unique to PRV.

Functions of PRV Glycoproteins

The best studied PRV glycoprotein is gIII, the HSV-1 gC homologue, which has been shown to be multifunctional. A role for gIII in adsorption was first suggested by Hampl et al. (1984) who demonstrated that monoclonal antibodies against gIII can neutralise PRV in vitro by blocking adsorption of virus to cells. Using such gIII-specific monoclonal antibodies, mutant viruses containing a deletion in the gIII gene were isolated, and these mutant viruses were able to successfully propagate in tissue culture, implying that gIII is not essential for viral

growth *in vitro* (Robbins *et al.*, 1986b). However, gIII is required for efficient viral growth since gIII$^-$ mutants grow much more slowly than wild-type virus (Whealy *et al.*, 1988). Two mutant viruses isolated which were resistant to neutralisation by gIII-specific monoclonal antibodies were also shown to be associated with a syncytial phenotype. Such mutants either did not express gIII or only expressed a truncated form (Wathen and Wathen, 1986). It therefore appears that gIII inhibits cell fusion. It was confirmed that gIII has an important role in the adsorption process as gIII$^-$ mutants are much less readily adsorbed to cells, producing a lower yield of infectious virus (Schreurs *et al.*, 1988). Since gIII$^-$ mutants are still infectious, it was suggested that the adsorption of virus to cells can also occur by another slower, gIII-independent process (Schreurs *et al.*, 1988), a proposal which was later confirmed in further studies which showed that this occurs by interaction with a cellular heparin-like receptor (Mettenleiter *et al.*, 1990). gIII is also thought to have a role in virus release from infected cells (Schreurs *et al.*, 1988). Thus, like HSV-1 gC, gIII is not essential for viral growth and inhibits cell fusion. However, no evidence exists that gIII can bind to the C3b component of complement as does HSV-1 gC, and by the same token HSV-1 gC has not been shown to play a role in virus adsorption to cells and virus release as has been suggested for gIII.

It is known that gX, which accumulates in large amounts in the medium of infected cells, is not essential for viral growth in tissue culture (Thomsen *et al.*, 1987). Since mutant viruses lacking gX are highly virulent (Thomsen *et al.*, 1987) and PRV can still kill animals in the presence of anti-gX antibodies, it

appears that gX does not contribute to PRV virulence. No specific functions for gX have yet been elucidated.

Very little is understood about the precise functions of gII and gp50. It appears that both gII and gp50 are involved in viral entry as antibodies specific for these species inhibit the penetration of adsorbed virus (Zuckermann et al., 1989). Such a role for these glycoproteins seems feasible in view of the fact that their HSV-1 homologues, gB and gD, also play a major role in viral penetration. gp50 is important in the immune response to viral infection in as much as monoclonal antibodies specific for gp50 can protect mice from virus challenge (Wathen et al., 1985) and it can elicit the production of complement-independent virus neutralising antibodies (Wathen and Wathen, 1984).

PRV gI has been demonstrated to play a major role in virus release and contributes to PRV virulence. Such functions were deduced from the the study of vaccine strains of PRV. Two such strains, Norden and Bartha, have deletions in U_S (Lomniczi et al., 1984a). Genes deleted from this region are necessary for virulence and the Bartha strain is also defective in other genes necessary for the expression of virulence (Lomniczi et al., 1984b). In a similar vein, Berns et al. (1985) demonstrated that genes responsible for virulence are located within U_S, and three avirulent strains of PRV contain a deletion in U_S and/or TR_S that results in no expression of gI and a 40kDa polypeptide (Mettenleiter et al., 1985b). The deletion in the Norden strain deletes all the gI coding region but gp63 coding sequences remain intact, although a modified form of gp63 is produced in infected cells (Petrovskis et al., 1986b). In the Bartha strain, the deletion removes all the gI coding region and all

but 89 amino acids of the gp63 coding sequence, and no gI or gp63 is detected in infected cells (Petrovskis et al., 1986b). Restoration of an intact U_S to the Bartha strain increases the extent of virus release and the size of plaques formed on rabbit kidney cells (Ben-Porat et al., 1986b). Mettenleiter et al. (1987a) investigated whether gI or gp63 plays a role in virus release by introducing deletions into the genomes of wild-type virus and a rescued Bartha strain ie. one which has an intact U_S restored. These deletions abolished gI or both gI and gp63 expression. Deletion mutants of the wild-type virus defective in gI or gI and gp63 showed similar levels of virus release and plaque size as wild-type virus on rabbit kidney cells, whereas the rescued Bartha strain with a deleted gI caused a large decrease in virus release and plaque size. Thus a role for gI in virus release was demonstrated, although this function was in conjunction with another viral function which was present in wild-type virus but absent from the Bartha strain. Only when this viral function is absent is gI essential for virus release. gI plays a vital role in virulence with the aid of other viral genes which are absent from the Bartha strain (Mettenleiter et al., 1987b). gI and gp63 are noncovalently linked to each other, though this complex does not possess Fc receptor activity as does its homologue, the gE-gI complex of HSV-1 (Zuckermann et al., 1988).

Glycoproteins of VZV

VZV specifies six to eight glycoproteins that are expressed on the surface of virions and virus-infected cells (Shemer et al., 1980; Grose et al., 1981). On the basis of their

serological reactivity with monoclonal antibodies, these glycoproteins have been divided into four distinct groups in accordance with a new common nomenclature (Davison et al., 1986). Analysis of the complete DNA sequence of the VZV genome has identified the genes encoding gpI to gpIV and a further glycoprotein gene, gpV (Davison and Scott, 1986a). However, determination of the exact functions of these species in the infectious process has been hampered by difficulties with the in vitro growth of VZV.

gpI, gpII and gpIII are the major glycoprotein species specified by VZV. gpI is the most abundant and immunogenic species (Keller et al., 1984) and stimulates the production of antibodies which neutralise virus infectivity in vitro in the presence of complement (Forghani et al., 1984; Keller et al., 1984). The gene for gpI has been mapped to the right end of U_S (Ellis et al., 1985) and shows limited homology to HSV-1 gE (Davison and McGeoch, 1986).

The gpII glycoprotein has been resolved as polypeptides in two size ranges, 115kDa to 140kDa and 57kDa to 66kDa, is the second most abundant and immunogenic species (Keller et al., 1984) and can elicit the production of complement-dependent and complement-independent virus neutralising antibodies (Keller et al., 1984; Edson et al., 1985; Wroblewska et al., 1985). Cross-reactive monoclonal antibodies have been isolated that recognise both a 63kDa species of gpII and HSV-1 gB, implying that these molecules share common antigenic epitopes (Edson et al., 1985). Mature gpII migrates as a doublet of about 60kDa under reducing conditions but as a single species of 120kDa to 140kDa under non-reducing conditions, and consequently gpII has been referred

to as a disulphide-linked dimer (Grose et al., 1984). gpII is highly homologous to HSV-1 gB (Keller et al., 1986) and, as such, can be expected to play similar roles in the infectious process.

The gene for gpIII has been mapped and sequenced (Keller et al., 1987) and its product is the least immunogenic of the three major glycoproteins (Keller et al., 1984). However, it contains at least one epitope that can stimulate production of antibodies which neutralise virus infectivity in the absence of complement (Keller et al., 1984; Montalvo and Grose, 1986). gpIII is the homologue of HSV-1 gH (McGeoch and Davison, 1986) and is essential for the cell-to-cell spread of virus, as monoclonal antibodies to gpIII inhibit plaque formation in infected cells (Keller et al., 1987). This function is shared with its HSV-1 homologue, gH.

gpIV has been identified as a 45kDa to 55kDa species which reacted with antibodies directed against a peptide from the carboxyl-terminus of the the 39kDa gene product from U_S (Davison et al., 1985), and is the the homologue of HSV-1 gI (Davison and McGeoch, 1986).

The fifth glycoprotein species, gpV, is the homologue of HSV-1 gC. However, this species remains to be characterised. The five distinct glycoprotein species encoded by VZV all possess homologues in HSV and PRV, although it remains to be determined whether such species have conserved functions.

Glycoproteins of BHV-1

BHV-1 is thought to specify up to 11 glycoproteins (Misra et al., 1981). The four major species are designated gI to gIV

as defined by a new nomenclature (van Drunen Littel-van den Hurk and Babiuk, 1986). The genes for gI and gIII have been mapped and sequenced (Lawrence et al., 1986; Misra et al., 1988; Whitbeck et al., 1988; Fitzpatrick et al., 1989). gI, gII, gIII and gIV are homologues of HSV-1 gB, gE, gC and gD, respectively. gI and gIII can elicit the formation of virus neutralising antibodies and antibodies that participate in the complement-mediated lysis of infected cells (van Drunen Littel-van den Hurk et al., 1984, 1985). Although gI was originally thought to play an important role in virus neutralisation and to contain a site for viral attachment (van Drunen Littel-van den Hurk et al., 1984), the work of Marshall et al. (1986) suggests that a lesser role is played by gI in this process. gII is of minor importance in immunity since antibodies against this species are unable to neutralise infectivity or to play a role in the immune lysis of the infected cell (van Drunen Littel-van den Hurk et al., 1984). The precise roles of these glycoproteins in the infectious process is still unclear. gI, like its HSV-1 gB counterpart, promotes cell fusion (Fitzpatrick et al., 1988) and gIII is thought to be the protein that mediates the attachment of virions to cells (Okazaki et al., 1987). Additionally, gI may interfere with cellular proteins associated with the cytopathic effects of BHV-1 infection, as plaques formed on gI-expressing fibroblasts infected with BHV-1 or HSV-1 are smaller than those observed on normal fibroblasts infected with these viruses (Chase et al., 1989). Since the actual number of plaques formed is the same in both normal and gI-expressing infected fibroblasts, gI does not appear to inhibit viral entry and cells expressing gIII are also fully susceptible to BHV-1 infection

(Chase et al., 1989).

Glycoproteins of HCMV

HCMV is known to specify several envelope glycoproteins (Farrar and Oram, 1984) that can elicit the production of antibodies that neutralise virus infectivity in vitro (Britt, 1984; Rasmussen et al., 1984, Kari et al., 1986) and in vivo (Rasmussen et al., 1985; Cranage et al., 1986; Gonczol et al., 1986). The most extensively studied and abundant species is a disulphide-linked heterodimer comprising two glycosylated proteins of molecular weight 55kDa and 116kDa (Britt, 1984), which is referred to as gp55-116. The gene for this glycoprotein has been sequenced (Cranage et al., 1986) and is homologous to the HSV-1 gB gene. The gp55-116 (gB) complex of HCMV is a very important target for the immune response since it is the most abundantly expressed glycoprotein species and can stimulate the production of virus neutralising antibodies (Britt, 1984; Cranage et al., 1986; Britt et al., 1988). HCMV specifies one more glycoprotein known to have a homologue in HSV, and that species is the counterpart of gH. The gene for the HCMV gH homologue has been sequenced and expressed in vaccinia virus (Cranage et al., 1988). gH was precipitated as an 86kDa species from cells infected with HCMV and from recombinant vaccinia virus-infected cells by a monoclonal antibody that neutralised virus infectivity, which indicates that gH is a neutralising target (Cranage et al., 1988). However, in contrast to results obtained with vaccinia virus expression of HCMV gB (Cranage et al., 1986), recombinant vaccinia virus-infected cells did not express gH on their surfaces, which indicates that the normal

transport of gH to the cell surface may require other HCMV-specific gene products.

Glycoproteins of EBV

The complete DNA sequence of the genome of EBV strain B95-8 predicted the existence of at least five glycoprotein genes, namely open reading frames (ORFs) BLLF1a, BLLF1b, BDLF3, BILF2 and BALF4 (Baer et al., 1984). Three major glycoprotein species have long been recognised: gp350, gp220 and gp85 (Strnad et al., 1982; Edson and Thorley-Lawson, 1983). The most prominent species, gp350 and gp220, were mapped to the BamHI-L region of the genome by hybrid-select translation (Hummel et al., 1984) and have both been shown to be encoded by ORF BLLF1 (Baer et al., 1984; Beisel et al., 1985). gp350 and gp220 are involved in attachment of virus to its receptor on B cells (Nemerow et al., 1987; Tanner et al., 1987). Antibodies against gp350 and gp220 can neutralise virus infectivity (Thorley-Lawson and Geilinger, 1980; Thorley-Lawson and Poodry, 1982) and epitope mapping of gp350/gp220 identified seven epitopes of which two could elicit the production of virus neutralising antibodies (Qualtiere et al., 1987). Antibodies to gp85 neutralise virus infectivity (Strnad et al., 1982) and gp85 appears to have a role in the fusion of EBV with B cell membranes since a monclonal antibody to gp85 inhibited cell fusion but not virus attachment (Miller and Hutt-Fletcher, 1988). gp85 is encoded by BXLF2 and is the homologue of glycoprotein gH of other herpesviruses (McGeoch and Davison, 1986). Since Baer et al. (1984) did not identify the product of BXLF2 as a glycoprotein, it appears that EBV may actually encode at least six glycoproteins. BALF4 encodes the

homologue of HSV-1 glycoprotein gB (Pellett et al., 1985a), which is known as gp110 (Gong et al., 1987). The glycoprotein encoded by BILF2 has been characterised and is known as gp78/55 (Mackett et al., 1990). However, the glycoprotein predicted to be encoded by BDLF3 remains to be characterised.

The glycoproteins specified by HSV (gB, gC, gD, gE, gG, gH and gI) and the nomenclature used for naming their known counterparts in PRV, VZV, BHV-1, HCMV and EBV is summarised in Table 1.1.

TABLE 1.1

Herpesvirus Glycoprotein Nomenclature

HSV	PRV	VZV	BHV-1	HCMV	EBV
gB	gII	gpII	gI	gp55-116	gp110
gC	gIII	gpV	gIII	-	-
gD	gp50	-	gIV	-	-
gE	gI	gpI	gII	-	-
gG	-	-	?	-	-
gH	gH	gpIII	?	gp85	gp85
gI	gp63	gpIV	?	-	-

Table 1.1 Nomenclature used to identify HSV glycoprotein homologues in PRV, VZV, BHV-1, HCMV and EBV. Glycoproteins which do not have known homologues in HSV are not included. (-), no homologue; (?), unknown.

THE EQUINE HERPESVIRUSES

Introduction

The equine herpesviruses are a group of antigenically distinct infectious agents which cause a wide range of natural infections. EHV-1, also known as equine abortion virus and equine rhinopneumonitis virus, is the best studied member of this group. It is now known that EHV-1 is not a single virus but two antigenically distinct subtype viruses, referred to as EHV-1 subtypes 1 and 2. EHV-1 subtype 1 is the major agent responsible for abortion and neonatal foal death and has also been implicated in respiratory disease and neurological disorders, whereas subtype 2 appears to be predominantly associated with respiratory disease (O'Callaghan et al., 1983; Allen and Bryans, 1986). EHV-1 subtypes 1 and 2 are now more commonly referred to as EHV-1 and EHV-4, respectively.

EHV-2 does not appear to cause any specific disease (Studdert, 1974) although the majority of horses appear to be infected (Roeder and Scott, 1975). Members of this group are slow growing with a limited host range in vitro, being restricted mainly to equine cells, although fast growing isolates have been reported (Karpas, 1966; Mumford and Thompson, 1978). Since the majority of strains are slow growing and remain cell-associated, EHV-2 has been classified as a cytomegalovirus (Wharton et al., 1981). Infection occurs during early life by inhalation of infectious virus and infected horses become persistent carriers and constantly shed virus (Studdert, 1974). EHV-2 has been isolated from a wide range of tissues including the vagina, mammary gland, bone marrow and kidney (Plummer and

Waterson, 1963; Kono and Kobayashi, 1964; Karpas, 1966; Studdert, 1974). EHV-2 has not been isolated from foetal tissue and does not therefore appear to cross the placental barrier (Studdert, 1974).

EHV-3 is the cause of a mild progenital disease in horses, known as equine coital exanthema. Lesions, which initially appear as small vesicles or papules which rapidly progress to pustules and ulcers, are localised mainly, but not exclusively, to the external genitalia and infected animals show no signs of systemic illness (Bryans and Allen, 1973; Studdert, 1974). Lesions may also appear on the conjuctivae, lips and nasal mucosa. The virus is usually spread venereally although other routes of transmission appear likely. Virus replication is restricted to equine cells and rabbit kidney cells.

Abortion Induced by EHV-1

Viral abortion as a clinical entity was first reported by Dimock and Edwards (1932) and further studies proceeded to define clinical aspects of disease and pathological lesions observed in aborted foals (Dimock, 1940; Dimock et al., 1942). Most mares abort during the last four months of gestation (Doll, 1952; Doll and Bryans, 1963) and, on rarer occasions, may abort again after an interval of more than two years (Dimock et al., 1942). Although viral abortion is usually a disease of late pregnancy, it can be induced experimentally as early as the third month of gestation (Doll et al., 1955). Horses are infected in early life and continue to be reinfected at intervals of 3 to 6 months throughout their life. The virus is transmitted via airborne secretions, contaminated food and water

and by close contact with mares and their aborted foetuses. Most pregnant mares previously exposed to the virus are relatively immune to abortion as a result of reinfection since they develop sufficiently high levels of virus neutralising antibodies, and reinfection is generally not observable (Bryans, 1969). Susceptible pregnant mares may abort 14 to 120 days after infection without displaying signs of disease (Doll and Bryans, 1962). The aborted foetus dies by suffocation due to placental separation from the endometrium or by the inability to respire after delivery due to pulmonary oedema and viral pneumonitis (Bryans and Prickett, 1970). Foetuses aborted prior to the 6th month of gestation are severely autolysed and their lesions are not associated with a local inflammatory response as is observed in older foetuses (Prickett, 1970). Those aborted after seven months gestation display a variety of macroscopic lesions as described by Dimock et al. (1942, 1947) which include jaundice, pulmonary oedema, splenic enlargement with prominent lymphoid follicles, necrotic foci in the liver and petechiation of mucous membranes. Microscopic lesions include pneumonitis, bronchiolitis and focal necrosis of liver and spleen. Foetuses aborted between the 6th and 7th month of gestation show a mixture of lesions characteristic of both early and late abortion. The lesions found in the foetus are therefore dependent upon the age of the foetus and the degree of maturity of the foetal immune system.

Respiratory Disease Caused by EHV-1

A number of investigators originally noted viral abortion was closely associated with respiratory disease (Manninger and

Csontos, 1941; Manninger, 1949). This disease was first studied experimentally by Doll et al. (1954b) who showed that equine abortion virus was a cause of respiratory disease in young horses and proceeded to demonstrate that a number of putative influenza virus isolates were identical to equine abortion virus (Doll and Kintner, 1954; Doll et al., 1954a). Further studies led to equine abortion virus being designated equine rhinopneumonitis virus (Doll et al., 1957) which was subsequently renamed EHV-1 on the basis of electron microscopy (Plummer and Waterson, 1963). Infection with either subtype of EHV-1 results in acute respiratory disease, mainly in young foals. Clinical signs include fever, coughing, anorexia, congested nasal mucosa and excessive nasal discharge which may become mucopurulent (Doll et al., 1954b; Coggins, 1979). A leucocyte-associated viraemia and neutropenia are associated with subtype 1 respiratory tract infections (Doll et al., 1954a; Bryans, 1969). Infected animals shed large amounts of virus (Burrows and Goodridge, 1975) which is recoverable for up to 12 days after infection from the nasopharynx (Bryans, 1969). Respiratory disease is rarely fatal. However, secondary bacterial infections may result in bronchopneumonia, due to virus reaching the lungs, and chronic respiratory infection (Bryans, 1964; Prickett, 1970). Infection of perinatal foals can lead to fatal bronchopneumonia (Bryans, 1969). Immunity to the disease is more short-lived than that to abortigenic disease and reinfection occurs every 3 to 6 months throughout the lifetime of the horse (Doll, 1961; Bryans, 1969, 1981), although clinical signs of the disease are rarely seen in the older horse. However, such animals continue to produce and shed virus and act

as a reservoir of infection for other horses. This disease appears to be associated with the 'poor performance' syndrome amongst racehorses (Mumford and Rossdale, 1980).

Neurological Disease Caused by EHV-1

Clinical manifestations of this disease were first reported by Manninger (1949) who noted that mares inoculated with EHV-1 recovered from aborted foetuses readily developed myelitis. The first true link of EHV-1 to neurological disease was demonstrated by Saxegaard (1966) who isolated EHV-1 from the nervous tissue of horses suffering from paralysis. Since then the disease has been widely reported in many countries containing large horse herds (Charlton et al., 1976; Dinter and Klingeborn, 1976; Greenwood and Simson, 1980; Batra et al., 1982). Although usually associated with abortion or respiratory disease, natural outbreaks of neurological disease have been reported to occur without concurrent abortion or respiratory disease (Dinter and Klingeborn, 1976; Thein, 1981). The disease has been reproduced experimentally by inoculation of virus both subcutaneously and intranasally (Jackson and Kendrick, 1971; Jackson et al., 1977). Clinical signs of infection include severe to mild ataxia, incoordination and, in more severe cases, complete paralysis of both fore and hind limbs. The disease has a variable histopathology. In most cases, vasculitis of small arteries and veins and degeneration of nervous tissue are the principal lesions (Jackson and Kendrick, 1971; Jackson et al., 1977). More severe lesions such as disseminated meningoencephalitis have been reported by Charlton et al. (1976), who were able to culture EHV-1 from brain and lung

tissue. Naturally occuring deaths are usually due to pneumonia, pulmonary congestion, bowel atony, ruptured urinary bladder or intussusception. Recovery from the disease may vary from a few days to up to three months, whilst some horses may continue to demonstrate slight but permanent signs of infection, such as incoordination. In common with other herpesviruses, EHV-1 is able to spread from the infected cell to contiguous cells without an extracellular phase. It is in this way that, in the presence of high levels of serum virus neutralising antibodies, the virus is able to infect endothelial cells via infected leucocytes circulating in the blood to the vascular structures of the central nervous system. This accounts for the severe and generalised vasculitis associated with EHV-1 infection (Jackson et al., 1977). Malacic foci develop subsequent to thrombosis of local vessels, and paralysis appears to be a direct result of ischaemia and metabolic changes in the nervous tissue (Jackson et al., 1977). The vascular damage and ischaemic injury to the nervous system is probably due to immunological reaction of the host to the presence of EHV-1 in the infected vascular epithelium (Platt et al., 1980).

Neonatal Foal Disease Caused by EHV-1

In recent years neonatal foal disease due to infection with EHV-1 in utero during the late stages of gestation has been reported (Dixon et al., 1978; Hartley and Dixon, 1979). Foals may be born alive but weak and depressed and die within 24 hours of birth. Other foals may appear normal at birth but develop severe respiratory distress soon after birth and die within 72 hours. The neonatal syndrome described by Bryans et al. (1977)

involves foals suffering from respiratory distress, weakness and severe diarrhoea during the first two weeks of life prior to succumbing to a variety of secondary bacterial infections. Generally, foals afflicted by this disease exhibit extensive lymphoreticular, pulmonary, hepatic and, in some cases, adrenal damage.

Differentiation Between EHV-1 Subtype Viruses

That EHV-1 comprises two subtypes was first suggested by Shimizu et al. (1959) who demonstrated that EHV-1 isolates could be grouped into two distinct yet cross-neutralising serotypes. It remained for Burrows and Goodridge (1973) to show that the majority of abortigenic and respiratory isolates belong to antigenic subtypes 1 and 2, respectively. Subsequent comparative studies showed that subtype 1 abortigenic isolates replicate much more rapidly than subtype 2 respiratory isolates in a wider range of host cells in vitro. Additionally, subtype 1 isolates are able to multiply rapidly in the nasopharynx of non-immune horses with extensive shedding of virus, readily produce a leucocyte-associated viraemia and are adaptable to hamsters (Burrows and Goodridge, 1973, 1975; Studdert and Blackney, 1979). None of these characteristics are shared with subtype 2 isolates. Plaques produced by subtype 1 isolates in tissue culture are smaller than those produced by subtype 2 isolates. Patel et al. (1982) showed by immunofluorescence studies in experimentally infected foals that a subtype 2 isolate could only replicate in the epithelial cells of the nasopharynx and was disseminated via macrophages into the lymph nodes draining the respiratory tract, and no further. In contrast, the

penetration of subtype 1 virus was more widespread, infecting both epithelial and endothelial cells of the nasopharynx and lungs in addition to circulating leucocytes. Furthermore, it has been shown that, unlike subtype 2 strains, subtype 1 strains are able to infect the brain of mice after intracerebral inoculation of virus (Patel and Edington, 1983).

Further support for the existence of two distinct subtypes came from the use of restriction endonuclease analysis of viral DNA to discriminate between abortigenic and respiratory isolates (Sabine et al., 1981; Studdert et al., 1981; Turtinen et al., 1981). This powerful method highlighted clear genetic variation between the two subtypes which possessed few, if any, common restriction endonuclease cleavage sites, whereas isolates of the same subtype shared a very similar pattern. The intersubtypic differences were so extensive it was proposed that EHV-1 subtypes 1 and 2 should no longer be regarded simply as antigenic variants of the same virus but as distinct equine herpesvirus species: EHV-1 abortigenic strains and EHV-4 respiratory strains, respectively (Studdert et al., 1981). This new nomenclature shall be used throughout the rest of this thesis. Application of this DNA fingerprinting methodology has confirmed that EHV-1 can cause respiratory and neurological disease in addition to abortion, whilst EHV-4 rarely causes abortion and neurological disease and is the predominant agent responsible for respiratory disease (Allen et al., 1983b; Studdert, 1983; Studdert et al., 1984). In addition, three EHV-1 strains which caused myeloencephalitis possessed different DNA fingerprint patterns to non-myeloencephalitis strains, which suggested the presence of a marker for neurovirulence at the DNA

level (Studdert et al., 1984).

Another approach to the differentiation of EHV-1 and EHV-4 has been employed by Yeargan et al. (1985), who constructed type-specific pools of monoclonal antibodies which were then used in enzyme immunofiltration and direct immunofluorescence assays to rapidly type isolates. This method can rapidly type many isolates at one time, is accomplished within 3 hours of virus isolation in tissue culture and remains a powerful tool for discriminating between EHV-1 and EHV-4.

Multiple passage of EHV-1 strains in non-equine cells in vitro and in hamsters in vivo gives rise to alterations in the genomic fingerprint pattern with the loss or gain of restriction endonuclease cleavage sites (Allen et al., 1983a). The passage of EHV-1 in equine cells has little effect on genome structure and stability (Allen et al., 1983a).

The use of a kinetic analysis of DNA-DNA reassociation has demonstrated that the EHV-1 and EHV-4 genomes share only 17% of their sequences (Allen and Turtinen, 1982), a value far removed from the 47% DNA homology predicted between HSV-1 and HSV-2 (Kieff et al., 1972). Furthermore, EHV-1 and EHV-4 show no detectable homology to EHV-2, which indicates that EHV-1 and EHV-4 have a common ancestral origin (Allen and Turtinen, 1982).

Intratypic Genetic Variation

The DNA fingerprinting technique has demonstrated significant intratypic variation amongst EHV-1 and EHV-4 isolates. The most notable study was that of Allen et al. (1983b) who analysed over 300 isolates recovered from 148 outbreaks of abortion and 24 outbreaks of respiratory disease

during a 22 year period in Kentucky. Using five different restriction endonucleases, it was shown that EHV-1 isolates could be grouped into at least 16 electropherotypes on the basis of their DNA fingerprint patterns, of which the two dominant electropherotypes caused over 90% of abortions during this period. EHV-1 isolates demonstrated relatively little genetic variation as 67% of abortions were due to EHV-1 isolates that had identical DNA fingerprint patterns. The genetic variability of EHV-1 isolates was actually less than has been observed for a number of human herpesviruses (Roizman and Buchman, 1979). In contrast, EHV-4 isolates exhibited extensive genetic variability with 13 distinct fingerprints identified amongst only 21 unrelated isolates (Allen et al., 1983b).

Genome Structure of EHV-1 and EHV-4

The genome structure of EHV-1 has been known for some time. It consists of a 145kb linear, double-stranded DNA molecule with a buoyant density of 1.716g/cm^3 and a G+C content of 57%. The genome is arranged as two covalently linked regions, L (111kb) and S (34kb). The S component comprises a short unique region (U_S) bounded by inverted repeats (IR_S, TR_S). The L segment is fixed and the S component can invert relative to the L component to give two isomeric forms, corresponding to a group D genome as described in Chapter 1. Restriction endonuclease cleavage maps of the genome for a number of enzymes have been derived for two EHV-1 strains: the low-passage tissue culture field isolate HVS-25 (Whalley et al., 1981) and the high-passage tissue culture KyA (L-M cell) strain (Henry et al., 1981; Baumann et al., 1986). Although the data of Henry et al.

F I G U R E 1.3

Physical Maps of the Genomes of EHV-1 and EHV-4

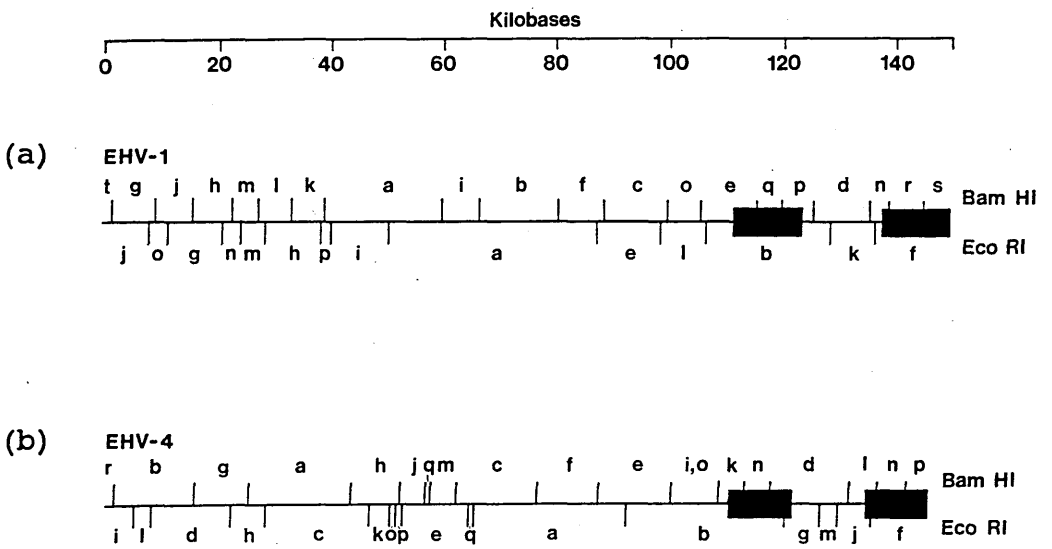


Figure 1.3 Physical maps of the EHV-1 and EHV-4 genomes showing the location of restriction sites for BamHI and EcoRI. (a) BamHI and EcoRI restriction maps of EHV-1 strain HVS-25. Derived by Whalley et al. (1981). (b) BamHI and EcoRI restriction maps of EHV-4 strain 1942. Derived by Cullinane et al. (1988).
The shaded boxes represent the repeat regions. Only one orientation of the S region is shown.

(1981) suggested that the KyA strain possesses a genome with an inverted region relative the HVS-25 strain, a subsequent reassessment has shown that the genomes of the two strains have a similar distribution of restriction endonuclease cleavage sites (Baumann et al., 1986). Recently it was shown that the EHV-4 (strain 1942) genome adopts a similar structure to that of EHV-1 strains (L, 109kb; S, 35kb) and restriction endonuclease cleavage maps have been derived for BamHI and EcoRI (Cullinane et al., 1988). The BamHI and EcoRI restriction maps of the EHV-1 (HVS-25) and EHV-4 (1942) genomes are shown in Figure 1.3. The arrangement of BamHI and EcoRI restriction sites is clearly different between the EHV-1 and EHV-4 genomes.

The EHV-1 genome is colinear with that of EHV-3 and the limited DNA homology (10%) is dispersed throughout U_L (Baumann et al., 1986). Although substantial homology exists between the inverted repeats of these two viruses, none is detectable in the U_S region.

Proteins of the EHV-1 and EHV-4 Virions

The EHV-1 and EHV-4 virions are morphologically indistinguishable from those of other herpesviruses ie. an internal DNA-containing core enclosed by an icosahedral capsid to form a nucleocapsid, which is immediately surrounded by the tegument and the loose outer envelope (Abodeely et al., 1970; O'Callaghan and Randall, 1976). Detailed studies on the morphology of the EHV-1 and EHV-4 virions and the proteins they contain have been carried out by a number of workers (Perdue et al., 1974, 1975, 1976; Kemp et al., 1974; Turtinen et al., 1981; Turtinen and Allen, 1982; Turtinen, 1983). The protein content

TABLE 1.2

Virion Proteins of EHV-1 and EHV-4

EHV-1 Virion Proteins				EHV-4 Virion Proteins			
Protein	Size (kDa)	Nature	Location	Protein	Size (kDa)	Nature	Location
1	250	G	E	1	250	G	E
**2	190-240	G(M)	E	**2	190-240	G(M)	E
9	140	-	NC	9	140	-	NC
9a	140	G	E				
10a	128	G	E				
10	124	G(M)	E	10	124	G(M)	E
11	117	-	T	11	117	-	T
12	110	-	T				
*13	96	G(M)	E	*13	110	G(M)	E
				13a	98	-	T
				14a	94	?	?
*14	90	G(M)	E	*14b	87	G(M)	E
				14c	84	?	?
15	82	-	T	15	82	-	T
16	74	G	E	16	74	G	E
				17a	70	-	T
17	68	G	E	17	68	G	E
				18a	64	-	T
*18	63	G(M)	E	*18	61	G(M)	E
19	60	-	T	19	60	-	T
20	54	-	NC	20	54	-	NC
21	45	G	E	21	45	G	E
22a	41	G(M)	E	22a	41	G(M)	E
22b	39	-	NC	22b	39	-	NC
23	36	-	T	23	36	-	T
23a	33	-	T	23a	33	G	E
*24a	31	-	NC	*24a	32	-	NC
24b	27	-	NC	24b	27	-	NC
*25	24	G	E	*25	25	G	E
*26a	18	-	T	*26a	19	-	T
*26b	17	-	NC	*26b	18	-	NC
27	16	-	T	27	16	-	T

Table 1.2 Virion proteins of EHV-1 and EHV-4. G, glycoprotein; G(M), major glycoprotein; E, envelope; T, tegument; NC, nucleocapsid; (-), not determined; (*), exhibits intertypic electrophoretic mobility differences; (**), exhibits intratypic electrophoretic mobility differences. From Allen and Bryans (1986).

of the EHV-1 and EHV-4 virions is summarised in Table 1.2. Although the envelope glycoproteins of HSV have been well characterised, the currently available information on those of EHV-1 and EHV-4 is limited. The important role of EHV-1 glycoproteins in the immune response to viral infection was first demonstrated by Papp-Vid and Derbyshire (1978, 1979). They showed that immunisation of hamsters with envelope-containing material offered protection against virus challenge. Furthermore, antibodies to the envelope could neutralise EHV-1 infectivity whereas those to the nucleocapsid showed no such neutralising activity. Thus the envelope glycoproteins were demonstrated to be important target antigens of the host immune response.

A number of comprehensive studies of the envelope glycoproteins have been carried out for both EHV-1 and EHV-4 (Turtinen and Allen, 1982; Turtinen, 1983). Six major and six minor glycoprotein species were identified for each virus type. The six major species were designated VGP2, 10, 13, 14, 18 and 22a. Intertypic mobility differences were demonstrated for VGP2, 13, 14, 18 and 25 and phosphoproteins VP24a, 26a and 26b (Table 1.2). Since the only species to exhibit intratypic mobility differences was VGP2, the electrophoretic protein profiles could be reliably used to distinguish between EHV-1 and EHV-4 isolates. Turtinen et al. (1981) investigated the serological relatedness between two EHV-4 (respiratory tract) isolates and the Army 183, KyA-ha and KyA-LM EHV-1 strains. Intertypic mobility differences were demonstrated for VGP13, 16, 17, 18, 23, 25 and 26a, whilst VGP8a was only detected in the KyA-ha strain and only small amounts of VP19 were present in the

respiratory tract isolates. The EHV-4 respiratory tract isolates showed little cross-neutralisation with the EHV-1 Army 183 and KyA-ha strains in plaque-reduction neutralisation tests.

The studies of Turtinen (1983) on the glycoproteins of EHV-1 and EHV-4 has yielded further valuable information. The bulk of virus-specific antibody is directed against VGP2, 10, 13, 14 and 22a and the serological cross-reactivity of EHV-1 and EHV-4 is due to type-common epitopes residing on VGP2, 10, 13 and 14. VGP2 and 14 appear to be the most cross-reactive species whilst VGP2, 10, 13 and 14 are the most immunogenic species. Only VGP18 and 22a were found to contain type-specific epitopes.

The nature of the epitopes on these glycoproteins was investigated using a panel of monoclonal antibodies specific for EHV-1 and EHV-4 glycoproteins (Yeargan et al., 1985). It was concluded from this study that four distinct types of epitopes can be found on EHV-1 and EHV-4 glycoproteins: type-specific epitopes common to all isolates of the same virus type, type-specific epitopes common to some isolates of the same virus type, type-common epitopes present on all isolates of both virus types and type-common epitopes present on some isolates of both virus types.

Intratypic Antigenic Variation

It is well documented that virtually all isolates of the homologous virus type are very closely related at the antigenic level (Turtinen et al., 1981; Turtinen, 1983). The only report to date which demonstrates antigenic variation within the same virus type is that of Yeargan et al. (1985) who used the powerful resolving ability of monoclonal antibodies specific to

EHV-1 and EHV-4 glycoproteins to group isolates into distinct monoclonal serotypes. Nine antigenically distinct serotypes were identified amongst 12 epizootically unrelated and genetically diverse EHV-1 isolates analysed, and this antigenic reactivity pattern did not correlate with the DNA fingerprint pattern (Allen and Bryans, 1986).

Cross-Protection Between EHV-1 and EHV-4 and the Nature of the Immune Response to Viral Infection

The fact that EHV-1 and EHV-4 are closely related at the antigenic level has prompted an assessment of the potential of a respiratory tract EHV-4 infection to cross-protect against subsequent infection with EHV-1 abortigenic strains (Allen and Bryans, 1986). Using the Syrian hamster model, it was demonstrated that a dose of EHV-4 ten-fold greater than EHV-1 was necessary to achieve the same level of protection against EHV-1 virus challenge. A single experimental infection of the respiratory tract of young foals with EHV-1 or EHV-4 offered little cross-protection between EHV-1 and EHV-4 since foals infected with either EHV-1 or EHV-4 were resistant to challenge by the homologous virus type, but susceptible to challenge with the heterologous virus type, after an interval of four weeks. However, repeated infections with EHV-4 at regular intervals ie. every six months, resulted in significant levels of immunity to infection with abortigenic EHV-1 isolates.

The nature of the immune response to EHV-1 and EHV-4 infection has been extensively studied. Such a response is short-lived as reinfecton is generally observable within three months of initial infection (Allen and Bryans, 1986). Although

horses infected with EHV-1 or EHV-4 usually develop antibodies to that virus, this antibody response does not always protect against reinfection (Bryans, 1969). However, the lack of such antibody is not usually associated with increased susceptibility to disease (Dutta and Shipley, 1975), indicating that cell-mediated immunity may play an important role in protecting horses against EHV-1 and EHV-4 infection. The nature of the immune response to EHV-1 and EHV-4 infection was further investigated using colostrum-deprived specific pathogen-free foals vaccinated with either EHV-1 or EHV-4 and then successively challenged with EHV-1 followed by EHV-4, or EHV-4 followed by EHV-1 (Fitzpatrick and Studdert, 1984). This study indicated that both humoral (antibody-mediated) immunity and cell-mediated immunity to EHV-1 and EHV-4 exists. The virus neutralising antibody response to EHV-1 was type-specific whereas that to EHV-4 was cross-reactive with equal amounts of antibody to both virus types being produced. The cellular immune responses involved were cross-reactive.

Additional evidence for the existence of immune mechanisms independent of virus neutralising antibody production came from the study of the ability of a commercially available vaccine (Pneumabort-K, inactivated EHV-1 Army 183 strain) to protect horses against challenge with EHV-1 strains (Burrows et al., 1984) and EHV-4 strains (Mumford and Bates, 1984). This vaccine did not protect against challenge with EHV-1 despite the presence of neutralising antibodies to EHV-1 in the sera of vaccinated animals, although limited protection was afforded against EHV-4 challenge.

Cell-mediated immunity has long been recognised as being

important in recovery from, and resistance to, viral infection and was first demonstrated as an immune mechanism to EHV-1 by Wilks and Coggins (1976, 1977). Cell-mediated immunity was principally examined using an *in vitro* lymphocyte transformation assay, a technique which is an indirect index of the cell-mediated immune response (Wilks and Coggins, 1976; Gerber *et al.*, 1977; Dutta *et al.*, 1980). Wilks and Coggins (1977) assessed the immune response of horses experimentally infected with EHV-1 by measuring cytotoxicity for EHV-1 infected target cells. Peripheral blood leucocytes from a horse recovered from EHV-1 infection were capable of lysing target cells. Cytotoxicity of both serum and peripheral blood leucocytes following experimental infection of specific pathogen-free foals was detected as early as one day post-infection (p.i.) and could persist for up to 20 days p.i. Cytotoxic antibodies or cells were therefore concluded to play an important role in the control of the spread of infection.

A most rigorous and elegant study on cell-mediated and antibody-mediated immune responses to EHV-1 was carried out by Gerber *et al.* (1977) who investigated the nature of these responses in young horses, older horses and pregnant mares. Following vaccination and revaccination five weeks later of young horses (6 to 8 months old) with a modified live vaccine of EHV-1, very little increase in virus neutralising antibody levels occured although a strong cell-mediated (lymphocytic) immune response developed. In contrast, older horses (18 to 21 months old) showed large increases in virus neutralising antibody levels at 7 days post-vaccination which remained elevated for 9 weeks post-vaccination (4 weeks post-

revaccination), but the cell-mediated immune response was suppressed during this time. The cell-mediated immune response only increased at 13 weeks post-vaccination (8 weeks post-revaccination) and correlated with a decrease in the level of virus neutralising antibodies. Thus, the level of the cell-mediated immune response increased in the absence of virus neutralising antibodies and decreased in the presence of virus neutralising antibodies. Pregnant mares during the late stages of gestation showed a heavily suppressed cell-mediated immune response but demonstrated a 16-fold increase in virus neutralising antibody levels after exposure to virulent EHV-1. Suppression of the cell-mediated immune response reaches a maximum during the late stages of pregnancy and accounts for the high incidence of abortion during this period (Doll and Bryans, 1963). In accordance with these studies, Dutta et al. (1980) also demonstrated a lymphocytic response to EHV-1 infection which appeared within 2 days and peaked at 7 to 10 days prior to decreasing.

More recently, Bridges and Edington (1987) developed a cytotoxic T lymphocyte (CTL) assay to investigate the immune response to EHV-1 and EHV-4 infection, a system which uses skin explants as targets for assaying CTL activity. They proceeded to demonstrate a genetically restricted CTL activity in ponies following an EHV-4 infection and this CTL activity was type-specific (Edington et al., 1988).

Two cellular immune mechanisms which have been the subject of investigation are antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent antibody lysis (CDL). ADCC is mediated by peripheral mononuclear cells which, when armed with

antibody, are able to specifically kill virus-infected cells. CDL is a result of activation of complement by the classical pathway following the attachment of virus-specific antibody to target antigens on the infected cell surface. Stokes and Wardley (1988) investigated the ability of ADCC and CDL to afford protection in the horse following vaccination or challenge with EHV-1. The sera from horses vaccinated with Pneumabort-K and that from non-vaccinated horses challenged with EHV-1 were capable of mediating ADCC and CDL to both EHV-1 and EHV-4. This was the first demonstration of these immune mechanisms operating *in vitro* after EHV-1 infection. However, the authors concluded that ADCC and CDL only appear to be of importance in recovery from EHV-1 infection and are not thought to play a role in protection following vaccination, and that other immune mechanisms may have a protective role.

A hamster model has recently been developed that will prove to be a useful system for studying the immune response to viral infection and evaluating the immunogenicity of individual EHV-1 and EHV-4 antigens (Stokes *et al.*, 1989). CDL appears to play a major role in protection in this model. In addition, the authors investigated the role of the six major glycoproteins of EHV-1 in the immune response to viral infection by the use of monoclonal antibodies specific for each of these six antigens (Allen and Yeargan, 1987). Hamsters inoculated with all six monoclonal antibodies were fully protected against virus challenge with EHV-1 (KyD strain) by the intranasal route and 80% were protected after challenge by the intraperitoneal route. More specifically, monoclonal antibodies to gp13, 14 and 17/18 were capable of eliciting passive protection individually. The

mechanism of protection is unknown since these monoclonal antibodies were unable to neutralise EHV-1 KyD *in vitro* and could only mediate ADCC when all six monoclonal antibodies were used, although it was suggested that either an ADCC or complement-mediated immune response may operate *in vivo* (Stokes *et al*., 1989).

A number of infected-cell polypeptides have been identified which are recognised by equine lymphocytes after an EHV-4 infection (Bridges *et al*., 1988). This cellular response was directed against the EHV-4 major glycoproteins gp10, 13, 14, 18 and 21/22a, and possibly gp9. Identical proteins of EHV-1 which share epitopes were also recognised by this response with the exception of gp18 which is antigenically type-specific. Hyperimmune equine serum from animals exposed to both EHV-1 and EHV-4 recognised ten polypeptides from both virus types of which four were also recognised by the cellular immune responses (Bridges *et al*., 1988).

Seven monoclonal antibodies have been characterised which recognise EHV-1 and EHV-4 (Sinclair *et al*., 1989). Of these, three were able to neutralise the EHV-1 Army 183 strain in the presence of complement but were unable to neutralise the EHV-4 MD strain. The target antigen for all three neutralising antibodies was an 83kDa EHV-1 glycoprotein which probably corresponds to gp13 (see Table 1.2). The remaining four non-neutralising antibodies recognised target antigens of molecular weights >205kDa, >205kDa, 97kDa and 13kDa. The two high molecular weight polypeptides probably correspond to the highly abundant envelope glycoprotein gp1/2 whose function is unknown and which were recognised in both EHV-1 and EHV-4. The 13kDa

species was recognised in both EHV-1 and EHV-4 and the 97kDa species was only recognised in EHV-4.

Latency Associated With EHV-1 and EHV-4 Infection

The ability of virus to persist in the host in a latent state is a serious problem associated with most herpesvirus infections, including those by HSV (Baringer and Swoveland, 1973; Stevens, 1975; Wildy et al., 1982), PRV (Sabo and Rajcani, 1976; Beran et al., 1980) and BHV-1 (Pastoret et al., 1984). Latent virus may be reactivated, usually under stress, at any time during the life of the host whereupon the virus is shed, leading to renewed outbreaks of disease in both the host and other susceptible individuals. It is well proven that the sensory ganglia are a site of latent infection for HSV (Baringer and Swoveland, 1973; Baringer, 1974), PRV (Beran et al., 1980) and BHV-1 (Homan and Easterday, 1980).

The evidence for the existence of latency for EHV-1 and EHV-4 based on field observations is purely circumstantial since the methods of tissue culture explantation and corticosteroid administration used to successfully demonstrate latency in other herpesviruses have failed to reactivate EHV-1 and EHV-4 from horses, with the exception of a single case where EHV-4 was detected in trigeminal ganglia (Gleeson and Coggins, 1980; Burrows and Goodridge, 1984; Allen and Bryans, 1986). Early indications for the reactivation of latent EHV-1/EHV-4 came from the vaccination of horses with African horse sickness virus, which subsequently developed respiratory illness (Erasmus, 1966). Stress-related activities such as weaning, castration and rehousing resulted in the shedding of virus from a number of

ponies which had had no contact with other horses for the last ten years (Burrows and Goodridge, 1984). Many of these ponies had previously developed significant increases in levels of EHV-1/EHV-4 neutralising antibodies although they showed no signs of illness (Burrows and Goodridge, 1978, 1979). However, infectious virus was undetectable following culture of tissue explants and corticosteroid administration.

The first experimental demonstration of EHV-1 latency was provided by Edington et al. (1985) who infected eight ponies with EHV-1 intranasally which were then maintained in isolation for three months, during which time levels of complement fixing antibodies decreased at least four-fold. All ponies demonstrated clinical and serological signs of infection and virus could be isolated from nasal swabs and leucocytes. The corticosteroids dexamethasone and prednisolone were administered and following this immunosuppression virus was recovered from six ponies within 14 days; five of these ponies were viraemic, three shed virus, only four showed significant increases in levels of complement fixing antibodies and two had raised levels of neutralising antibodies. The detection of viraemia without nasal shedding in three animals suggested a reactivation of latent virus by the corticosteroids as opposed to a secondary infection. The conclusion that latent EHV-1 could be reactivated following immunosuppression with corticosteroids in the presence of circulating antibodies correlated well with previous reports of increased levels of virus neutralising antibodies and shedding of EHV-1 in stressful situations (Burrows and Goodridge, 1978, 1984). Using a similar approach, reactivation of latent EHV-4 has also been demonstrated

(Browning et al., 1988).

EHV-1 has been successfully recovered from leucocytes following respiratory infection with the virus (Bryans, 1969; Gleeson and Coggins, 1980). Since virus can only be isolated from viable, undisrupted leucocytes it seems likely that the virus may be present in a noninfectious form, and that this cell-associated viraemia represents a latent infection of equine leucocytes by EHV-1 (Gleeson and Coggins, 1980; Scott et al., 1983).

Lytic Infection With EHV-1

EHV-1 replicates rapidly in both the mouse L-M cell line (O'Callaghan et al., 1968b, 1983) and the Syrian hamster (Bracken and Randall, 1957; O'Callaghan et al., 1972) with 'one-step' growth kinetics. Following infection, maximum virus titres are obtained after 10 to 12hr in the hamster and 18 to 24hr in the L-M cell line. Following inoculation of Syrian hamsters with 10^8 LD_{50} of virus, all animals die after approximately 12hr and display a marked viraemia with 95% of the hepatic parenchymal cells showing a typical intranuclear inclusion. In the L-M cell line only 5% of virus remains cell-associated.

Initiation of Infection

The virus envelope has a major role in the early stages of infection ie. the attachment and penetration of virus into the host cell. It is now widely accepted that following adsorption of virus to the host cell, the virion envelope fuses with the cell surface membrane to liberate the nucleocapsid into the cytoplasm (Morgan et al., 1968). This hypothesis for viral entry

is supported by electron microscopic studies on the intracellular distribution of nucleocapsids after exposure of cells to infectious virions or non-infectious nucleocapsids (Abodeely et al., 1970). For HSV, adsorption and penetration of infectious virus is mediated by three glycoproteins, gB, gD and gH, as has been reviewed in Chapter 1.

The attachment of EHV-1 to L-M cells occurs within 5min of exposure to virus and penetration is complete after 15min as is demonstrated by the presence of virions in cytoplasmic vacuoles. The fact that the virus contained inside cytoplasmic vacuoles is enveloped suggests that EHV-1 gains entry by viropexis (Abodeely et al., 1970). Uncoating of virus is complete within 4hr p.i. as is demonstrated by transcriptional studies.

Transcription and Viral Protein Synthesis

Huang et al. (1971) used competitive hybridisation techniques to first demonstrate that EHV-1 mRNA synthesis (gene expression) is temporally regulated. EHV-1-specific mRNA was present by 4hr p.i. of L-M cells and by 12hr p.i. additional mRNA species appeared, thus demonstrating that the synthesis of viral transcripts varied during infection. Cohen et al. (1975b) identified two classes of EHV-1 transcripts whose ratio varied at different times p.i. within infected cells. In L-M cells infected with EHV-1 in the presence of the viral DNA synthesis inhibitor 5'-fluoro-2-deoxyuridine, the synthesis of a major portion of the transcripts comprising the major RNA class was inhibited, indicating that the transcriptional regulation of EHV-1 was linked to the onset of viral DNA replication (Cohen et al., 1977b). These initial observations strongly suggested that

EHV-1 gene expression is highly regulated and sequentially ordered in a manner similar to that described for HSV-1.

The synthesis of HSV polypeptides has been shown to be coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974, 1975). On the basis of temporal order and metabolic requirements for their synthesis, these polypeptides fall into three major groups: α (immediate-early, IE), β (early) and γ (late). The IE polypeptides are the first species synthesised and transcription of their genes occurs in the absence of protein synthesis and is mediated by host cell RNA polymerase II which recognises unique IE promoters (Costanzo et al., 1977; Mackem and Roizman, 1980, 1981). These IE polypeptides are defined as those which are expressed immediately after the withdrawal of a protein synthesis block eg. by puromycin or cycloheximide, which was present prior to infection. The synthesis of IE polypeptides reaches a peak at 2 to 4hr p.i. The synthesis of early polypeptides is dependent upon the presence of functional IE polypeptides and occurs prior to, and independent of, viral DNA synthesis and their synthesis reaches a peak at 5 to 7hr p.i. The late polypeptides are only synthesised during the late stages of infection and their synthesis requires the presence of functional IE and early polypeptides and is dependent on viral DNA synthesis. The late polypeptides comprise mainly structural proteins whose peak synthetic rate is at 15 to 18hr p.i. The onset of synthesis of early polypeptides inhibits further synthesis of the IE polypeptides and, in a similar manner, early polypeptide synthesis is inhibited by the commencement of late polypeptide synthesis.

The first demonstration that EHV-1 infected-cell polypeptide (ICP) synthesis is temporally regulated in a manner similar to HSV was carried out by examining ICP synthesis during a productive EHV-1 infection in rabbit kidney (RK) cells and the effect of metabolic inhibitors on gene expression (Caughman et al., 1985). At least 34 ICP species in the range 16.5kDa to 213kDa were identified of which 22 comigrated with virion structural proteins. Four ICPs (203kDa, 176kDa, 151kDa, 129kDa) were abundantly synthesised in infected RK cells labelled in the presence or absence of actinomycin D immediately after release from a 4hr protein synthesis block with cycloheximide. These ICPs were not detected in unblocked infected cells and were classed as IE polypeptides. 17 ICPs were classed as early proteins of which at least three (137kDa, 74kDa, 31.5kDa) accumulated in larger amounts in cultures treated with the viral DNA synthesis inhibitor phosphonoacetic acid (PAA) than in untreated cultures, suggesting that the decline in the synthesis of these species may be partly regulated by the onset of viral DNA replication. 12 ICPs were classified as late proteins on the basis of their greatly reduced synthesis in cultures in which viral DNA replication was inhibited by PAA. All but one of these species corresponded to virion structural proteins.

Gray et al. (1987a) characterised the temporal regulation of EHV-1 transcription in infected RK cells using metabolic inhibitors and demonstrated that transcription is coordinately regulated and sequentially ordered into IE, early and late phases, which supports the findings of Caughman et al. (1985) that EHV-1 ICP synthesis is also temporally regulated in such a manner. Sequences in the S component of the genome were the

first to be transcribed and it is interesting that only a single IE transcript (6.0kb) was identified that could be synthesised in the absence of protein synthesis, although 4 IE polypeptides have been detected. This IE transcript mapped within the inverted repeats at 0.78-0.83 and 0.95-1.00 m.u. Following IE gene expression, early genes in the S region were expressed and sequences at 0.84-0.87 and 0.91-0.94 m.u. were highly transcribed during the early stages of infection and a 1.0kb transcript was synthesised from these sequences. Early genes in the L region were the next to be expressed and during late viral transcription all regions of the genome were transcriptionally active.

It was subsequently shown that EHV-1 gene expression is regulated at the level of transcription although regulation at the translational level is also possible (Gray et al., 1987b). The authors also constructed a transcriptional map of EHV-1 IE, early and late mRNAs; the presence of a single 6.0kb transcript which mapped to the inverted repeats was confirmed and a 4.4kb early transcript was found that could be transcribed from sequences involved in the synthesis of the 6.0kb IE transcript, which suggested that these sequences are differentially regulated during IE and early gene expression. 41 to 45 early transcripts (0.8kb to 6.4kb) and 18 to 20 late transcripts (0.8kb to 10.0kb) were also identified and mapped to individual restriction endonuclease fragments of the EHV-1 genome. The use of in vitro translation of hybrid-selected messages has mapped 10 distinct viral proteins to the S region of the EHV-1 genome (Robertson et al., 1988a).

Further studies have been concerned with the

characterisation of the EHV-1 IE proteins. Caughman et al. (1988) investigated the relationship between the four IE proteins (IE1, IE2, IE3, IE4) by analysis of their limited proteolytic digestion profiles, antigenic cross-reactivity and pulse-chase patterns. It was concluded that the four IE species are closely related as they shared very similar peptide profiles, were antigenically cross-reactive and were either produced simultaneously from the 6.0kb IE transcript or rapidly processed to give rise to the individual forms. IE1 was the most abundant and most highly phosphorylated species. The in vitro translation products of the EHV-1 IE mRNA were analysed by Robertson et al. (1988b) who demonstrated that the family of IE polypeptides generated by in vitro translation of the 6.0kb IE mRNA were of the same size and antigenicity as those detected in infected cells. A series of time-course and pulse-chase experiments showed that synthesis of IE1 (193kDa), IE3 (166kDa) and IE4 (130kDa) occurred concomitantly, none of these species could be chased into another and at least two minor species were processed following synthesis. Since the major IE proteins were all successfully produced in vitro and none of the larger species could be chased into the smaller species, it was concluded that random proteolytic processing did not account for the production of the smaller IE proteins in vitro and in vivo. Furthermore, it was demonstrated that the 6.0kb IE mRNA species isolated during the early and late stages of infection could be translated to produce all of the major IE proteins, indicating that their synthesis was not dependent on the accumulation of IE mRNA which occurs during a cycloheximide protein block (Robertson et al., 1988b).

EHV-1 DNA Synthesis

The kinetics of EHV-1 DNA synthesis has been investigated in L-M cells (O'Callaghan et al., 1968a,b) and in the Syrian hamster (O'Callaghan et al., 1972) by pulse-labelling of infected cells or animals with [^{3}H]-deoxythymidine at different times during the infectious cycle and quantitation of the amount of radioactivity incorporated into viral DNA. This method showed that in infected L-M cells, EHV-1 DNA synthesis begins at 4 to 6hr p.i., increases rapidly and reaches a peak at 12hr p.i., following which DNA synthesis decreases rapidly. In EHV-1-infected Syrian hamsters, EHV-1 DNA synthesis begins at 3 to 4hr p.i. and continues to increase until 8hr p.i., after which DNA synthesis decreases and animals die at 12 to 14hr p.i.

Enzymatic Activities Associated With EHV-1 Infection

EHV-1 has been demonstrated to encode a number of enzymes. These include a TK (Allen et al., 1978b, 1979; McGowan et al., 1979), DNA polymerase (Kemp et al., 1975; Allen et al., 1977) and a ribonucleotide reductase (Cohen et al., 1975a; Cohen et al., 1977a; Allen et al., 1978a). EHV-1 virions have also been reported to contain a protein kinase, identifiable on the basis of its ability to phosphorylate viral proteins (Randall et al., 1972). It has been speculated that EHV-1 may also encode a deoxyribonuclease (Stock and Gentry, 1969).

Effect of EHV-1 Infection on Host Macromolecular Synthesis

In common with other herpesviruses, EHV-1 has a profound effect on macromolecular synthesis in the host. Inhibition of cellular DNA synthesis in EHV-1-infected L-M cells commences at

approximately 6hr p.i. and progresses to a maximum inhibition of 95% at 12hr p.i., the time of maximum EHV-1 DNA replication (O'Callaghan et al., 1968a,b). In contrast, infection of Syrian hamsters with EHV-1 has little effect on cellular DNA synthesis in resting liver cells and a low level of DNA synthesis is maintained during the infectious cycle (O'Callaghan et al., 1972). Inhibition of DNA synthesis is associated with a dramatic decrease in the activity of host cell DNA polymerase (Cohen et al., 1975a; Kemp et al., 1975). A marked inhibition of cellular RNA synthesis is evident in EHV-1-infected cells which commences at 2hr p.i. with the onset of IE transcription and reaches a maximum inhibition level of 95% at 12hr p.i., the time of maximum EHV-1 DNA replication. (O'Callaghan et al., 1968b; Lawrence, 1971). The analysis of EHV-1 ICP synthesis during productive infection suggests that the shut-off of host protein synthesis is a gradual but progressive process (Caughman et al., 1985).

Assembly and Envelopment of EHV-1 Capsids

In common with other herpesviruses, the assembly of EHV-1 nucleocapsids takes place in the nucleus of the infected cell and the site of envelopment appears to be the inner nuclear membrane (O'Callaghan and Randall, 1976; O'Callaghan et al., 1978; Spear and Roizman, 1980).

Three morphological forms of capsids have been purified and characterised from EHV-1-infected cells (Perdue et al., 1974, 1975, 1976; O'Callaghan and Randall, 1976; O'Callaghan et al., 1977, 1978). The light (L) capsids are essentially hollow shells since they contain no internal core structure and are devoid of

viral DNA, intermediate (I) capsids do not possess a mature core structure but exhibit an internal cross-like structure and heavy (H) capsids are mature capsid species rich in DNA which exhibit the morphology of core-containing nucleocapsids. Pulse-labelling and pulse-chase experiments in EHV-1-infected L-M cells suggested that the I capsids are the chief precursor to the H capsids and the L capsids are defective by-products that accumulate during infection (O'Callaghan and Randall, 1976; Perdue et al., 1976). Upon acquisition of viral DNA by the I capsids, the DNA becomes associated with the two major core proteins (45kDa, 30kDa) and is subsequently collapsed and packaged into a toroidal structure to form the mature H capsid. The detection of polyglutamic acid in mature H capsids has led to the suggestion that it may play a role in the collapse and packaging of viral DNA (O'Callaghan and Randall , 1976; Perdue et al., 1976).

The envelopment of EHV-1 nucleocapsids at the nuclear membrane occurs by the mechanisms of budding or invagination of the nuclear membrane (Darlington and Moss, 1968; O'Callaghan and Randall, 1976; O'Callaghan et al., 1978).

Oncogenic Transformation and Persistent Infection by EHV-1

Preparations of EHV-1 enriched for defective interfering particles (DIPs) (Robinson et al., 1980b; Dauenhauer et al., 1982), and UV-irradiated EHV-1 virus (Robinson et al., 1980a), have been shown to oncogenically transform primary hamster embryo fibroblasts in culture. The inoculation of EHV-1-transformed cells into adult syngeneic hamsters leads to the formation of highly metastatic tumours (Robinson et al., 1980a;

Dauenhauer et al., 1982). Transformed and tumour cell lines have been established which express viral proteins and contain stably integrated viral DNA sequences (Robinson et al., 1981; O'Callaghan et al., 1983; Robinson and O'Callaghan, 1983). The region of the EHV-1 genome that is stably integrated corresponds to 0.32 to 0.38 m.u., implying that integration of EHV-1 sequences is associated with oncogenic transformation (Robinson et al., 1981; Robinson and O'Callaghan, 1983). In addition to establishing oncogenic transformation, EHV-1 DIPs are also associated with the establishment of persistent infection (Robinson et al., 1980b; Dauenhauer et al., 1982). Persistently infected cell lines (DI cells) have been established which, unlike the tumour cell lines, contain a small proportion of cells that continue to release standard EHV-1 virus and DIPs even after extensive passage in culture (Robinson et al., 1980b; Dauenhauer et al., 1982). The vast majority of DI cells are oncogenically transformed and, in common with EHV-1-transformed cells, can produce tumours in adult syngeneic hamsters. Viral transcription in EHV-1 DIP-enriched infections is temporally regulated in an IE, early and late manner as in a standard EHV-1 infection (Gray et al., 1989).

CHAPTER 2

General Materials and Methods

The materials and methods described in this chapter are those routinely used throughout the experiments presented in this thesis. Methods specifically related to work in individual chapters are described in detail in the Materials and Methods section of that chapter.

MATERIALS

Materials used during the course of the studies presented in this thesis were obtained from the sources listed below.

DNA

Plasmids pUC8 and pBR322, λ-HindIII size markers:- Bethesda Research Laboratories (BRL), Paisley, U.K.

Bluescript plasmid and Bluescript-specific primers:- Northumbria Biologicals Limited (NBL), Cramlington, U.K.

Plasmid pGEX-2T:- Pharmacia LKB Biotechnology, Milton Keynes, U.K.

Plasmid pACYC-EcoRI(F):- kindly provided by Dr. J. B. Clements, MRC Institute of Virology, Glasgow, U.K.

Plasmid pUR288:- kindly provided by Mr. Robert MacFarlane, Beatson Institute for Cancer Research, Glasgow, U.K.

EHV-4 genomic library cloned in pUC9:- kindly provided by Dr. Ann Cullinane, Irish Equine Centre, Naas, Eire.

Restriction Endonucleases/Modifying Enzymes

Restriction endonucleases, T4 DNA ligase:- BRL.

Calf intestinal alkaline phosphatase (molecular biology grade), Klenow fragment of T4 DNA polymerase I, proteinase K, DNase I,

RNase A:- Boehringer Corporation Limited (BCL), Lewes, U.K.

Nick translation kit:- Amersham International plc, Amersham, U.K.

Bacterial Strains

Escherichia coli (E.coli) strains JM101 and Y1090:- NBL.

Chemicals

Tris-equilibrated phenol:- Rathburn Chemicals Limited, Walkerburn, U.K.

Formamide:- Fluka Chemicals Limited, Glossop, U.K.

Ampicillin, tetracycline, caesium chloride, deoxynucleotide triphosphates, dideoxynucleotide triphosphates:- BCL.

Agarose, low melting point agarose, acrylamide, methylene-bisacrylamide, tetramethylethylenediamine (TEMED), ammonium persulphate:- BRL.

Sephadex G-50, bromophenol blue, xylene cyanol, Bind-Silane, Repel-Silane:- Pharmacia-LKB Biotechnology.

Tryptone, yeast extract, bacteriological agar, bactopeptone:- Oxoid Limited, Basingstoke, U.K.

PBS tablets:- Flow Laboratories, Rickmansworth, U.K.

Radioisotopes

[α-^{35}S]dATP, [α-^{32}P]dCTP:- Amersham International plc.

Miscellaneous Items

Polaroid type 57 film:- Polaroid (U.K.) Limited, St. Albans, U.K.

X-ray film (Hyperfilm):- Amersham International plc.

Spin-X filter centrifuge tubes:- NBL.

Whatman 3MM paper:- Whatman International Limited, Maidstone, U.K.

Gene Screen hybridisation membranes:- DuPont (U.K.) Limited, Stevenage, U.K.

All other materials were obtained from BDH Limited, Poole, U.K. or Sigma Chemical Company Limited, Poole, U.K.

METHODS

Growth and Storage of Parental Plasmids

The E.coli strain JM101 carrying the appropriate plasmid was inoculated into 5ml of L broth containing the appropriate antibiotic at the standard working concentration (Table 2.1). All plasmids used carried the ampicillin resistance (Amp^R) gene with the exception of plasmid pACYC184-EcoRI(F) which carried the gene for tetracycline resistance (Tet^R). Bacterial cultures were grown by shaking in an orbital incubator for 16 to 20hr at 37°C. 1ml of this culture was used to prepare a glycerol stock by adding an equal volume of 80% v/v glycerol/2% w/v bactopeptone, and this stock was then stored at -20°C until required. Of the remaining culture, 1ml was inoculated into 500ml of L broth containing antibiotic and incubated in an orbital shaker for 16 to 20hr at 37°C. Bacterial cells were pelleted by centrifugation (7000rpm/10min/4°C) in a Beckman JS7.5 rotor.

Large Scale Isolation of Plasmid DNA

Plasmid DNA was isolated by a modification of the alkaline lysis procedure previously described by Birnboim and Doly (1979). The pellet of a 500ml bacterial culture carrying recombinant plasmid was resuspended in 50ml of solution I (Table 2.1) and the lysate was left on ice for 30min. 80ml of solution II (Table 2.1) was added with gentle mixing to the lysate and kept on ice for 10min. 40ml of solution III (Table 2.1) was then added with gentle mixing and the lysate incubated on ice for a further 15min. The lysate was centrifuged

(7000rpm/20min/4°C) in a Beckman JS7.5 rotor to pellet bacterial cell debris. The supernatant was retained and filtered through sterile cotton gauze, if necessary, to remove any floating debris. Plasmid DNA was precipitated by the addition of 100ml (0.6 volume) of isopropanol followed by centrifugation (7000rpm/20min/0°C) in a Beckman JS7.5 rotor. The pellet was resuspended in 19ml of TE buffer (10mM TrisHCl pH 8.0, 1mM EDTA), to which was added 20g of caesium chloride and 1.8ml of 3mg/ml ethidium bromide. The refractive index of the solution was checked (should be 1.388 to 1.390), the sample loaded into two 12ml Beckman 'Quick-Seal' centrifuge tubes and the tubes sealed using a Beckman heat sealing apparatus. Samples were centrifuged in a Beckman fixed angle 50Ti rotor at 40000rpm and 18°C for 40 to 48hr. Using such an isopycnic gradient RNA pellets at the bottom of the tube and any protein present forms a pellet at the top of the tube. Two bands formed on the gradient- the lower, largest band was the desired closed circular plasmid DNA and the upper, very faint band consisted of linear and nicked circular plasmid DNA. The closed circular plasmid DNA was harvested by insertion of a 19-gauge syringe needle into the side of the tube and withdrawal of the band into a 2ml syringe. Ethidium bromide was removed from the DNA by addition of an equal volume (1 to 2ml) of isopropanol, vigorous mixing and retention of the lower aqueous phase. This was repeated at least four times, with 0.2ml of distilled water (dH_2O) being added to the aqueous phase between extractions to maintain the volume, until all traces of ethidium bromide were removed from the aqueous phase. Finally, the DNA was dialysed against 0.1xTE buffer for 3 x 2hr to remove caesium chloride,

aliquoted into 1.5ml centrifuge tubes and precipitated with two volumes of ethanol in the presence of 0.3M sodium acetate (pH 6.0) at -70°C for 30min. The DNA was pelleted by centrifugation in a benchtop microcentrifuge at 13000rpm for 10min, briefly washed with 70% ethanol and dried in a vacuum dessicator. DNA was resuspended in dH_2O to a final concentration of 0.5mg/ml and stored at -20°C until required.

Restriction Endonuclease Digestion of Plasmid DNA

The structure of plasmid DNA was routinely confirmed by digestion with several restriction endonucleases which were used according to the suppliers instructions. Typically, 1 to 3ug of plasmid DNA was added to 0.1 volume of the appropriate 10x restriction endonuclease buffer (BRL, React buffer system). dH_2O was added to bring the buffer to the working concentration and spermidine (Table 2.1) was added to a final concentration of 1mM. The appropriate restriction endonuclease was added (3 units/ug of DNA), the contents gently mixed and incubated at 37°C for 3hr. Digestion was terminated by the addition of 0.1 volume of sample loading dye (50% w/v glycerol, 0.1% w/v bromophenol blue).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out on horizontal agarose gel slabs submerged in 1xTBE electrophoresis buffer (Table 2.1). Agarose (0.8g) was dissolved to a final concentration of 0.8% in 1xTBE buffer (100ml) by heating in a microwave oven. After cooling to 50°C, the molten agarose was poured into the gel box and the well-forming comb inserted.

After the gel had solidified, the comb was removed and the gel submerged in electrophoresis buffer. All agarose gels shown in this thesis were 0.8% and run in 1xTBE buffer. Samples were loaded into the wells and gels run at a constant voltage of 2 to 5V/cm for 1hr to overnight. Gels were stained with ethidium bromide in dH_2O at 1ug/ml for 30min and destained in dH_2O for 30min. DNA was visualised on a 302nm UV transilluminator and photographed using Polaroid type 57 film.

Purification of DNA Fragments from Agarose Gels

Plasmid DNA digested with restriction endonucleases was run on an agarose gel in 1xTBE buffer. The gel was stained and destained as described previously and the appropriate DNA fragments were excised using a sterile scalpel. Excised gel slices were transferred to Spin-X filter centrifuge tubes, stored at -20°C for 20min and then centrifuged at 13000rpm in a benchtop microcentrifuge for 30min. The eluate was successively extracted with an equal volume of phenol (phenol is equilibrated with 1M TrisHCl, pH 8.0), phenol/chloroform (1:1), chloroform and then ether and the DNA was precipitated with two volumes of ethanol in the presence of 0.3M sodium acetate (pH 6.0) at -70°C for 30min. The DNA was pelleted by centrifugation, washed briefly with 70% ethanol and dried in a vacuum dessicator. This precipitation procedure was repeated once more, the DNA was finally resuspended in 20 to 50ul of dH_2O and stored at -20°C until required.

Alternatively, where larger amounts of DNA fragment were required DNA was purified through low melting point agarose gels. In this case the desired DNA fragment was excised from the

gel, the gel melted by incubation at 65°C for 10min and diluted with two volumes of dH_2O (5 to 10ml). Residual agarose was removed by three successive phenol extractions and one chloroform extraction and the volume was reduced to about 0.5ml by several extractions with butanol. Finally, the DNA was precipitated and recovered as described above. In each case, the purity of the recovered DNA fragment was checked by running a small aliquot on an agarose gel.

Construction of Recombinant Plasmids

When a restriction fragment with homologous ends was to be cloned, alkaline phosphatase treatment of linearised vector DNA was carried out to prevent recircularisation of vector DNA during the ligation reaction. 5ug of plasmid DNA was digested with the appropriate restriction endonuclease for 3hr using standard conditions. At the end of the digestion 8 units of calf intestinal alkaline phosphatase (molecular biology grade) was added to the digested DNA and the incubation continued at 37°C for a further 30min. The DNA was extracted with equal volumes of phenol, phenol/chloroform (1:1), chloroform and ether, and then precipitated with two volumes of ethanol in the presence of 0.3M sodium acetate (pH 6.0) at -70°C for 30min. DNA was pelleted by centrifugation, washed with 70% ethanol, dried in a vacuum dessicator, resuspended in 20 to 50ul of dH_2O and its purity checked by agarose gel electrophoresis.

Alkaline phosphatase treatment was not necessary when vector DNA was digested with two restriction endonucleases giving non-homologous termini, since the possibility of recircularisation of vector DNA in this case is minimal.

However, it was necessary to purify vector DNA digested in this manner by agarose gel electrophoresis to remove the smaller DNA fragment which would otherwise interfere with the ligation reaction.

Recombinant plasmids were constructed by ligation of vector and insert DNA fragments as follows. Generally, 100ng of vector DNA was ligated to a three molar excess of insert DNA with compatible termini using 2 units of T4 DNA ligase in the presence of 50mM TrisHCl pH 7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% w/v PEG-8000 (T4 DNA ligase buffer, BRL), in a total volume of 20ul, at 14°C for 16 to 20hr. For each ligation reaction 100ng of vector DNA was tested for the ability to self-ligate in a separate reaction. This acted as a negative control and served to indicate the efficiency of preparation of the vector DNA.

Transformation of Competent E.Coli Cells With Plasmid DNA

E.coli cells competent for DNA uptake were prepared by the calcium chloride method (Maniatis et al., 1982). Essentially, 1ml of an overnight culture of E.coli strain JM101 was inoculated into 100ml of L broth and grown at 37°C with constant shaking in an orbital incubator until the optical density (OD) reading at 600nm was approximately 0.4 (took 2.5 to 3hr). Bacterial cells were pelleted by centrifugation in 50ml polypropylene conical tubes in a benchtop centrifuge at 2500rpm for 5min at 4°C, resuspended in 30ml of 0.1M $MgSO_4$ and placed on ice for 30min. Cells were pelleted by centrifugation as above, resuspended in 30ml of 0.05M $CaCl_2$ and incubated on ice for 15min. Cells were again recovered by centrifugation,

resuspended in 2ml of 0.05M $CaCl_2$ and stored on ice if required within 24hr or stored in 15% glycerol at -70°C for use at a later date.

The contents of each ligation reaction were added to 0.2ml of competent E.coli cells prepared as described above and incubated on ice for 30min. This cell-DNA mixture was heat shocked by incubating at 37°C for 5min to facilitate entry of DNA into the bacterial cells. To the heat shocked mixture was added 1ml of L broth and the bacteria were incubated at 37°C for 1.5hr to allow expression of antibiotic resistance genes. Cells were spread onto five L agar plates (Table 2.1) containing the appropriate antibiotic and plates were incubated for 15min at room temperature to allow the liquid to be absorbed. Plates were inverted and incubated at 37°C for 16 to 20hr. Transformants appeared as separate, well-defined colonies on the surface of the agar plates.

Small Scale Preparation of Plasmid DNA by the Boiling Method

Transformants were screened for the presence of the desired recombinant plasmid by small scale isolation of plasmid DNA according to the boiling method (Holmes and Quigley, 1981). 8 to 24 bacterial colonies were picked from agar plates into 3ml of L broth containing ampicillin using sterile cocktail sticks. Cultures were grown in an orbital shaker at 37°C for 16 to 20hr. The cells from 1ml of each culture were pelleted by centrifugation at 13000rpm for 1min in a benchtop microcentrifuge, the remaining 2ml being retained as a stock preparation. Cells were resuspended in 75ul of STET buffer (Table 2.1) and 25ul of a freshly prepared solution of lysozyme

(10mg/ml in dH_2O) was added. Samples were mixed by gentle vortexing, boiled in a water bath for 40 seconds and the lysates were centrifuged at 13000rpm for 10min in a benchtop microcentrifuge. Plasmid DNA was precipitated from the supernatants by addition of an equal volume (100ul) of isopropanol and storage at -70°C for 30min. DNA was pelleted by centrifugation at 13000rpm for 10min in a benchtop centrifuge, washed with 70% ethanol, dried in a vacuum dessicator and resuspended in 30ul of dH_2O. 10ul of DNA was digested in each restriction endonuclease reaction as previously described. Using this method, yields of 1ug DNA/ml of culture were routinely obtained.

TABLE 2.1

General Stock Solutions and Buffers

L Broth Medium

tryptone	10g
yeast extract	5g
sodium chloride	10g
d_2O	to 1 litre

Sterilise by autoclaving.

L Agar (1.5%)

L broth	200ml
agar	3g

Sterilise by autoclaving.

NZCYM Medium

NZ amine (Type-A hydrolysate of casein)	10g
NaCl	5g
yeast extract	5g
casamino acids	1g
$MgSO_4.7H_2O$	2g
dH_2O	to 1 litre

Sterilise by autoclaving.

Ampicillin

Ampicillin (sodium salt) at 100mg/ml in dH_2O. Sterilise by filtration. Store at -20°C. Use at final concentration of 100ug/ml.

Tetracycline

Tetracycline hydrochloride at 12.5ug/ml in 50% ethanol. Sterilise by filtration. Store at -20°C. Use at final concentration of 12.5ug/ml.

Solution I

0.2M EDTA (pH 8.0)	2.5ml
1M TrisHCl (pH 8.0)	1.25ml
1M glucose	2.5ml
dH_2O	43.75ml

Solution II

5M NaOH	3.2ml
10% SDS	8.0ml
dH_2O	68.8ml

Solution III

5M potassium acetate	60ml
glacial acetic acid	11.5ml
dH_2O	28.5ml

STET Buffer

0.2M EDTA (pH 8.0)	25ml
1M TrisHCl (pH 8.0)	5ml
Triton X-100	5ml
sucrose	8g
dH_2O	to 100ml

10xTBE

Tris base	108g
boric acid	55g
0.2M EDTA (pH 8.0)	50ml
dH_2O	to 1 litre

Use at final concentration of 1x.

3M Sodium Acetate

sodium acetate trihydrate 40.81g.
Dissolve in 80ml dH_2O. Adjust to pH 6.0 with glacial acetic acid. Make up volume to 100ml with dH_2O. Sterilise by autoclaving.

0.1M Spermidine

145mg spermidine in 10ml dH_2O.
Sterilise by filtration.

1M Tris

Tris base	121.1g
dH_2O	800ml

Adjust to required pH with concentrated HCl. Make up volume to 1 litre with dH_2O. Sterilise by autoclaving.

0.2M EDTA (pH 8.0)

EDTA	74.4g
dH_2O	800ml

Adjust to pH 8.0 with NaOH. Make up volume to 1 litre with dH_2O. Sterilise by autoclaving.

5M NaCl

NaCl	292.2g
dH_2O	to 1 litre

Sterilise by autoclaving.

5M NaOH

NaOH	200g
dH_2O	to 1 litre

Sterilise by filtration.

10% SDS

sodium dodecyl sulphate	100g
dH_2O	to 1 litre

Ethidium Bromide (10mg/ml)

ethidium bromide	1g
dH_2O	to 1 litre

Store away from light.

SM

NaCl	5.8g
$MgSO_4.7H_2O$	2.0g
1M TrisHCl (pH 7.5)	50ml
2% gelatin	5ml
dH_2O	to 1 litre

Sterilise by autoclaving.

Proteinase K

Proteinase K at 20mg/ml in dH_2O. Store at -20^oC. Use at final concentration of 50ug/ml.

RNase

RNase A at 10mg/ml in dH_2O. Boil for 10min. Store at -20^oC. Use at final concentration of 1ug/ml.

DNase

DNase I at 3mg/ml in dH_2O. Store at -20^oC. Use at final concentration of 1ug/ml.

CHAPTER 3

Identification and Restriction Endonuclease Mapping of the EHV-4 Glycoprotein gB Gene

INTRODUCTION

Currently available vaccines licensed to protect against EHV-induced abortion and respiratory disease are generally considered to be disappointing from the standpoint of efficacy and safety. Consequently, in recent years emphasis has been placed on the development of safer, more effective vaccines by the use of recombinant DNA technology. The first step in the development of such vaccines involves the characterisation of the individual glycoprotein species of the virus envelope. Antigens that elicit a protective immune response could be developed for use as subunit vaccines by their expression in prokaryotic vector systems.

At the onset of the studies presented in this thesis no glycoprotein genes of EHV-4 had been sequenced. In this chapter data is presented on the genomic location of the glycoprotein gB gene of EHV-4. This particular envelope component was chosen for further study for a number of reasons. As discussed in Chapter 1 of this thesis, extensive studies on the gB component of HSV-1 have demonstrated essential roles for gB in viral infectivity (Sarmiento et al., 1979; Little et al., 1981) and cell fusion (Manservigi et al., 1977; DeLuca et al., 1982). Moreover, gB can invoke both humoral (Norrild, 1980; Glorioso et al., 1984; Eberle et al., 1985; Blacklaws et al., 1987; Cantin et al., 1987) and cell-mediated (Lawman et al., 1980; Carter et al., 1981; Chan et al., 1985; Zarling et al., 1986a; Blacklaws et al., 1987) immune responses, and can confer protective immunity (Chan et al., 1985; Blacklaws et al., 1987; Cantin et al., 1987). As such, gB is an attractive virion envelope component

for vaccine development.

At the onset of the studies presented in this thesis it was known that gB homologues were encoded by the betaherpesvirus HCMV (Cranage et al., 1986) and the gammaherpesvirus EBV (Baer et al., 1984; Pellett et al., 1985a) in addition to the alphaherpesviruses HSV-1 (Bzik et al., 1984a; Pellett et al., 1985b), HSV-2 (Bzik et al., 1986; Stuve et al., 1987) and VZV (Davison and Scott, 1986a; Keller et al., 1986). The fact that gB is encoded by representatives of all three herpesvirus subgroups (α, β and γ) suggested that this glycoprotein may have important conserved functions. It thus seemed likely that EHV-1 (and therefore EHV-4) would also specify gB on the surface of their envelopes. Unequivocal evidence for this was presented by Snowden et al. (1985) who used an antiserum to the oligomeric form of HSV-1 gB strain 17 to immunoprecipitate gB-related protein species from cells infected with EHV-1. The tryptic peptide profiles of the EHV-1 and HSV-1 gB proteins were similar, confirming that the two proteins share a high degree of structural homology. In a separate study, gB-related polypeptides were detected by Western blotting in HSV-1-, HSV-2-, BHV-2- and EHV-1-infected cells using polyvalent rabbit antiserum against EHV-1 (Snowden and Halliburton, 1985).

Thus, given the essential role of gB in viral infectivity and immunity and evidence for its presence in EHV-1 virions, it was undertaken to determine the genomic location of gB in EHV-4 strain 1942. The gene for EHV-4 gB was identified within a genomic library by a combination of dot blot and Southern blot hybridisation analysis using probes derived from the HSV-1 gB gene. The derivation of probes from the HSV-1 gB DNA sequence

was facilitated by the available mapping and DNA sequence data for the genomic region of HSV-1 which encodes the gB gene. Analysis of HSV-1 mutants temperature-sensitive for growth and accumulation of gB localised the gB gene to between 0.345 and 0.368 m.u. (Little et al., 1981; DeLuca et al., 1982; Holland et al., 1983b). Independent studies mapped a mRNA of approximately 3.1 to 3.3kb in this region (Bzik et al., 1984a; Holland et al., 1984a) and this mRNA was confirmed to be that for gB by Rafield and Knipe (1984), who showed that the polypeptide produced by in vitro translation of this species could be immunoprecipitated with monoclonal antibodies to gB. Nucleotide sequence analysis of the gB gene has been carried out for HSV-1 strain KOS (Bzik et al., 1984a) and strain F (Pellett et al., 1985b). Localisation of the EHV-4 gB gene was facilitated by the known colinearity of the EHV-4 genome with the I_L-I_{SL} arrangement of the HSV-1 genome (Cullinane et al., 1988). The available map position for the HSV-1 gB gene allowed a prediction of the EHV-4 genomic region that may contain the gB gene and the 5′ and 3′ boundaries were determined using 5′ and 3′ HSV-1 gB DNA probes. Hybridisation data was confirmed by restriction endonuclease mapping of the genomic region to which the HSV-1 gB sequences hybridised. The restriction endonuclease map of this region identified a suitable fragment for subcloning into a plasmid vector for subsequent detailed sequence analysis of this gene.

MATERIALS AND METHODS

Cloning of HSV-1 gB DNA Sequences

The plasmid pACYC-EcoRI(F) was double-digested with KpnI/XhoI, the products of digestion separated on a 0.8% agarose gel and the 3.3kb fragment excised and purified through Spin-X tubes as previously described. This fragment was ligated between the KpnI and XhoI sites of plasmid vector pIC20R using techniques described previously to generate the plasmid pICgB.

Restriction Endonuclease Mapping of EHV-4 DNA

Restriction endonucleases were used according to the suppliers instructions (BRL). The products of restriction endonuclease digests were separated by electrophoresis through 0.8% agarose gels run in 1xTBE buffer. Bacteriophage lambda (λ) DNA fragments generated by digestion with HindIII were used as size markers.

Dot Blot Analysis

The C, F and M viral restriction fragments from an EHV-4 library cloned in the BamHI site of pUC9 were excised with BamHI and purified through low melting point agarose gels as previously described. Purified fragments were spotted onto Gene Screen hybridisation membranes, previously wetted in Gene Screen transfer buffer (Table 3.1), in 100ng, 200ng and 400ng amounts. In a subsequent experiment, the four subfragments of BamHI-C obtained by digestion with SmaI were spotted onto membranes as described above. In all experiments, 100ng of plasmid vectors pBR322 and pUC8 were used as negative controls and 100ng of

unlabelled probe DNA was used as a positive control. The DNA was left to dry onto membranes for 5min at room temperature. Membranes were placed, DNA facing upwards, on a sheet of Whatman 3MM paper saturated with denaturation solution (0.5M NaOH/1.5M NaCl) for 2 x 5min at room temperature, ensuring that the uppermost surface of the membranes did not become covered in solution. DNA was neutralised by 2 x 5min exposures of membranes to 1M TrisHCl pH 8.0/1.5M NaCl as described above. Membranes were briefly air dried and DNA was immobilised by baking membranes between two sheets of Whatman 3MM paper at 80°C for 2hr.

Southern Blot Analysis

The 13.6kb BamHI-C fragment of EHV-4 (which was cloned in pUC9) was subjected to Southern blot analysis (Southern, 1975). 2ug of plasmid DNA was digested with restriction endonuclease BamHI and also double-digested with BamHI and each of the restriction endonucleases BglI, EcoRI, PstI, PvuII, and SmaI to release viral DNA sequences from vector sequences, using standard conditions. Double digests were carried out in a single step in React 2 buffer (BRL) (50mM TrisHCl pH 8.0, 10mM $MgCl_2$, 50mM NaCl) except for BamHI/SmaI digestion which required sequential digestions (SmaI will not operate in React 2 buffer and requires its own buffer, React 4: 20mM TrisHCl pH 7.4, 5mM $MgCl_2$, 50mM KCl). Digested DNA was electrophoresed on 0.8% gels in 1xTBE buffer, stained with ethidium bromide and photographed under UV light as previously described. The gel was placed in a glass baking dish and any unused areas of the gel were removed with a sharp scalpel. The DNA was denatured by soaking the gel

in 250ml of 0.5M NaOH/1.5M NaCl for 2 x 15min at room temperature with gentle shaking. The gel was rinsed with dH_2O and then neutralised by soaking in 250ml of 1M TrisHCl pH 8.0/ 1.5M NaCl for 2 x 15min at room temperature with gentle shaking. The gel was rinsed with dH_2O and then soaked for 3 x 20min in Gene Screen transfer buffer at room temperature with gentle shaking. The gel was now ready for Southern transfer to a hybridisation membrane. A suitably sized glass plate (to act as a support) was placed on top of four rubber bungs in a glass dish (6 to 8cm in depth) - this glass dish acted as a buffer reservoir during transfer. A sheet of Whatman 3MM paper (58cm x 68cm) was cut into quarters and these four sheets were wrapped around the glass plate support, so that the overhanging edges could act as wicks for buffer transfer. Gene Screen transfer buffer was added to the dish until the level was just below the glass support and any air bubbles present in the 3MM paper were removed. The gel was placed onto the saturated 3MM paper and air bubbles between the gel and the 3MM paper were removed. A piece of Gene Screen membrane, cut to the required size, was briefly soaked in transfer buffer and placed in position on the gel, and all air bubbles between the gel and the membrane were removed. Four sheets of 3MM paper, cut to the same size as the gel, were wet in transfer buffer and placed on top of the membrane and all air bubbles were removed. A stack of paper towels (6 to 8cm high) was placed on the 3MM paper and a glass plate was put on the top of the stack. A light weight was applied to the stack to ensure even contact during transfer. Finally, the area around the gel was surrounded with Saran wrap to prevent the short-circuiting of fluid between the 3MM

paper under the gel and the paper towels. Transfer was allowed to proceed for 16 to 20hr, the membrane was carefully removed and soaked in transfer buffer for 5min. Excess fluid was drained from the membrane which was then briefly air dried at room temperature. The membrane was placed between two sheets of 3MM paper and baked at 80°C for 2hr to immobilise DNA.

Preparation of DNA Probes (Nick Translation)

All probes were prepared by the method of nick translation in which DNA is labelled to high specific activity by E.coli DNA polymerase I (Rigby et al., 1977). Generally, 0.5 to 1ug of DNA fragment previously purified through low melting point agarose gels or Spin-X tubes was used in a nick translation reaction. The reaction was set up as follows: 10ul 5x nick translation nucleotide/buffer solution (Amersham), 5ul enzyme solution (Amersham), 10ul (100uCi) [α-^{32}P]dCTP (S.A.>800 Ci/mmole), 0.5 to 1ug DNA in TE, dH_2O to 50ul. The contents were mixed by pipetting and the reaction was incubated at 14°C for 2.5hr. The reaction was terminated by the addition of 5ul of 0.2M EDTA. Unincorporated label was removed from incorporated label by separation through a Sephadex G-50 column. Sephadex G-50 was prepared by adding 30g of powder to 250ml of TE and heating at 65°C for 2hr. After cooling to room temperature, the supernatant was replaced with an equal volume of TE. A Sephadex G-50 column was prepared by packing a 5ml glass pipette plugged with glass wool with Sephadex G-50 prepared as described above, and the column was washed with two column volumes of TE. The DNA sample was applied to the column and once the sample had entered the matrix a reservoir of TE was connected to the

column. The separation of labelled DNA from free labelled nucleotide was monitored with a Geiger counter- two peaks were observed, the leading peak corresponding to the labelled DNA. The labelled DNA was collected into a 1.5ml centrifuge tube (an eluate of 1 to 1.5ml), the radioactivity measured by counting in a liquid scintillation counter (a specific activity of 1 to 2 x 10^8 cpm/ug of DNA was routinely obtained) and the labelled DNA stored at -20°C until required. Labelled DNA was denatured prior to use in hybridisations by boiling for 10min.

Hybridisation of Immobilised DNA to Labelled DNA Probes

Membranes containing immobilised DNA were hybridised to radioactively labelled DNA probes as follows. Membranes were wet in transfer buffer and then prehybridised in 10ml of hybridisation buffer (Table 3.1) at 42°C for 16 to 20hr, with constant agitation, in heat-sealable plastic bags. All air bubbles were expelled from the bags prior to sealing. Denatured probe DNA was added to the prehybridised membranes at a concentration of 10ng/ml of hybridisation buffer (10^6 cpm/ml of hybridisation buffer for a probe of specific activity 10^8cpm/ug of DNA). This was accomplished by mixing the appropriate volume of probe DNA with 2ml of hybridisation buffer, adding this to the bags containing the membranes in hybridisation buffer, mixing evenly by hand and expelling all air bubbles prior to resealing the bags. Hybridisation was carried out at 42°C for 16 to 20hr with constant shaking. Following hybridisation, membranes were carefully removed from the bags and prewashed in 2xSSC for 2 x 15min at room temperature. Unbound probe was removed from membranes by washing

in either 2xSSC-0.1% SDS, 1xSSC-0.1% SDS, or 0.1xSSC-0.1% SDS for 3 x 20min at 65^{o}C with constant shaking. Finally, membranes were rinsed in 0.1xSSC, air dried, sealed in plastic bags, and exposed to X-ray film at -70^{o}C for 18 to 24hr.

TABLE 3.1

Hybridisation Stock Solutions and Buffers

Gene Screen Transfer Buffer (40x)

NaH_2PO_4	156g
Na_2HPO_4	178g
dH_2O	800ml

Adjust to pH 6.5 with HCl. Make up volume to 1 litre with dH_2O. Use at final concentration of 1x.

20xSSC

NaCl	175.3g
sodium citrate	88.2g
dH_2O	800ml

Adjust to pH 7.0 with NaOH. Make up volume to 1 litre with dH_2O.

Denatured Salmon Sperm DNA

Salmon sperm DNA at 10mg/ml in dH_2O. Shear by repeated passage through a 21-gauge needle. Boil for 10min. Store at -20°C. Boil for 5min just before use.

Dextran Sulphate (50%)

50g dextran sulphate in 80ml dH_2O. Dissolve by vigorous stirring. Make up volume to 100ml with dH_2O. Store at -20°C.

Denhardt's Solution (50x)

Ficoll	5g
polyvinlypyrrolidone	5g
bovine serum albumin (Fraction V)	5g
dH_2O	to 500ml

Store at -20°C.

Hybridisation Buffer

40% formamide
5xDenhardt's solution
5xSSC
10% dextran sulphate
2xGene Screen transfer buffer
0.1% SDS
100ug/ml denatured salmon sperm DNA

RESULTS

Derivation of HSV-1 gB DNA Hybridisation Probes

The derivation of probes was aided by an available restriction endonuclease map of the HSV-1 EcoRI-F fragment which contains the gB gene (Figure 3.1). The 3.3kb KpnI/XhoI fragment (the smallest subfragment of EcoRI-F which contains the entire gB gene) was excised from plasmid pACYC-EcoRI(F) (Figures 3.2 and 3.3) and inserted between the KpnI and XhoI sites of plasmid vector pIC20R to generate the recombinant plasmid pICgB (Figure 3.4). Analysis of the HSV-1 gB DNA sequence demonstrated the fortuitous location of NarI and PstI restriction endonuclease sites that would allow the generation of 5′ and 3′ probes. Thus, digestion of pICgB with NarI would release a 650bp fragment encoding amino acids 121 to 337 of the gB protein. This was called the 5′ NarI probe (Figure 3.4). Digestion of pICgB with PstI would release an 810bp fragment encoding amino acids 531 to 800 of the gB protein. This was called the 3′ PstI probe (Figure 3.4). The restriction endonuclease analysis of pICgB is shown in Figure 3.5. The salient features of the HSV-1 gB gene and the regions used as probes are shown in Figure 3.6.

Dot Blot Analysis

Initial experiments were carried out to unequivocally identify the EHV-4 BamHI genomic fragment which contains the gB gene by dot blot analysis. On the basis of previous colinearity studies between EHV-4 and HSV-1 (Cullinane *et al.*, 1988), the EHV-4 gB gene was predicted to map within BamHI-C. The contiguous BamHI-C, F and M fragments of the EHV-4 genome were

used in dot blot analysis. The 5′ NarI probe hybridised only to BamHI-C (Figure 3.7). The 3′ PstI probe also hybridised only to BamHI-C (Figure 3.8). Since BamHI-C is a large fragment (13.6kb), it was necessary to identify the exact region of this fragment to which the HSV-1 gB probes hybridised.

The structure of the plasmid pUC9 containing the EHV-4 BamHI-C fragment is shown in Figure 3.9. Restriction endonuclease analysis of this plasmid with SmaI and BamHI/SmaI revealed that three SmaI sites are present in BamHI-C which dissect it into four subfragments of approximately 6.5kb, 3.4kb, 2.3kb and 1.4kb, and that the rightmost SmaI site occurs 3.4kb from the right terminus of BamHI-C (Figure 3.10a, lanes 1 and 2). As such, each of these four subfragments of BamHI-C could then be analysed by dot blotting to determine which subfragment contains the gB gene. Prior to this it was necessary to obtain a restriction map of BamHI-C complete for SmaI. This was constructed by a series of digests shown in Figure 3.10a (lanes 3 to 8). There is a single KpnI site in BamHI-C giving two fragments of 9.4kb and 4.2kb (lane 3). To determine the order of the two remaining SmaI sites and the single KpnI site, purified 9.4kb and 4.2kb BamHI/KpnI subfragments of BamHI-C were digested with EcoRI, since the position of the EcoRI sites within BamHI-C was known. Since the 9.4kb BamHI/KpnI and 6.5kb SmaI-generated fragments contained the two EcoRI sites (lanes 4 and 6) and the 4.2kb BamHI/KpnI fragment did not (lane 5), it was concluded that the first SmaI site occurs 6.5kb, and the first KpnI site 9.4kb, from the left terminus of BamHI-C. The position of the remaining SmaI site was determined by digestion of the 9.4kb and 4.2kb BamHI/KpnI fragments with SmaI (lanes 7 and 8). The

restriction map of BamHI-C complete for EcoRI, KpnI and SmaI, consistent with the mapping data in Figure 3.10a, is shown in Figure 3.10b.

Dot blot analysis of the four subfragments of EHV-4 BamHI-C generated by BamHI/SmaI digestion demonstrated that both the 5′ NarI and 3′ PstI HSV-1 gB probes hybridised only to the 6.5kb BamHI/SmaI left-terminal subfragment (Figures 3.11 and 3.12).

Southern Blot Analysis

The plasmid pUC9 containing EHV-4 BamHI-C was digested with BamHI and double-digested with BamHI and each of the restriction endonucleases BglI, EcoRI, PstI, PvuII and SmaI to release EHV-4 sequences from vector sequences. A gel showing the fractionated fragments is presented in Figure 3.13 and the estimated sizes of these fragments are presented in Table 3.2. The results of hybridisation of the HSV-1 gB probes to the transferred fragments are shown in Figures 3.14 and 3.15 and the EHV-4 fragments to which each probe hybridised are denoted in Table 3.3. In conclusion, these results demonstrate that the major portion of the EHV-4 gB gene resides within the 2.9kb BamHI/EcoRI subfragment at the left terminus of BamHI-C (Figure 3.10b), the smallest EHV-4 fragment demonstrated to contain the regions to which both HSV-1 gB probes hybridised.

Derivation of a Restriction Map of the 2.9kb Fragment Containing the EHV-4 gB Gene

A restriction map of the 2.9kb BamHI/EcoRI fragment for BglI and PvuII is shown in Figure 3.16b. This was deduced by restriction endonuclease analysis of subfragments of, and

intact, EHV-4 BamHI-C (Figure 3.16a) and is consistent with the Southern blot hybridisation data presented. As can be seen from Table 3.3 and Figures 3.14 and 3.15, the only differences between the hybridisation pattern of the two probes is that the 5′ NarI probe hybridised to 0.4kb and 4.2kb fragments in the BamHI/BglI lane and to a 0.6kb fragment in the BamHI/PvuII lane, whereas the 3′ PstI probe only hybridised to a 4.2kb fragment in the BamHI/BglI lane and to a 2.7kb fragment in the BamHI/PvuII lane. The genomic mapping of the EHV-4 gB gene and the region to which the HSV-1 gB probes hybridised are summarised in Figure 3.17.

F I G U R E 3.1

Restriction Map of HSV-1 EcoRI-F

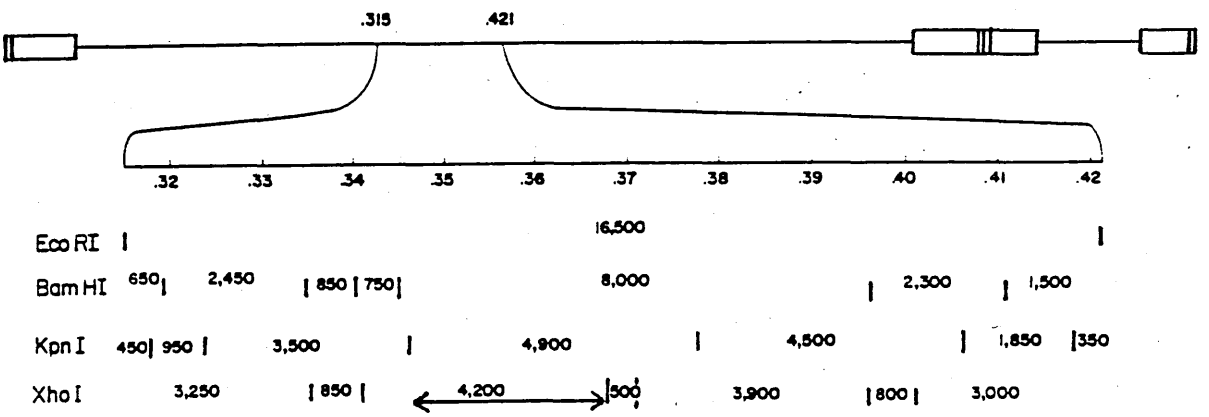


Figure 3.1 Map of restriction endonuclease fragments for BamHI, KpnI and XhoI within the HSV-1 EcoRI-F fragment. Fragment sizes are shown in base pairs. The smallest fragment containing the entire HSV-1 gB gene is indicated by an arrow. Adapted from Holland et al. (1984a).

F I G U R E 3.2

Physical Map of Plasmid pACYC-EcoRI(F)

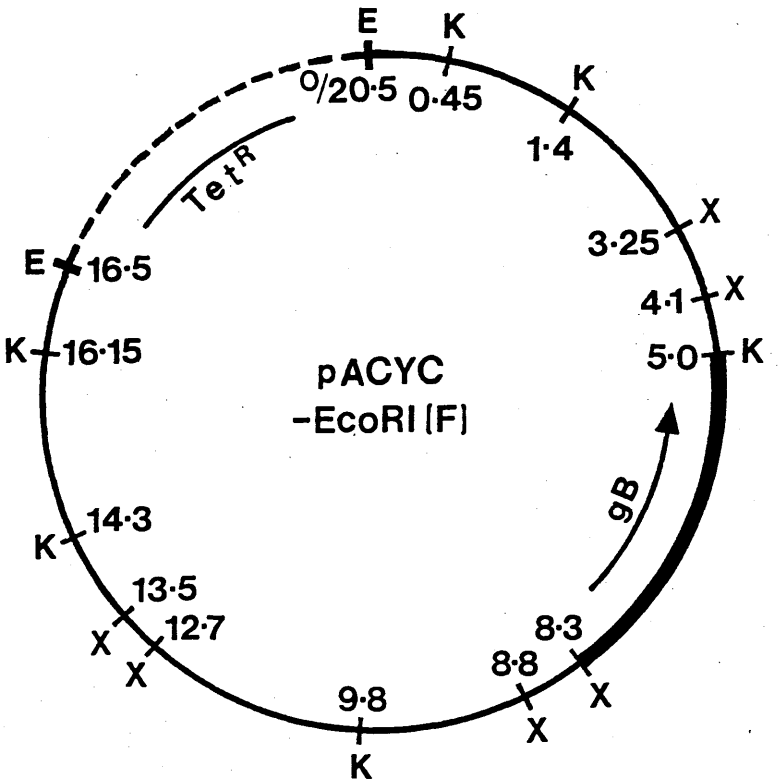


Figure 3.2 Physical map of plasmid pACYC-EcoRI (F). The broken line represents pACYC184 DNA and the solid line represents the 16.5kb HSV-1 EcoRI-F fragment. Sizes are shown in kilobases. The EcoRI (E), KpnI (K) and XhoI (X) sites are indicated. The 3.3kb KpnI/XhoI fragment which contains the entire HSV-1 gB gene is denoted by a thick bar.

F I G U R E 3.3

Restriction Endonuclease Analysis
of Plasmid pACYC-EcoRI(F)

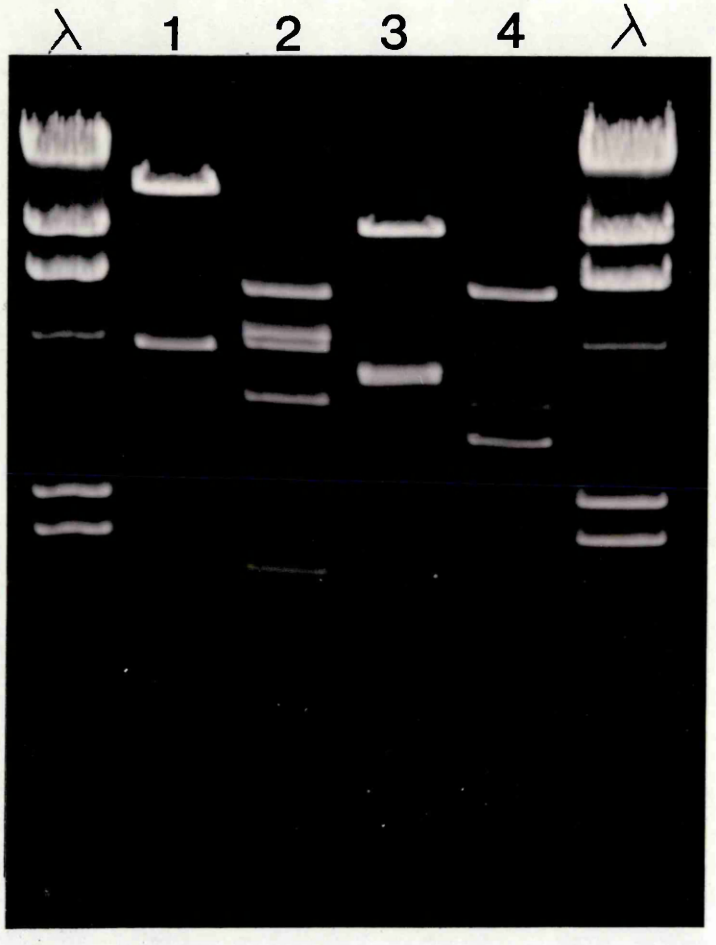


Figure 3.3 Restriction endonuclease analysis of plasmid pACYC-EcoRI(F). The plasmid was digested with EcoRI (lane 1), KpnI (lane 2), XhoI (lane 3) and KpnI/XhoI (lane 4). HindIII digested λ DNA fragments were used as size markers.

F I G U R E 3.4

Physical Map of Plasmid pICgB

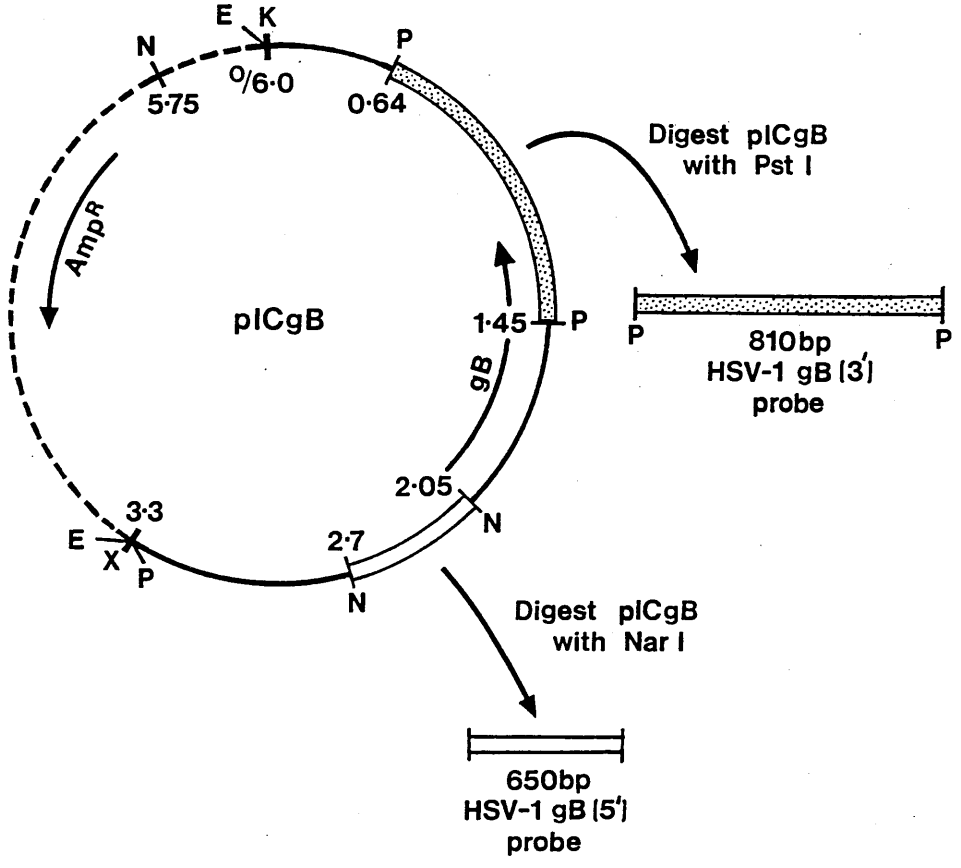


Figure 3.4 Physical map of plasmid pICgB. The broken line represents pIC20R DNA and the solid line represents HSV-1 gB DNA. Sizes are shown in kilobases. The EcoRI (E), KpnI (K), NarI (N), PstI (P) and XhoI (X) sites are indicated. The direction of transcription of the HSV-1 gB gene is denoted by an arrow. The regions of the plasmid from which the 5′ and 3′ HSV-1 gB DNA probes were derived are also indicated.

F I G U R E 3.5

Restriction Endonuclease Analysis of Plasmid pICgB

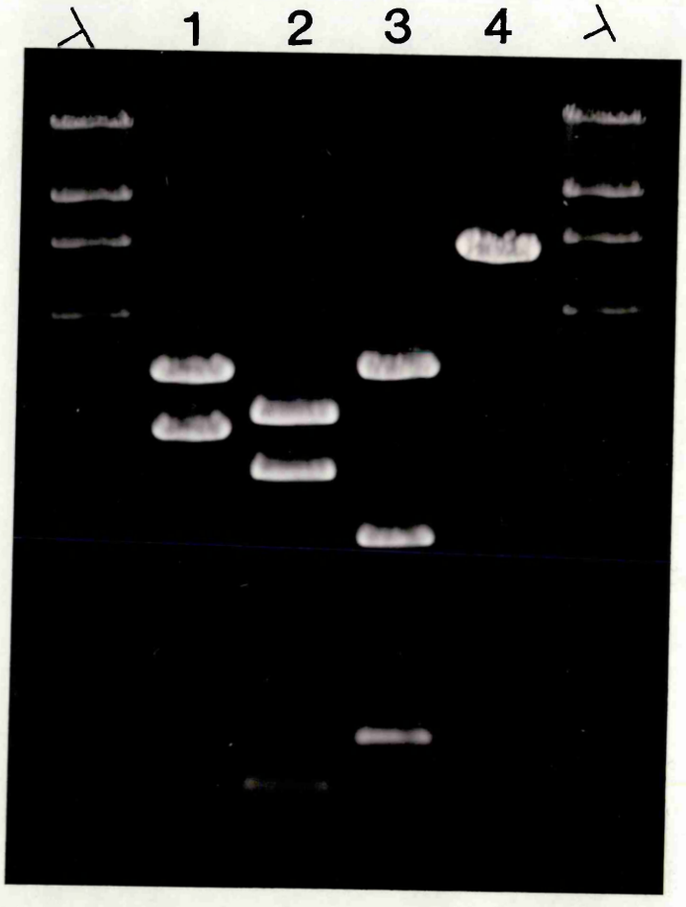


Figure 3.5 Restriction endonuclease analysis of plasmid pICgB. The plasmid was digested with EcoRI (lane 1), NarI (lane 2), PstI (lane 3) and XhoI (lane 4). HindIII digested λ DNA fragments were used as size markers.

F I G U R E 3.6

Salient Features of the HSV-1 gB Gene

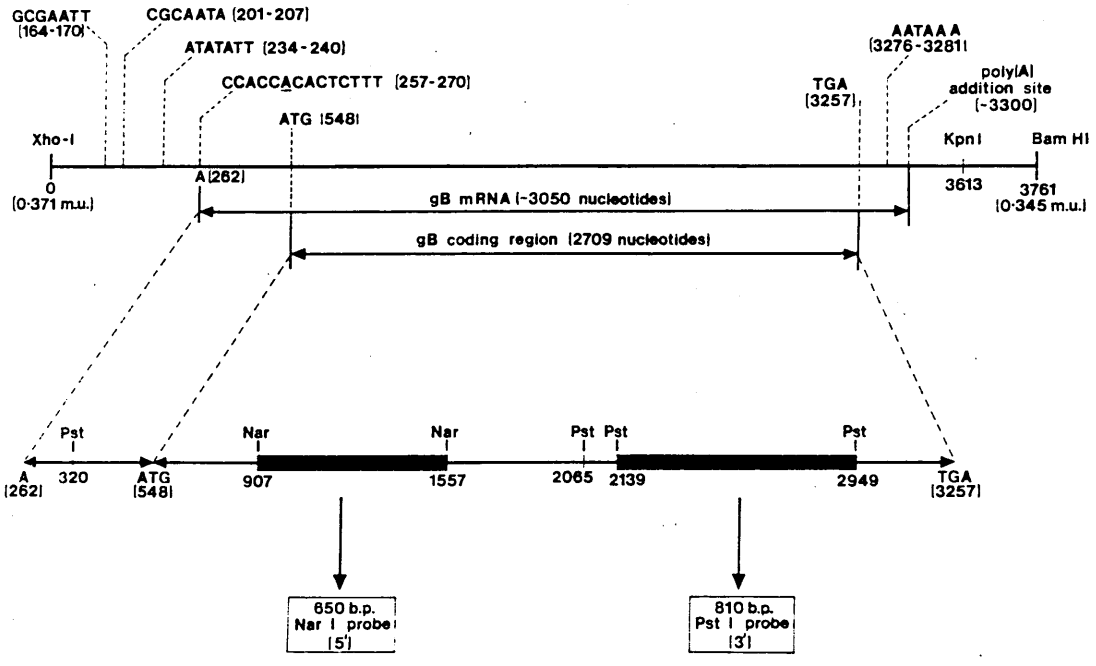


Figure 3.6 Salient features of the HSV-1 gB gene. The putative TATA box, CAT box, mRNA initiation site, initiation codon, termination codon, polyadenylation signal and poly (A) addition site are shown according to Bzik et al. (1984a) and Pellett et al. (1985b). The relative positions of the 5′ and 3′ HSV-1 gB DNA fragments used as hybridisation probes are also indicated.

F I G U R E 3.7

Dot Blot Analysis of EHV-4 DNA
Using the 5′ HSV-1 gB Probe

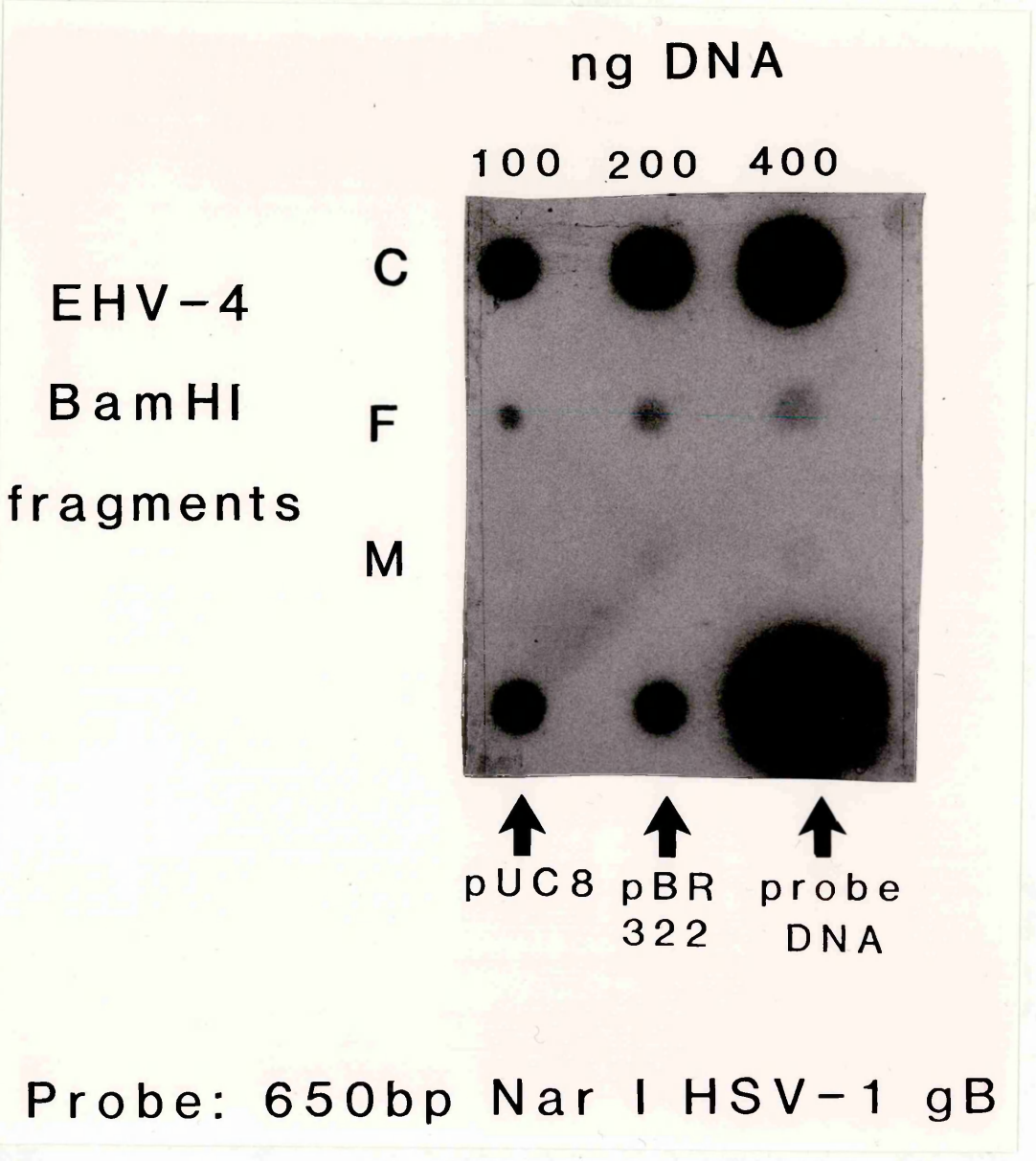


Figure 3.7 Dot blot analysis of EHV-4 DNA. The EHV-4 BamHI-C, F and M fragments were probed with ^{32}P-labelled 650bp NarI HSV-1 gB (5′) DNA. Hybridisation was only observed when membranes were washed at low stringency (2xSSC/0.1% SDS at 65^{o}C). Some hybridisation of probe to vector DNA (pUC8, pBR322) was observed and was probably due to cross-hybridisation of G+C-rich sequences.

F I G U R E 3.8

Dot Blot Analysis of EHV-4 DNA
Using the 3′ HSV-1 gB Probe

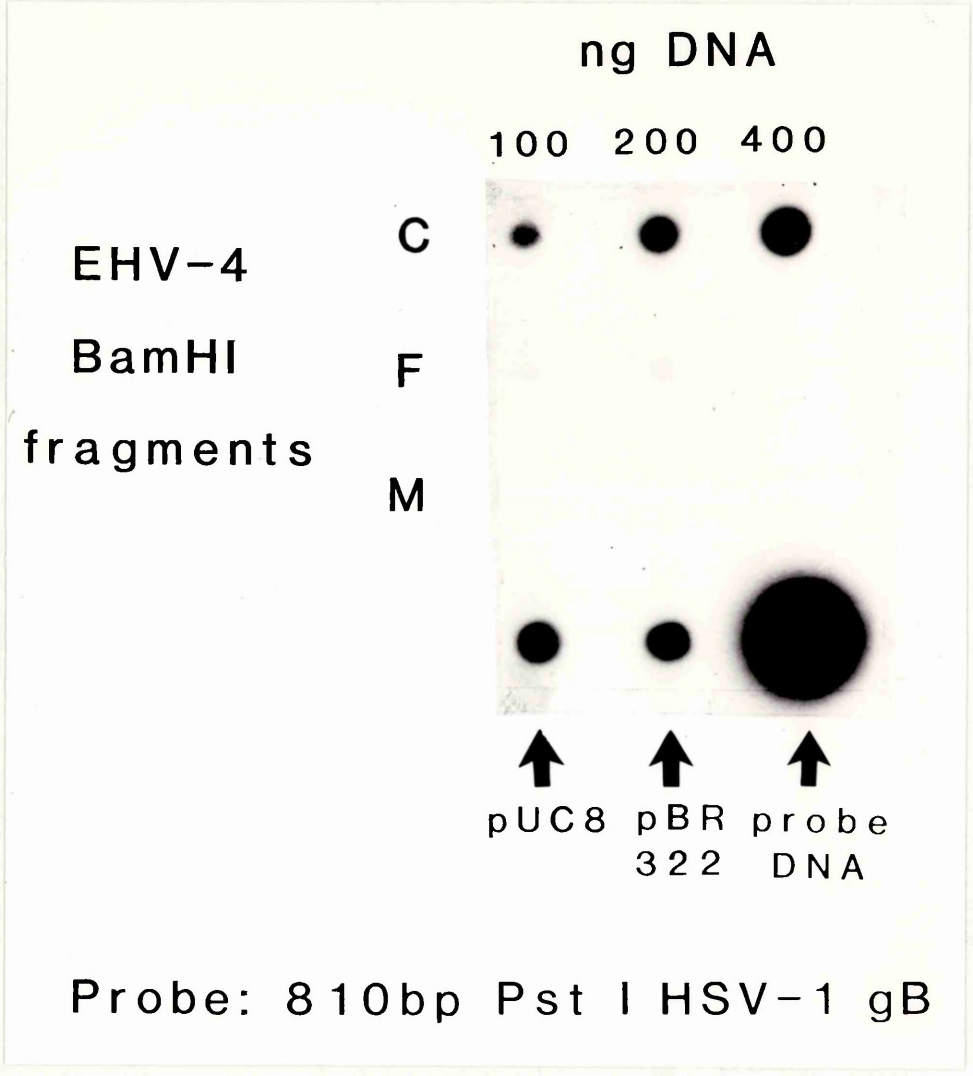


Figure 3.8 Dot blot analysis of EHV-4 DNA. As in Figure 3.7 but with the 810bp PstI HSV-1 gB (3′) DNA as a hybridisation probe.

F I G U R E 3.9

Physical Map of Plasmid pUC9/EHV-4 BamHI-C

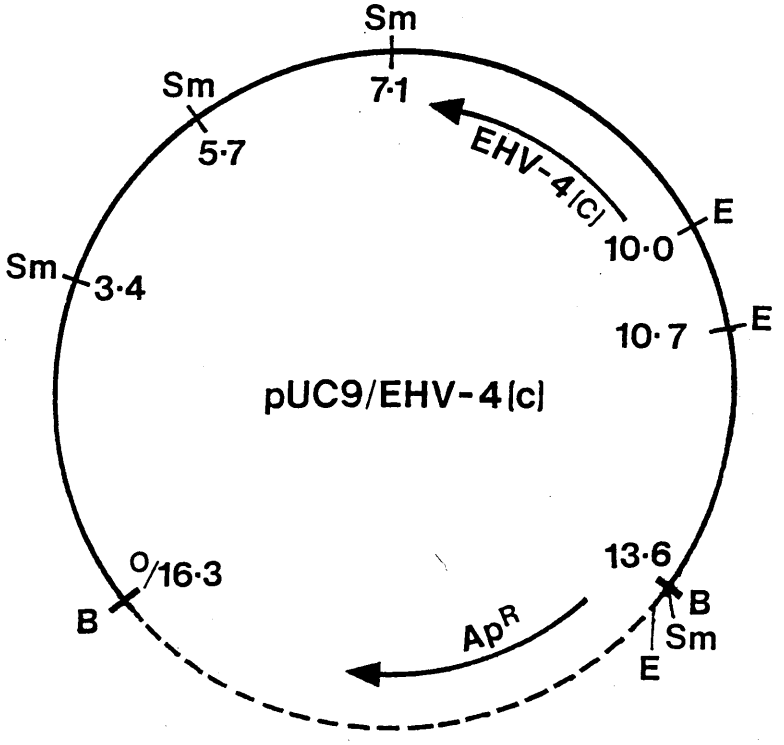
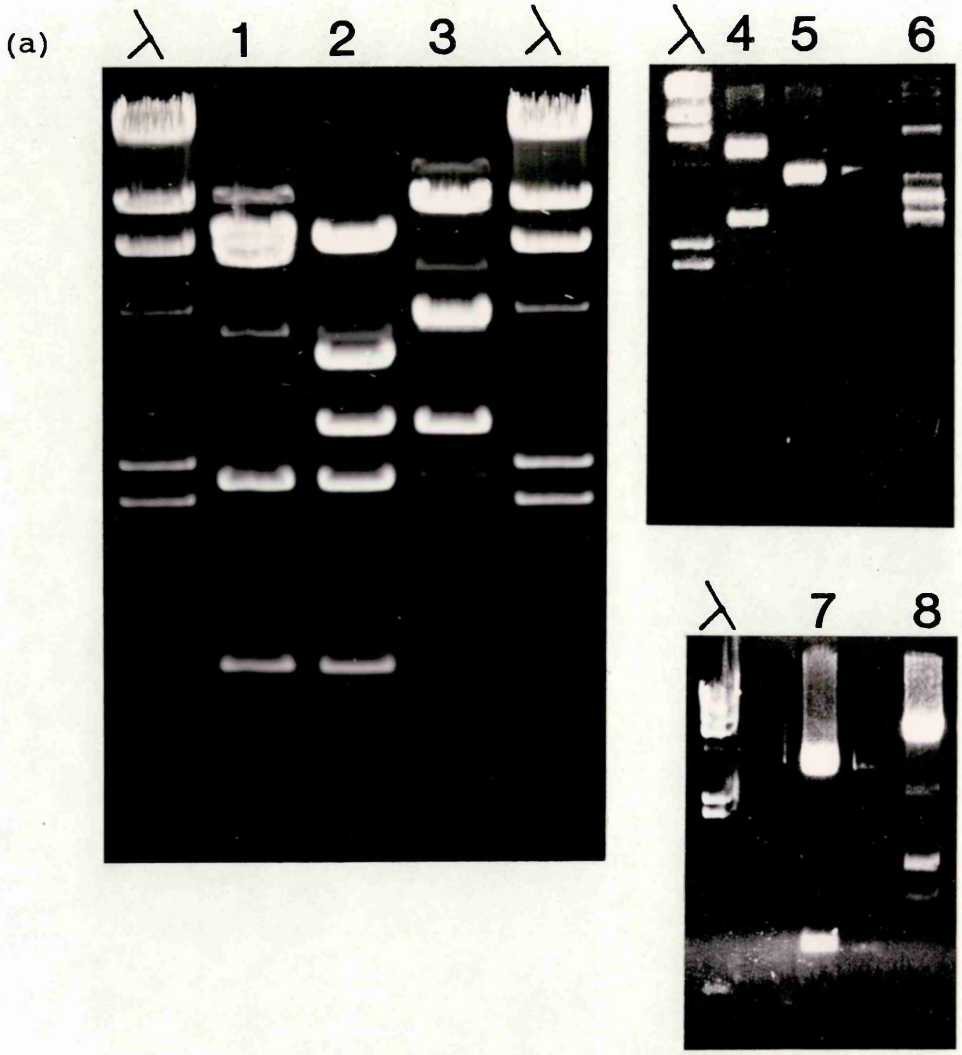


Figure 3.9 Physical map of plasmid pUC9/EHV-4 BamHI-C. The broken line represents pUC9 DNA and the solid line represents EHV-4 BamHI-C DNA. Sizes are shown in kilobases. The BamHI (B), EcoRI (E) and SmaI (Sm) sites are indicated. The orientation of the EHV-4 DNA insert is denoted by an arrow.

Figure 3.10 Mapping of the KpnI and SmaI sites in EHV-4 BamHI-C by restriction endonuclease analysis. (a) Agarose gels showing the products of digestion of intact BamHI-C and various subfragments of BamHI-C with EcoRI, KpnI or SmaI. The samples shown are as follows:-
BamHI-C digested with SmaI (lane 1), BamHI/SmaI (lane 2) and BamHI/KpnI (lane 3); 9.4kb BamHI/KpnI subfragment of BamHI-C digested with EcoRI (lane 4) and SmaI (lane 8); 4.2kb BamHI/KpnI subfragment of BamHI-C digested with EcoRI (lane 5) and SmaI (lane 7); 6.5kb BamHI/SmaI subfragment of BamHI-C digested with EcoRI (lane 6). HindIII digested λ DNA fragments were used as size markers.
(b) Deduced restriction map of BamHI-C showing the location of sites for EcoRI (E), KpnI (K) and SmaI (Sm). B, BamHI.

FIGURE 3.10

Mapping the KpnI and SmaI Sites in EHV-4 BamHI-C



F I G U R E 3.11

Dot Blot Analysis of EHV-4 BamHI-C DNA Using the 5′ HSV-1 gB Probe

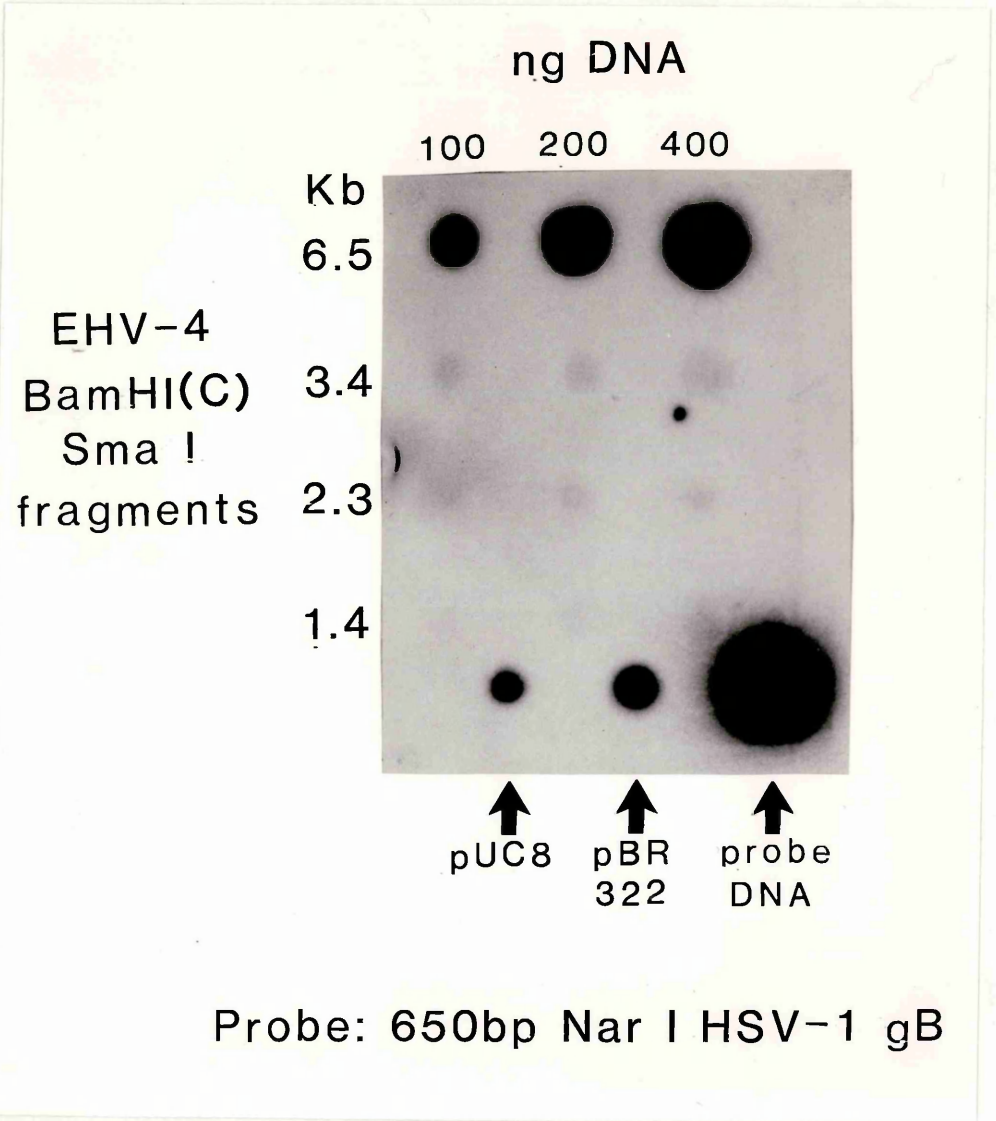


Figure 3.11 Dot blot analysis of EHV-4 BamHI-C DNA. The 6.5kb, 3.4kb, 2.3kb and 1.4kb subfragments of EHV-4 BamHI-C generated by digestion with SmaI were probed with ^{32}P-labelled 650bp NarI HSV-1 gB (5′) DNA. Hybridisation was only observed when membranes were washed at low stringency (2xSSC/0.1% SDS at 65^{o}C).

F I G U R E 3.12

Dot Blot Analysis of EHV-4 BamHI-C DNA Using the 3' HSV-1 gB Probe

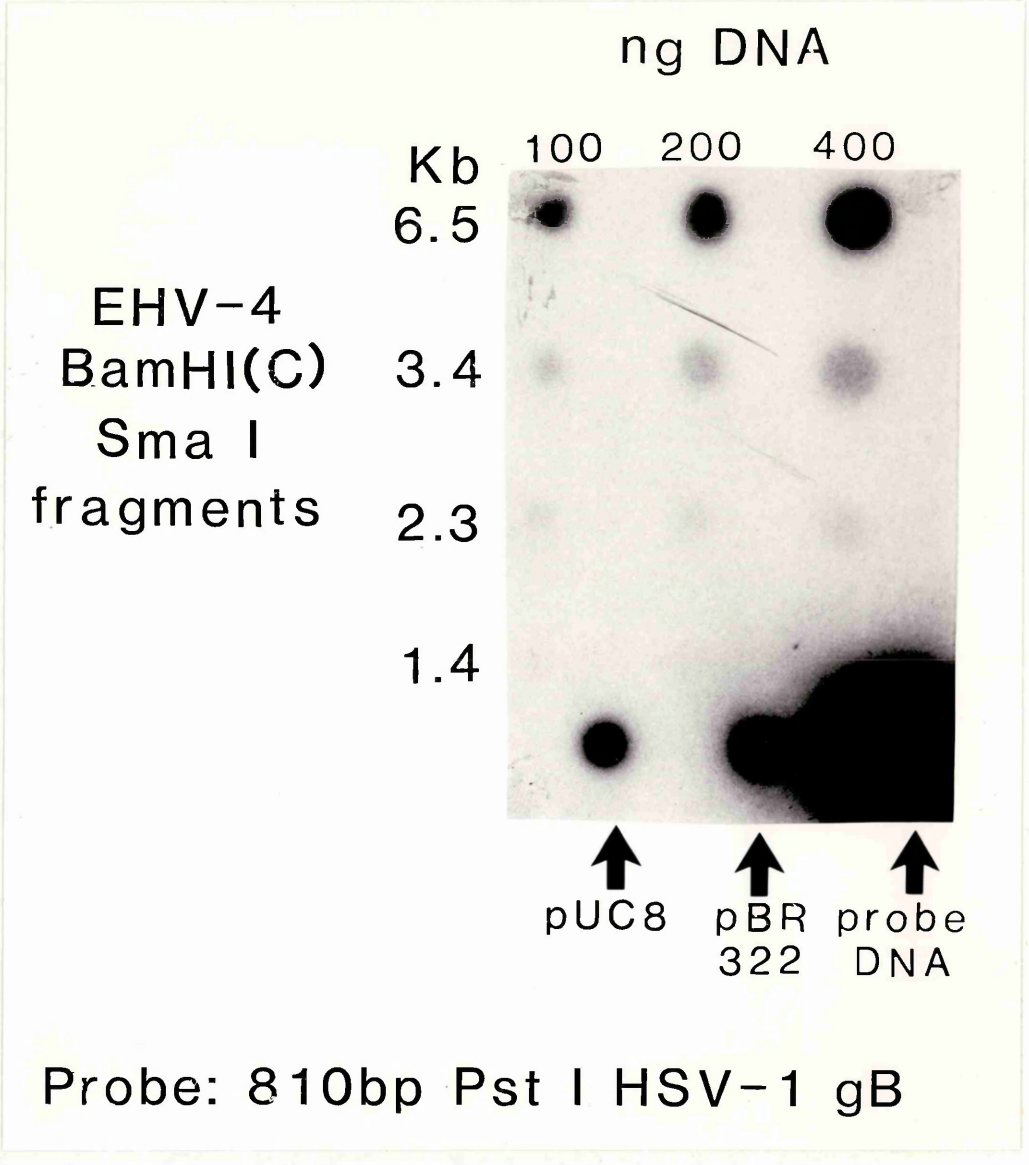


Figure 3.12 As in Figure 3.11 but with the 810bp PstI HSV-1 gB (3') DNA as a hybridisation probe.

Figure 3.13 Restriction endonuclease analysis of plasmid pUC9/EHV-4 BamHI-C. Plasmid DNA was digested with BamHI (lane 1), BamHI/BglII (lane 2), BamHI/EcoRI (lane 3), BamHI/PstI (lane 4), BamHI/PvuII (lane 5) and BamHI/SmaI (lane 6). Samples were run in duplicate prior to being separately transferred to membranes for hybridisation to 5′ and 3′ HSV-1 gB DNA probes. Gels were run in 0.8% agarose and 1xTBE buffer. ^{32}P-labelled HindIII digested λ DNA fragments were used as size markers.

F I G U R E 3.13

Restriction Endonuclease Analysis of
Plasmid pUC9/EHV-4 BamHI-C

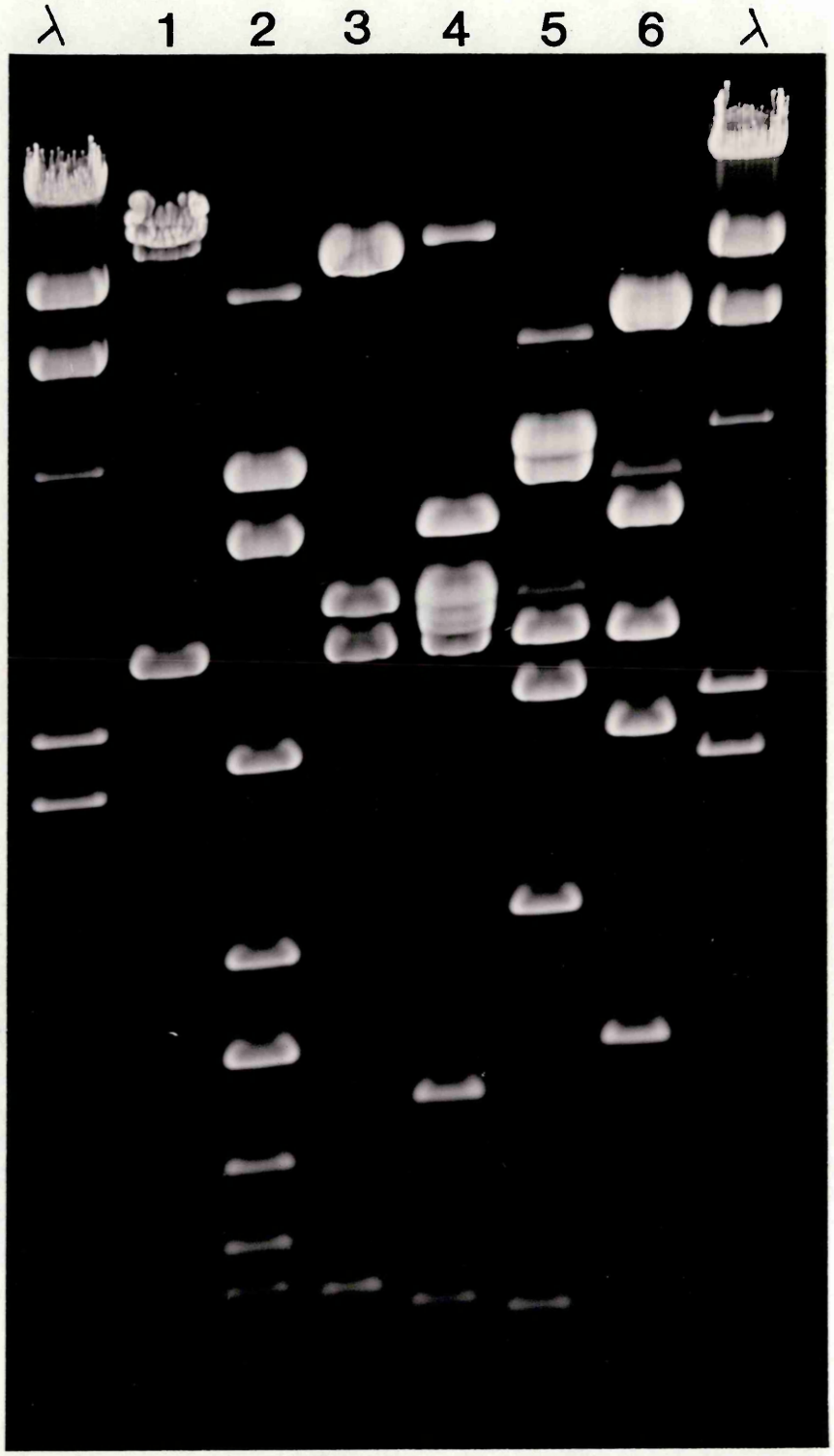


TABLE 3.2

Sizes of Fragments Generated by Digestion of EHV-4 BamHI-C DNA With the Restriction Endonucleases BglI, EcoRI, PstI, PvuII and SmaI

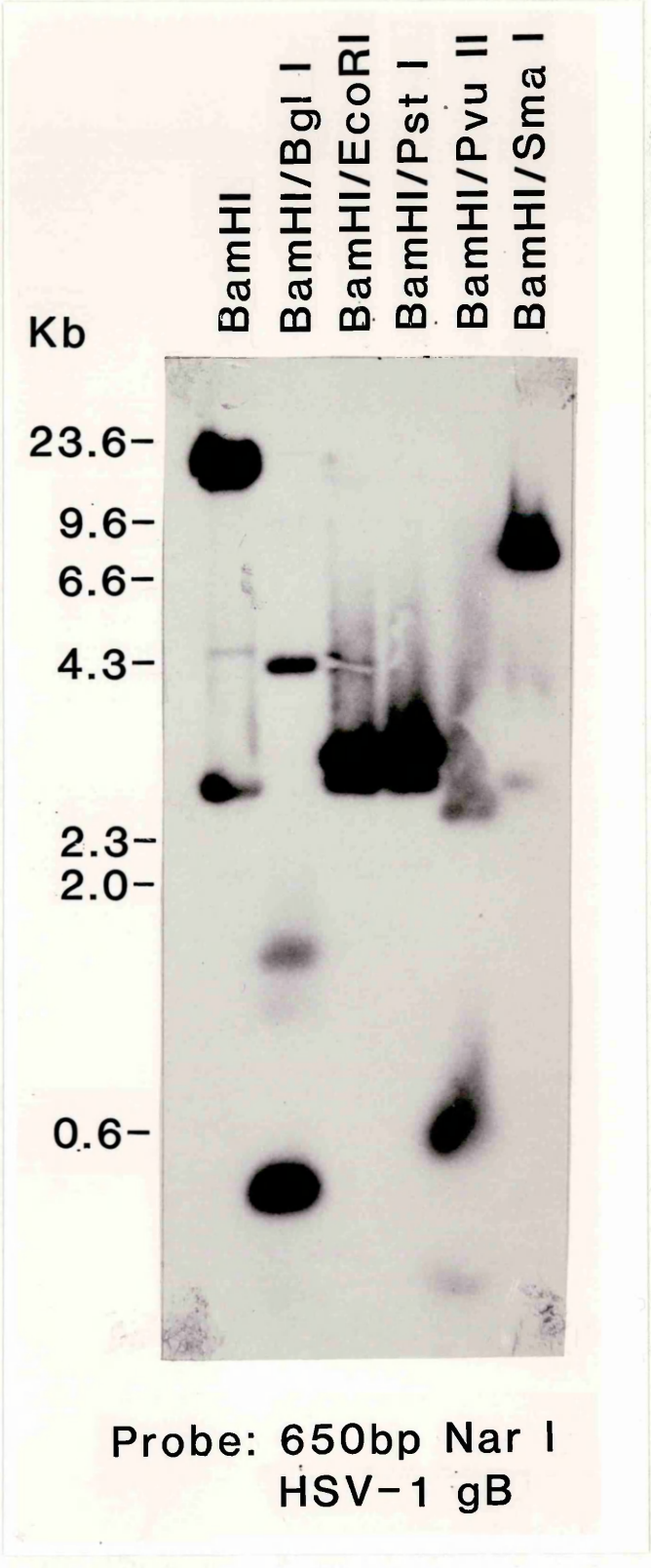
Restriction Endonuclease	Size of EHV-4 Fragments (kb)	Size of pUC9 Fragments (kb)
BglI	4.2	1.4
	3.4	1.2
	2.2	0.1
	1.2	
	0.8	
	0.7	
	0.6	
	0.4	
	0.1	
EcoRI	10.0	2.7
	2.9	
	0.7	
PstI	3.4	2.7
	3.0	
	2.8	
	2.8	
	1.0	
	0.6	
PvuII	4.3	2.4
	4.0	0.2
	2.7	0.1
	1.5	
	0.6	
	0.4	
	0.1	
SmaI	6.5	2.7
	3.4	
	2.3	
	1.4	

Table 3.2 Approximate sizes of fragments generated by digestion of the pUC9 plasmid containing EHV-4 BamHI-C DNA with the restriction endonucleases BglI, EcoRI, PstI, PvuII and SmaI. Fragment sizes were predominantly estimated from electrophoretic mobilities in the 0.8% agarose gel shown in Figure 3.13. The sizes of the pUC9 vector fragments generated are also indicated.

Figure 3.14 Southern blot analysis of EHV-4 BamHI-C DNA. Fragments generated by restriction endonuclease digestion, separated by electrophoresis and transferred to hybridisation membranes were probed with ^{32}P-labelled 650bp NarI HSV-1 gB (5') DNA. Hybridisation was only observed when membranes were washed at low stringency (2xSSC/0.1%SDS at 65^{o}C).

F I G U R E 3.14

Southern Blot Analysis of EHV-4 BamHI-C DNA Using the 5′ HSV-1 gB Probe



F I G U R E 3.15

Southern Blot Analysis of EHV-4 BamHI-C DNA Using the 3′ HSV-1 gB Probe

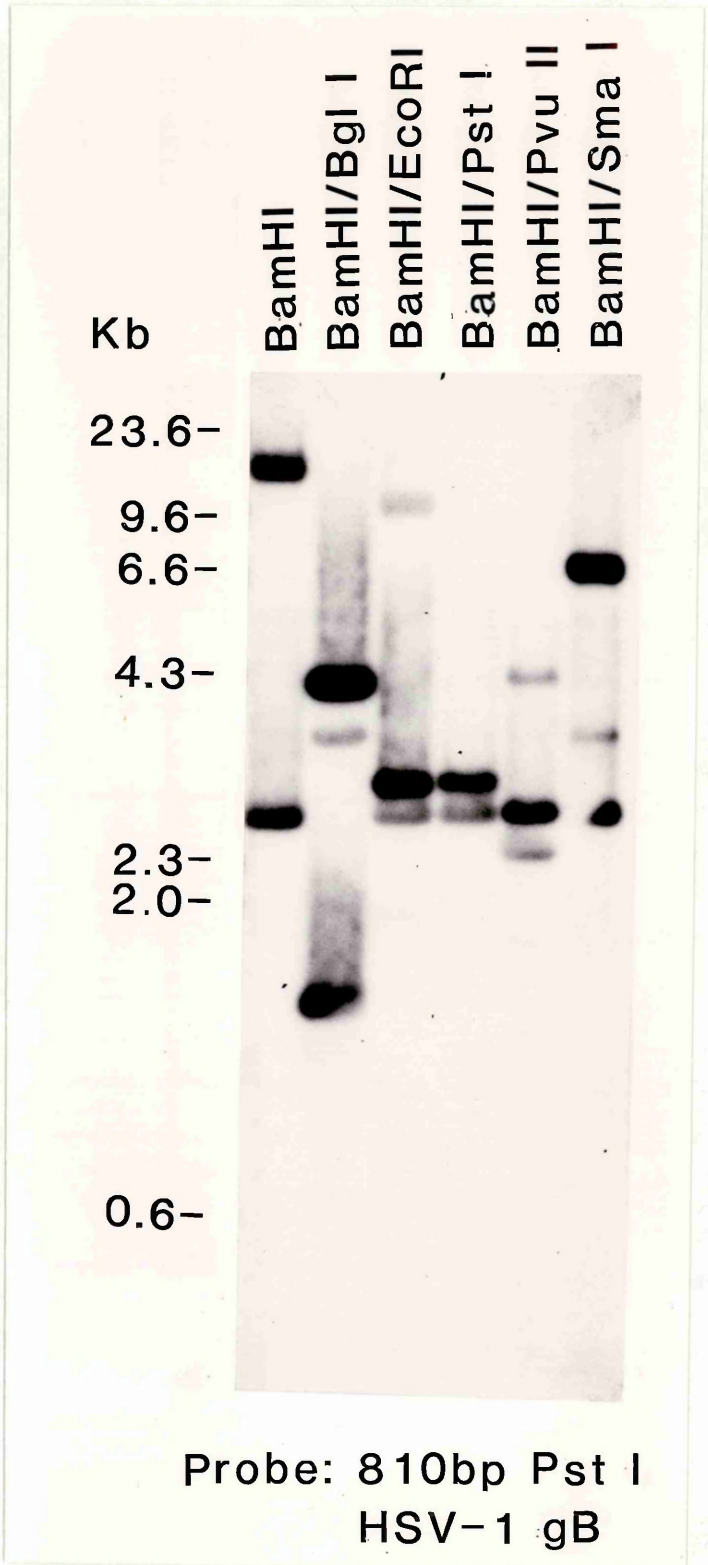


Figure 3.15 As in Figure 3.14 but with the 810bp PstI HSV-1 gB (3′) DNA as a hybridisation probe.

TABLE 3.3

Summary of Hybridisation of HSV-1 gB DNA Probes to EHV-4 BamHI-C DNA

HSV-1 gB Probe	Size of EHV-4 Restriction Endonuclease Fragment (kb)					
	BamHI	BglI	EcoRI	PstI	PvuII	SmaI
650bp NarI (5′)	13.6	4.2 0.4	2.9	3.0	0.6	6.5
810bp PstI (3′)	13.6	4.2	2.9	3.0	2.7	6.5

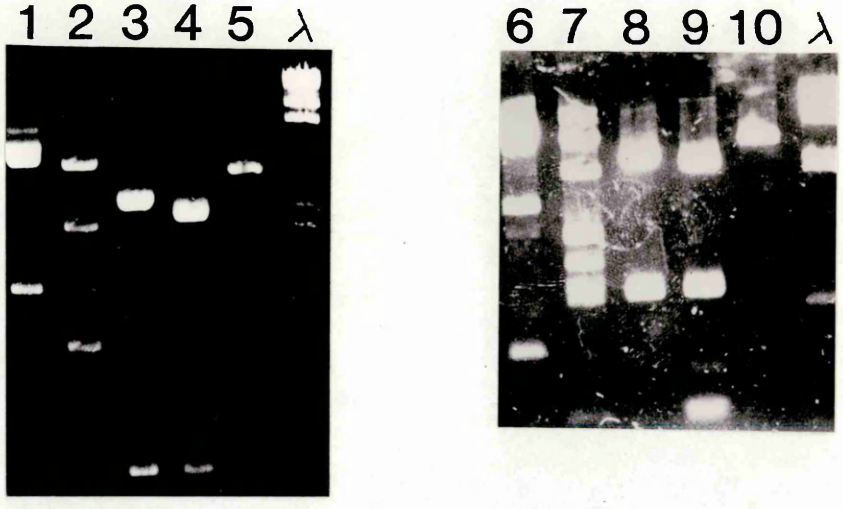
Table 3.3 Pattern of hybridisation of the HSV-1 gB DNA probes to EHV-4 BamHI-C restriction endonuclease fragments. Weak hybridisation of the probes to pUC9 vector fragments was also observed and was possibly due to cross-hybridisation of G+C-rich sequences.

Figure 3.16 Derivation of a restriction map of the 2.9kb BamHI/EcoRI left-terminal subfragment of EHV-4 BamHI-C that contains the gB gene. (a) Restriction endonuclease analysis of the 2.9kb and 10.0kb BamHI/EcoRI subfragments of BamHI-C. The samples shown on the gel are as follows:- 10.0kb BamHI/EcoRI subfragment of BamHI-C digested with PvuII (lane 1) and BglI (lane 2); 2.9kb BamHI/EcoRI subfragment of BamHI-C digested with BglI (lane 3), BglI/PvuII (lane 4) and PstI (lane 5). Lanes 6 to 10 are the same as lanes 1 to 5 except that the samples were run for a shorter time to allow visualisation of particularly small fragments. HindIII digested λ DNA fragments were used as size markers.
(b) Deduced restriction map of the 2.9kb BamHI/EcoRI left-terminal subfragment of BamHI-C showing the sites for BglI (Bg) and PvuII (Pv). The first BglI, EcoRI (E) and PvuII sites to the right of the 2.9kb BamHI/EcoRI subfragment are shown. The arrow indicates the direction of transcription of the gB gene. B, BamHI.

F I G U R E 3.16

Restriction Mapping of the 2.9kb BamHI/EcoRI Subfragment of EHV-4 BamHI-C That Contains the gB Gene

(a)



(b)

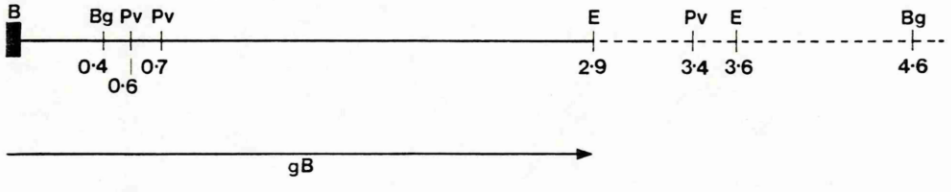
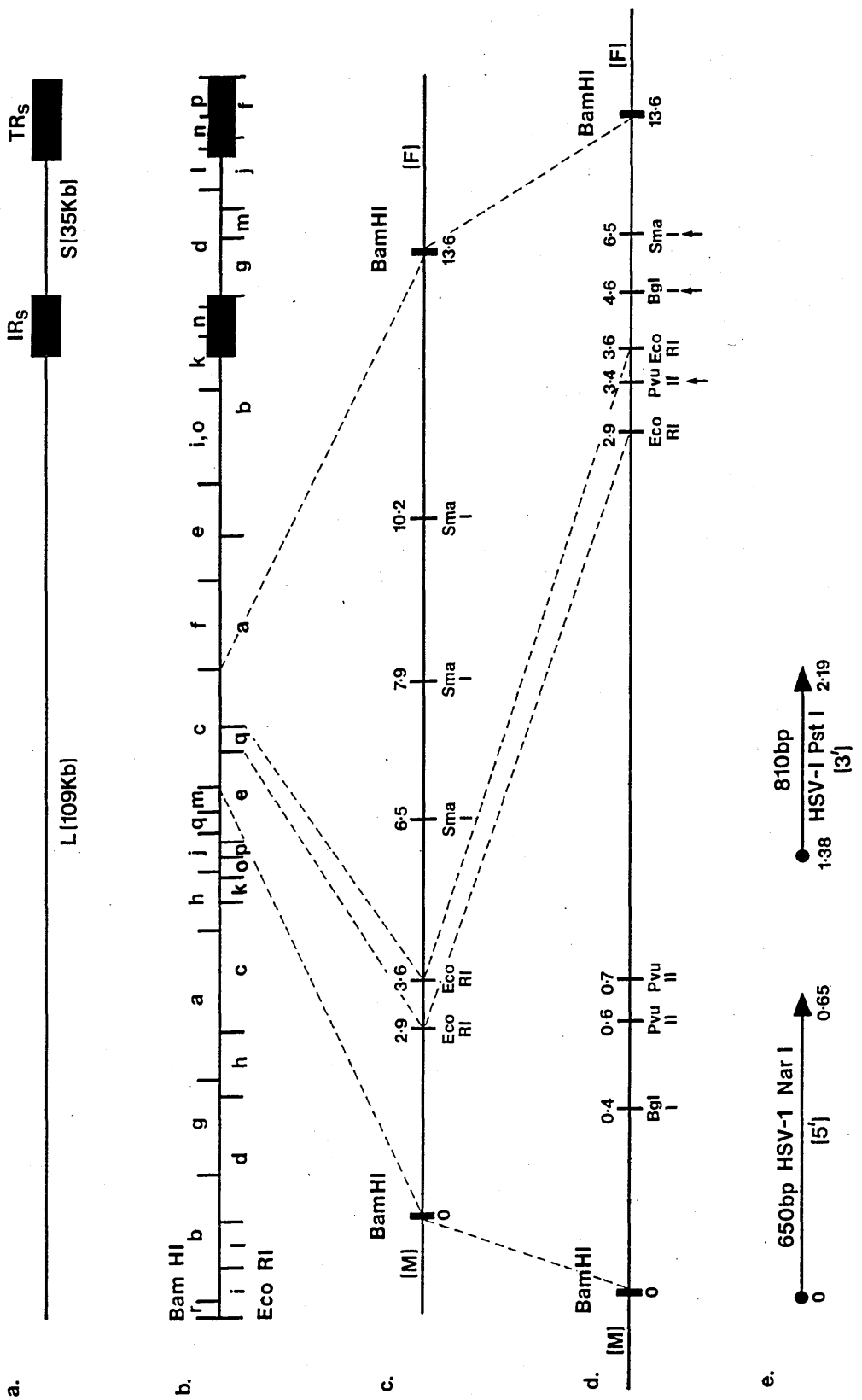


Figure 3.17 Map of the EHV-4 genome showing restriction endonuclease sites and DNA fragments used in studying the glycoprotein gB gene. (a) Structure of the EHV-4 genome. (b) Arrangement of BamHI and EcoRI sites along the genome. (c) Location of EcoRI and SmaI sites in the 13.6kb BamHI-C fragment of the genome. (d) Detailed restriction endonuclease mapping of the the 2.9kb BamHI/EcoRI subfragment of BamHI-C, which contains the major portion of the gB gene. The first BglI, PvuII and SmaI sites to the right of the 2.9kb BamHI/EcoRI subfragment are indicated by arrows. (e) Arrows showing the regions of the 2.9kb BamHI/EcoRI subfragment of BamHI-C to which the 5′ and 3′ HSV-1 gB DNA probes hybridised. bp, Base pairs.

F I G U R E 3.17

Summary of Genomic Mapping of the EHV-4 gB Gene



D I S C U S S I O N

The major portion of the EHV-4 gB gene has been successfully mapped using molecular hybridisation techniques to a 2.9kb BamHI/EcoRI fragment at the left terminus of the BamHI-C genomic fragment, corresponding to 0.408 to 0.438 m.u., and is transcribed in a rightward direction. Since the 5′ NarI HSV-1 gB probe did not contain the first 357 nucleotides of the HSV-1 gB gene, it was predicted that a corresponding portion of the EHV-4 gB gene and its transcriptional control domains will reside in the BamHI-M fragment immediately to the left of BamHI-C. The genomic location and orientation of the EHV-4 gB gene are consistent with the general colinearity observed between the EHV-4 and HSV-1 genomes (Cullinane et al., 1988). This approach to the detection of homologous genes in herpesvirus genomes was particularly well exploited by Davison and Wilkie (1983) who used molecular hybridisation to investigate homologous regions in the genomes of HSV-1, HSV-2, VZV, PRV and EHV-1. The use of cloned HSV-1 probes demonstrated high levels of conservation of the major DNA binding protein, major capsid protein, DNA polymerase, immediate-early protein V_{mw}IE175 and one or both of the early proteins V_{mw}136 and V_{mw}38, which are the two subunits of HSV-1 ribonucleotide reductase, between these alphaherpesviruses. Using HSV-1 gene probes, the genes for the major DNA binding protein, major capsid protein, immediate-early protein V_{mw}IE175 and the two subunits of ribonucleotide reductase were mapped to the BamHI-M, BamHI-F, BamHI-K/P and BamHI-A fragments of the EHV-4 genome, respectively (Cullinane et al., 1988). However, this method cannot be used for the

identification of genes with poor homology. For example, attempts to identify PRV glycoproteins by using cloned HSV-1 gB, gC, gD and gE glycoprotein genes as probes only permitted the detection of the homologue of the well conserved glycoprotein gB in the PRV genome (Robbins et al., 1987).

A novel approach to the identification of poorly conserved genes has recently been reported. Using short, degenerate oligonucleotide primers in a polymerase chain reaction, the TK homologue in feline herpesvirus 1 (FHV-1) has been identified (Nunberg et al., 1989). The oligonucleotide primers were derived from the best conserved regions, identified by alignment, of several alphaherpesvirus TK proteins. An amplified 210bp fragment was then successfully used as a hybridisation probe to identify the genomic region of FHV-1 which encodes the TK gene. This method will prove useful in identifying new members of other viral and cellular gene families and will facilitate the isolation of other poorly conserved gene homologues in the herpesviruses.

Allen and Yeargan (1987) were the first workers to map EHV-1 glycoproteins, and did so by utilising the strategy of Young and Davis (1983a,b) to determine the genomic location of the coding sequences of proteins for which monoclonal antibodies are available. By immunoscreening an EHV-1 genomic library constructed in the λgt11 expression vector with monoclonal antibodies specific for each of the six major EHV-1 glycoproteins (gp2, 10, 13, 14, 17/18, 21/22a), recombinant bacteriophage which expressed EHV-1 glycoprotein epitopes as fusion products with β-galactosidase were readily identified. The DNA contained in the recombinant phage was used as a

hybridisation probe to map the insert sequences on the viral genome. All glycoproteins mapped to the U_L region with the exception of gp17/18 which was mapped to the U_S region (Figure 3.18). The genes for gp13 and gp14 mapped to positions colinear with the gC and gB homologues, respectively, of other alphaherpesviruses, and the map location of gp14 corresponds to that presented here for EHV-4 gB. It was subsequently proposed that gp17/18 represents the homologue of HSV-1 gE (Allen and Coogle, 1988). The study of Allen and Yeargan (1987) also identified three highly abundant glycoproteins (gp2, 10, 21/22a) for which no counterparts are present in HSV-1. On the otherhand, none of the EHV-1 glycoproteins mapped corresponded to the minor HSV-1 glycoprotein, gH. It was suggested that some of the low-abundance glycoproteins, and also possible additional unidentified glycoproteins, of EHV-1 may map to the U_S region of the genome. This remains the most comprehensive study on the mapping of EHV-1 glycoproteins to date, and provides valuable information which could be applied to the further characterisation of these important virion components.

F I G U R E 3.18

Mapping of the Major EHV-1 Glycoprotein Genes

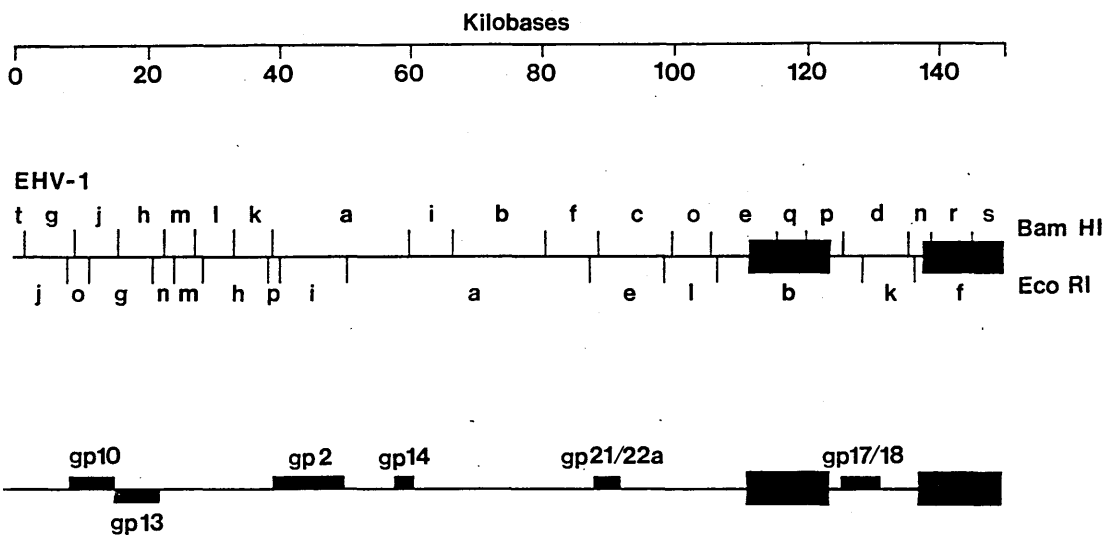


Figure 3.18 Physical map of the EHV-1 genome showing the genomic location of the genes encoding the six major EHV-1 glycoproteins gp2, gp10, gp13, gp14, gp17/18 and gp21/22a. The shaded boxes indicate the smallest genomic fragments that encode the EHV-1 glycoprotein epitopes immunoreactive with EHV-1 monoclonal antibodies to those glycoproteins. Adapted from Allen and Yeargan (1987).

CHAPTER 4

DNA Sequence of the EHV-4 Glycoprotein gB Gene and Analysis of the Predicted Gene Product

INTRODUCTION

The identification of EHV-4 genomic fragments containing the EHV-4 gB gene was reported in the previous chapter. This chapter is therefore concerned with DNA sequence analysis of the 2.9kb BamHI/EcoRI fragment at the left-hand end of the BamHI-C, and the rightmost end of the adjacent BamHI-M, EHV-4 genomic fragments. Sequence analysis would identify the precise limits of the gB gene and yield valuable information on the structure of the predicted gene product. The EHV-4 gB gene product could then be compared with the gB-like glycoproteins of other herpesviruses and domains which may have important conserved functions identified.

It has only been over the last couple of years that information on the DNA sequence of EHV-1 glycoprotein genes has begun to emerge. A 6kb BamHI/EcoRI fragment between 0.114 and 0.148 m.u. on the EHV-1 (Kentucky T431) genome was preliminary identified as encoding the major glycoprotein gp13, analogous to the gC-like glycoproteins of other herpesviruses (Allen and Yeargan, 1987). Further characterisation of this region more accurately localised the gp13 gene to a 1765bp AccI/EcoRI fragment (0.136 to 0.148 m.u.), which was subsequently sequenced (Allen and Coogle, 1988). The gp13 gene was identified as a 1404bp ORF encoding a 468 amino acid (51kDa) protein with features characteristic of a membrane-spanning glycoprotein, showing limited but significant homology at the amino acid level to the gC-like glycoproteins of HSV-1, HSV-2, VZV and PRV.

In parallel with the studies presented in this chapter, Whalley *et al*. (1989) identified the EHV-1 (strain HVS-25) gB

homologue within a 4.3kb ClaI/PstI fragment spanning the BamHI-A and BamHI-I genomic fragments at 0.40 to 0.43 m.u. The DNA sequence of this fragment identified a 2940bp ORF encoding gB. The position of the EHV-1 gB gene was identical to that of the gene encoding EHV-1 gp14 (Allen and Yeargan, 1987) and to that encoding EHV-4 gB as demonstrated in Chapter 3 of this thesis.

Virtually no sequence data had been published for the glycoprotein genes of EHV-4 at the start of the studies presented in this thesis. The only information available was reported by Cullinane et al. (1988) who determined the DNA sequence of EHV-4 BamHI-L, located at the right-hand end of U_S and extending into TR_S. The authors identified a partial ORF at the left-hand end of BamHI-L which encodes the carboxyl-terminal 255 amino acids of a protein homologous to HSV-1 glycoprotein gE (McGeoch et al., 1985) and VZV glycoprotein gpI (Ellis et al., 1985; Davison and Scott, 1986a).

Therefore, the DNA sequence of the EHV-4 gB gene presented in this chapter represents the first piece of work which describes the complete structural analysis of any EHV-4 glycoprotein gene. A paper reporting this work has been published (Riggio et al., 1989), a bound copy of which can be found at the end of this thesis.

MATERIALS AND METHODS

DNA Sequencing

DNA was sequenced by the dideoxy chain termination method (Sanger et al., 1977), using the double-stranded DNA sequencing technique in which single-stranded DNA template was produced by alkaline denaturation of plasmid DNA. DNA fragments to be sequenced were cloned into Bluescript M13+ (2.9kb subfragment of EHV-4 BamHI-C) or were already contained within pUC9 (right-hand end of EHV-4 BamHI-M). Stock solutions and buffers were prepared as described in Table 4.1.

Sequencing Primers

Commercially available primers (M13, Reverse, KS and T7) were diluted to 5ng/ul. Custom made EHV-4-specific primers were synthesised on an Applied Biosystems 381A DNA synthesiser. The oligonucleotide was cleaved from the synthesis column by 4 x 30min treatments with 10M ammonium hydroxide in a total volume of 2ml and deprotected at 55°C for 12 to 18hr. DNA was precipitated as previously described and diluted in dH_2O to a final concentration of 5ng/ul. All primers were stored at -20°C until required.

Radioactive Label

The radioactive label used for all sequencing reactions was deoxyadenosine 5-[α-^{35}S] thiotriphosphate triethylammonium salt (dATP, Sp isomer) which was purchased from Amersham. The specific activity of the [α-^{35}S]dATP label was >1200Ci/mmol and the concentration was 10mCi/ml.

Denaturation Reaction

To 1 picomole of plasmid DNA (1 picomole of DNA is 2ug of a 3kb plasmid) was added 4ul of 1M NaOH/1mM EDTA and dH_2O to a final volume of 20ul, and the reaction incubated for 5min at room temperature. Samples were immediately placed on ice and neutralised by the addition of 2ul of 2M ammonium acetate (pH 5.3). DNA was precipitated by the addition of 55ul of ethanol, followed by incubation at -70°C for 30min. DNA was recovered by centrifugation at 13000rpm for 10min in a benchtop microcentrifuge, briefly washed with 70% ethanol, dried in a vacuum dessicator and resuspended in 8.5ul of TE buffer. This DNA template was used immediately in the annealing reaction.

Annealing Reaction

The annealing reaction was composed of 8.5ul DNA template, 1.5ul 10 x annealing buffer, 2ul sequencing primer and 3ul (30uCi) [α-^{35}S]dATP. The contents of the annealing reaction were mixed well by pipetting and incubated at 37°C for 15min. This constituted the template/primer/label mix (TPL mix).

Sequencing Reaction

1ul (1 unit) of Klenow enzyme was added to the TPL mix and mixed by gentle pipetting. This constituted the template/primer/label/enzyme mix (TPLE mix). 3ul of the TPLE mix was added to the side wall of each of four 1.5ml microcentrifuge tubes labelled A, C, G and T, which contained 2ul of A, C, G and T sequencing mix, respectively. The reactions were started by spinning briefly in a benchtop microcentrifuge and the samples were incubated at 37°C for 20min. 2ul of chase mix was added to

the side wall of each tube, mixed by spinning and the reactions continued at 37°C for a further 15min. Reactions were terminated by the addition of 5ul of stop mix and samples were stored at -70°C until required. Samples were boiled for 5min prior to loading onto a sequencing gel. 5ul of each sample was loaded onto the gel for a long run and another 5ul of each reaction loaded, following reboiling, after about 3hr for a short run.

Polyacrylamide Sequencing Gel

Solutions used in preparation of the gel are shown in Table 4.2. The concentration of acrylamide used was 6%. The Macrophor gel sequencing system was used according to the manufacturers instructions (Pharmacia LKB). Wedge-shaped gels were routinely used. To allow for easier handling, gels were bound to the notched glass plate by treatment with Bind-Silane. Treatment of the thermostatic plate with Repel-Silane prevented sticking of this plate to the notched glass plate containing the bound gel. After casting of the gels and insertion of the well-forming combs, they were allowed to polymerise for at least 1hr at room temperature. Gels were pre-electrophoresed for 30min at 2000V and 60°C in 1xTBE running buffer. Sample wells were flushed with running buffer prior to loading of samples which were applied to the gels using drawn-out capillary tubes. Gels were run at 2000V and 60°C until the bromophenol blue band had reached the bottom of the gels. When a long run was also required, the remaining samples were loaded at this point and electrophoresis continued until the bromophenol blue band from this second loading had reached the bottom of the gels. Gels were soaked in gel fixing solution for 30min prior to drying onto the notched glass plate

with a hair drier and then subjected to autoradiography in a light-proof exposure box for 8 to 24hr at -70°C.

Analysis of DNA Sequence Data

DNA sequence data were compiled using the Microgenie programme package (Queen and Korn, 1984). Pairwise alignments of amino acid sequences were carried out using this programme package and multiple alignments were compiled manually and guided by previous pairwise alignments. Protein structure was analysed using PEPTIDESTRUCTURE and PLOTSTRUCTURE from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984) and dot matrix similarity analyses were carried out using COMPARE and DOTPLOT from this group of programmes.

T A B L E 4.1

DNA Sequencing Stock Solutions and Buffers

DeoxyNTP Stock Solutions

Stock solutions (10mM) of dATP, dCTP, dGTP and dTTP were prepared in dH_2O and stored at -20^oC.

dNTP Working Solutions

DeoxyNTP stock solutions were diluted (1:20) in dH_2O to a final concentration of 0.5mM and stored at -20^oC.

dNTP Mixes

(for sequencing with [α-35S]dATP)

	A^o	C^o	G^o	T^o
0.5mM dCTP	20ul	1ul	20ul	20ul
0.5mM dGTP	20ul	20ul	1ul	20ul
0.5mM dTTP	20ul	20ul	20ul	1ul
TE buffer (pH 8.0)	20ul	20ul	20ul	20ul

DideoxyNTP Stock Solutions

Stock solutions (10mM) of ddATP, ddCTP, ddGTP and ddTTP were prepared in dH_2O and stored at -20^oC.

ddNTP Working Solutions

ddATP 0.15mM (3ul stock solution in 200ul H_2O)

ddATP 0.20mM (10ul stock solution in 500ul H_2O)

ddGTP 0.10mM (4ul stock solution in 400ul H_2O)

ddTTP 0.50mM (5ul stock solution in 100ul H_2O)

dNTP/ddNTP Mixes

A mix	25ul A^o	+	25ul 0.15mM ddATP
C mix	25ul C^o	+	25ul 0.20mM ddCTP
G mix	25ul G^o	+	25ul 0.10mM ddGTP
T mix	25ul T^o	+	25ul 0.50mM ddTTP

Chase Mix

10mM dATP	5ul
10mM dCTP	5ul
10mM dGTP	5ul
10mM dTTP	5ul
dH_2O	80ul

10 x Annealing Buffer

100mM TrisHCl (pH 8.0)

50mM $MgCl_2$

Stop Mix

Gently stir 100ml formamide with 5g Amberlite Mixed Bed resin for 30min. Filter to remove resin. Add 0.1g xylene cyanol, 0.1g bromophenol blue and 10ml 0.2M EDTA. Store at -20°C.

Gel Fixing Solution

acetic acid	200ml
methanol	200ml
dH_2O	1600ml

T A B L E 4.2

Sequencing Gel Stock Solutions and Mixes

Stock Acrylamide (40%)

acrylamide	38g
methylene-bisacrylamide	2g
dH_2O	to 100ml

Urea Mix

stock acrylamide	15ml
10xTBE	10ml
urea	42g
dH_2O	to 100ml

10% Ammonium Persulphate (APS)

APS	0.2g
dH_2O	to 2ml

Prepare fresh when required.

Sequencing Gel Mix

urea mix	60ml
10% APS	0.4ml
TEMED	40ul

R E S U L T S

Molecular Cloning of the EHV-4 gB Gene

The 2.9kb BamHI/EcoRI subfragment of EHV-4 BamHI-C was excised as a 2.9kb EcoRI fragment from the plasmid pUC9/BamHI-C and inserted into the EcoRI site in the polylinker of Bluescript M13+ (Figure 4.1). The structure of the resulting recombinant plasmid pBSgB is shown in Figure 4.2. The orientation of the EHV-4 DNA insert in pBSgB was determined by restriction endonuclease analysis of plasmid DNA (Figure 4.3).

Determination of the DNA Sequence of the EHV-4 gB Gene

To initiate sequencing of the DNA insert of pBSgB, T7 and KS primers were initially used. Thereafter, extensive use was made of EHV-4-specific oligonucleotide primers in order to rapidly generate sequence data. Since BamHI-M was predicted to contain the start of the gB gene, the right-hand end of this fragment was also sequenced. The relative orientation of BamHI-M in pUC9 was initially unknown and it was thus necessary to sequence both ends of BamHI-M (Figure 4.4) using the M13 and Reverse primers. The strategy used for determination of the DNA sequence of the EHV-4 gB gene is shown in Figure 4.5. An autoradiograph of a typical sequencing gel obtained is shown in Figure 4.6.

Analysis of the DNA Sequence

The DNA sequence obtained represents 3636 nucleotides. Translation of the DNA sequence in all three reading frames identified only one ORF of sufficient length to code for a

protein of the size expected for gB. The DNA sequence of this region and the predicted amino acid sequence of the EHV-4 gB protein are shown in Figure 4.7. The gB ORF is 2925 nucleotides in length, with an ATG initiation codon at position 270 and a chain termination codon at position 3195 of the sequence, and its predicted translation product would be a 975 amino acid (110kDa) protein. This compares with a 2709 nucleotide ORF encoding a 903 amino acid (100.3kDa) protein for HSV-1 gB.

Comparison of the EHV-4 gB and HSV-1 gB Genes

Alignment of the DNA sequences of the EHV-4 gB and HSV-1 gB genes revealed that the two genes are well conserved, demonstrating DNA homology of 52% (Figure 4.8). The putative transcriptional and translational control domains pertaining to these genes are particularly well conserved. The putative TATA box (Corden et al., 1980) of the EHV-4 gB gene, AATATAT, occurs at nucleotides 119 to 125 and is almost in perfect alignment with the HSV-1 gB TATA box, ATATATT, at nucleotides 234 to 240 in the HSV-1 gB sequence. The first transcribed base of eukaryotic mRNA (mRNA initiation site) is usually an A residue surrounded by pyrimidine residues (Busslinger et al., 1980; Breathnach and Chambon, 1981). From analysis of the EHV-4 sequence, the A residue at position 150 (within the sequence CAGT) is the most likely candidate for the mRNA initiation site of the gB gene since it is located 25 nucleotides downstream of the TATA box. This is consistent with the typical 24- to 32-nucleotide spacing between the TATA box and the mRNA initiation site (Busslinger et al., 1980; Breathnach and Chambon, 1981). Furthermore, this putative mRNA initiation site aligns well with

that predicted for the HSV-1 gB gene and is consistent with the transcript mapping reported for the EHV-1 gB gene (Bell et al., 1990). The sequence ATTG at nucleotides 38 to 41 of the EHV-4 gB sequence reads CAAT on the opposite strand and, since it is about 100 nucleotides upstream of the putative mRNA initiation site, may function as a CAT box (Breathnach and Chambon, 1981). This CAT box is identical to, and in perfect alignment with, that predicted for the HSV-1 gB gene.

Although the HSV-1 gB gene possesses a decanucleotide just upstream of the CAT box which matches the consensus binding sequence for the cellular transcription factor Sp1 (Kadonaga et al., 1986) at 8 out of 10 positions (nucleotides 130 to 139), no such sequence is observed in the EHV-4 gB sequence.

The TATA and CAT elements are conserved in all alphaherpesvirus gB-like genes sequenced to date, with the exception of the BHV-1 gI gene which does not possess a sequence resembling a CAT box. However, this gene is unusual in possessing three putative Sp1 binding sites, two of which are located around the position where the CAT box would have been expected to occur (Whitbeck et al., 1988). As such, the BHV-1 gI gene appears to be the only alphaherpesvirus gB-like gene other than HSV-1 gB to possess an Sp1 binding site.

The modified scanning hypothesis of translation states that the first ATG following the mRNA initiation site will act as the initiation codon, assuming that the local environment will allow this to occur (Kozak, 1981, 1983). The first such ATG in the EHV-4 gB gene occurs at nucleotide 270 and is in-frame with two closely located ATGs: the second ATG at nucleotide 412 is immediately followed by an in-frame termination codon and the

next available ATG is at nucleotide 438. The sequences flanking the first ATG codon (ACGTCATGT) are favourable for efficient translation initiation at this site as proposed by Kozak (1984, 1986), namely a purine (G) at -3, C at -1 and -4, although the favoured G at +4 is replaced by a T. The sequences surrounding the second possible initiation site at nucleotide 438 (CCCCCATGT) suggest that this site is less likely to be used since there is no purine at position -3 or a G at +4, although C residues are present at -1 and -4. The first ATG at nucleotide 270 is the most likely initiation codon for the EHV-4 gB gene.

The termination codon of the EHV-4 gB gene is at nucleotide 3195, 9 nucleotides prior to a consensus polyadenylation signal, AATAAA (Berget, 1984). The poly(A) addition site of eukaryotic mRNAs is usually found 10 to 30 nucleotides downstream of the polyadenylation signal (Fitzgerald and Shenk, 1981; Birnstiel et al., 1985). Since the poly(A) addition site is usually at the dinucleotide CA or TA, the putative poly(A) addition site of the EHV-4 gB gene may be at nucleotide 3221 or 3225. An additional element, the GT-rich cluster, is usually found about 30 nucleotides downstream of the polyadenylation signal (Birnstiel et al., 1985) which often conforms to the consensus sequence YGTGTTYY (Y=pyrimidine) (McLauchlan et al., 1985) and is associated with 3' processing of polyadenylated mRNAs. The only GT-rich cluster sufficiently close to the EHV-4 gB polyadenylation signal is the sequence CTTGTGGGTTG at nucleotides 3226 to 3236.

Identification of the putative mRNA initiation and termination sites from the EHV-4 gB DNA sequence predicts that the mRNA for this gene would be about 3070 nucleotides prior to

polyadenylation, which is similar in size to the 3050 nucleotides predicted for the HSV-1 gB mRNA (Bzik et al., 1984a).

Comparison of the EHV-4 gB and EHV-1 gB Genes

An alignment of the EHV-4 gB and EHV-1 gB genes is shown in Figure 4.9. The two genes show a remarkably high degree of homology (80%) with only the 5′ region of the genes displaying albeit a limited degree of divergence. A similarly high level of gene conservation (86%) has also been reported for the gB genes of the closely related herpesviruses HSV-1 and HSV-2 (Stuve et al., 1987).

Identification of an Upstream ORF That Overlaps the 5′ End of the EHV-4 gB Coding Sequences

Analysis of the EHV-4 DNA sequence identified the termination codon of a partial upstream ORF at nucleotide 406 which overlaps the gB coding sequences by 136 nucleotides at its 5′ end (Figure 4.7). This upstream ORF is the homologue of the HSV-1 ICP18.5 gene, whose product is involved in the transport of viral glycoproteins (Pellett et al., 1986). Such an overlap has also been reported for PRV (Robbins et al., 1987) and BHV-1 (Whitbeck et al., 1988), with overlaps of 132 and 141 nucleotides, respectively. However, in HSV-1 the ICP18.5 gene terminates 10 nucleotides prior to the gB initiation codon (nucleotide 538, Figure 4.8) and inspection of the MDV (Ross et al., 1989) and VZV (Davison and Scott, 1986a) DNA sequences revealed that the upstream ORF terminates 77 and 47 nucleotides prior to the gB initiation codon, respectively. No

polyadenylation signal is evident at the 3′ end of the ORFs encoding the ICP18.5 gene homologue in any of the alphaherpesviruses examined, which suggests that the 3′ end of the transcript may be coterminal with that of the gB gene as reported for HSV-1 (Holland et al., 1984a).

Analysis of the EHV-4 gB Gene Product and Comparison With the HSV-1 gB Protein

An alignment of the predicted amino acid sequences of the EHV-4 gB and HSV-1 gB proteins is presented in Figure 4.10. The unprocessed EHV-4 gB protein is predicted to be 975 amino acids (110kDa) as compared to 903 amino acids (100.3kDa) for HSV-1 gB. In this alignment, 47% of the amino acids are perfectly matched between the two proteins. The codon usage and amino acid composition of the EHV-4 gB protein are shown in Table 4.3. The G+C content of the first, second and third bases of the codons is 51, 41 and 50%, respectively. The strong preference for a G or C in the third base of the codons noted for HSV-1 gB (Bzik et al., 1984a) is not evident for EHV-4 gB, and is a reflection of the lower G+C content of the EHV-4 gB coding region (47%) when compared to that of HSV-1 gB (66%).

Hydropathic analysis of the EHV-4 gB protein revealed that it possesses features characteristic of envelope glycoproteins: a hydrophobic signal sequence near the amino-terminus, a large hydrophilic external domain, a strongly hydrophobic membrane-spanning domain near the carboxyl-terminus and a predominantly hydrophilic cytoplasmic anchor domain at the carboxyl-terminus. The hydropathic profiles and secondary structure analyses for the EHV-4 gB and HSV-1 gB proteins are presented in Figure 4.11.

They strongly suggest that the two proteins have a very similar overall structure.

In the following analysis of the EHV-4 gB protein each domain shall be described separately. The residue numbers referred to correspond to those in Figure 4.10.

i) Signal sequence domain

Unlike the HSV-1 gB signal sequence, that of EHV-4 gB is unusually long (84 amino acids). The 'true' EHV-4 gB signal sequence is preceded by an additional 56 amino acids which are generally hydrophilic and are evident as a distinct hydrophilic peak in the hydropathic profile (Figure 4.11). A similar hydrophilic region has also been reported for the gB homologues of PRV (Robbins et al., 1987) and BHV-1 (Whitbeck et al., 1988) which have unusually long signal sequences of 53 and 67 amino acids, respectively. It has been suggested that such unusually long signal sequences are a reflection of constraints imposed upon the DNA sequence to encode both a functional signal sequence and the carboxyl-terminal amino acids of the ICP18.5 protein homologue encoded by the upstream ORF (Robbins et al., 1987).

Signal sequences are characterised by a hydrophobic core sequence preceded by positively charged residues (Perlman and Halvorson, 1983) and immediately followed by a signal peptidase cleavage site near a predicted beta turn (von Heijne, 1984). A strongly hydrophobic sequence from Ile_{13} to Val_{26} represents the hydrophobic core which would be of sufficient length to span the membrane but not to serve as an anchor sequence. Cleavage of the EHV-4 gB signal sequence probably occurs after Ala_{28}, which is immediately preceded by a beta turn. The proposal of this as the

cleavage site is supported by the observation that a helix-breaking residue (Gly) occurs at position 22 and a polar residue (Glu) at position 27, which is consistent with the general presence of these types of residues within the eight residues prior to the cleavage site (Watson, 1984). The EHV-4 gB signal sequence carries an Ala at its carboxyl-terminus, one of the most popular residues found in this position (von Heijne, 1983; Watson, 1984). The predicted signal sequence also conforms to the -3, -1 rule (von Heijne, 1983, 1984) ie. an Ala at -1 and a small, neutral residue (Val) at -3. The predicted EHV-4 gB cleavage site is in perfect alignment with that of HSV-1 gB.

ii) Hydrophilic surface domain

By analogy with HSV-1 gB, the hydrophilic surface domain of EHV-4 gB is predicted to extend from Val_{29} to Asp_{740}, is exposed on the outer surface of the viral envelope and contains epitopes for virus neutralising antibodies (Pellett et al., 1985b). EHV-4 gB is predicted to possess 11 potential N-linked glycosylation sites (Asn-X-Ser/Thr, where X is not Pro) in the external domain. Four of the six potential N-linked glycosylation sites in HSV-1 gB are perfectly conserved with EHV-4 gB which, taken together with the fact that all ten cysteine residues in the external domain are also conserved, suggests that the two proteins possess similar secondary and tertiary structures. The secondary structure analysis of Chou and Fasman (1978) indicates that, as previously predicted for HSV-1 gB (Pellett et al., 1985b), most of the glycosylation sites in EHV-4 gB are located at the junction of alpha-helical and beta-sheet domains (Figure 4.11), thereby orientating these sites to the outer surface of the molecule. A potential proteolytic cleavage site, not found

in HSV-1 gB, was identified in the EHV-4 gB sequence (Arg-Arg/Ser, residues 459 to 461).

iii) Hydrophobic transmembrane domain

The three hydrophobic peaks towards the carboxyl-terminus in the hydropathic profile of EHV-4 gB (Figure 4.11) represent, by analogy with HSV-1 gB, the transmembrane domain. This domain consists of 69 amino acids (residues 741 to 809) and is identical in size to that found in HSV-1 gB. This hydrophobic domain is predicted to contain three antiparallel segments connected to each other by a very short turn region which traverse the membrane (Pellett et al., 1985b).

iv) Cytoplasmic anchor domain

The remaining 110 amino acids of EHV-4 gB (residues 810 to 919) are hydrophilic and predicted to adopt an alpha-helical conformation. This region shares primary amino acid homology of 49% with HSV-1 gB; two particular regions of EHV-4 gB (residues 810 to 828 and residues 849 to 878) show local similarities with HSV-1 gB of 84% and 74%, respectively. Of the 110 amino acids in the cytoplasmic domain of EHV-4 gB, 24 are positively charged and 17 are negatively charged, giving an overall positive charge as in HSV-1 gB.

Comparison of the EHV-4 gB and EHV-1 gB Proteins

An alignment of the EHV-4 gB and EHV-1 gB proteins is shown in Figure 4.12. The two proteins are highly homologous, sharing 88% of their sequences. This compares with a value of 86% reported for the gB proteins of HSV-1 and HSV-2 (Stuve et al., 1987). The signal sequences show greatest diversity (55% identity) and all ten cysteine residues in the surface domains

are conserved between the two proteins, although EHV-1 gB contains an extra cysteine residue at position 259 which is probably not involved in secondary structure formation. The nine N-linked glycosylation sites in EHV-1 gB are conserved with EHV-4 gB, but EHV-4 gB is somewhat unusual in possessing three such sites in succession (EHV-4 gB residues 549 to 557) with only one of these sites being conserved with EHV-1 gB. This conserved site may be the only one of these three sites utilised in EHV-4 gB.

Some of the amino acid changes elsewhere between the EHV-4 gB and EHV-1 gB proteins may represent epitope sites which can lead to altered antigenic specificity, in particular EHV-4 gB residues 91 to 103 and 256 to 262. The high amino acid conservation between the EHV-4 gB and EHV-1 gB proteins is consistent with the type-common nature of their antigenic epitopes (Allen and Bryans, 1986).

An identical internal proteolytic cleavage site that aligns with that of EHV-4 gB is present in EHV-1 gB (RR/S, EHV-1 gB residues 520 to 522).

Comparison of EHV-4 gB to the gB-Like Proteins of Other Herpesviruses

The salient features of the gB-like proteins of the eight herpesviruses compared in this section are summarised in Table 4.4. Alignment of EHV-4 gB with the gB-like proteins of the alphaherpesviruses PRV, BHV-1, MDV, VZV and HSV-1, the betaherpesvirus HCMV and the gammaherpesvirus EBV (Figure 4.13) clearly demonstrates the highly conserved nature of gB across the herpesviruses, with only the amino- and carboxyl-termini

displaying marked levels of dissimilarity. The conservation of all ten cysteine residues on the external domain of the molecules across the eight viruses indicates that the proteins adopt similar secondary, and possibly tertiary, structures. The predicted similarity of tertiary structure is reinforced by the conservation of several N-linked glycosylation sites on the proteins. For example, EHV-4 gB shares three sites with PRV, BHV-1 and EBV, and four with MDV, HSV-1 and VZV, with five of the sites in EHV-4 gB being shared with at least three other gB proteins. Some of these sites are particularly well conserved: that at EHV-4 gB position 162 is only absent in MDV and HCMV, another corresponding to HSV-1 gB position 429 is only absent in EHV-4 and EBV, whilst the best conserved site is at EHV-4 gB position 744 and is only absent in HCMV. Several short motifs are well conserved between the molecules, the most noteable examples being CYSRP (EHV-4 gB residues 663 to 667) and GQLG (EHV-4 gB residues 685 to 688) which are perfectly aligned in all the gB proteins and may thus be of functional significance. Amino acids other than cysteine are also conserved: nine phenylalanine residues are aligned in all eight proteins and 15 proline residues are conserved in the six alphaherpesvirus proteins.

VZV gpII exists as a disulphide-linked dimer composed of two species, each of approximately 60kDa (Grose et al., 1984). The mature species is a heterodimer generated by in vivo proteolytic cleavage after the two consecutive arginine residues in the sequence R-R/S near the centre of the molecule (Keller et al., 1986). It can be seen from Figure 4.13 that a similar site is also present in a similar position in the gB-like proteins of

EHV-4 (R-R/S), PRV (R-R/S), HCMV (R-R/S), BHV-1 (R-R/A), MDV (R-R/D) and EBV (R-R/D), but not in HSV-1 gB which is not cleaved. Post-translational proteolytic cleavage during processing has also been reported to occur for HCMV gB (Spaete et al., 1988; Britt and Vugler, 1989), PRV gII (Lukacs et al., 1985; Mettenleiter et al., 1986), BHV-1 gI (van Drunen Littel-van den Hurk and Babiuk, 1986), and EHV-1 gB and EHV-4 gB (Meredith et al., 1989; Sullivan et al., 1989). MDV gB also appears to undergo internal proteolytic cleavage (Ross et al., 1989). Although EBV gB contains a putative proteolytic cleavage site, it does not appear to be cleaved (Gong et al., 1987). The predicted proteolytic cleavage sites are located within a region of great diversity and such local divergence of amino acid sequence may be a direct result of processing at these sites. The cleavage sites are generally contained within a stretch of amino acids which have no counterparts in HSV-1 gB. For example, EHV-4 gB contains a 47 amino acid insertion relative to HSV-1 gB whilst the insertion in BHV-1 gI is 24 amino acids.

The extent of similarity between EHV-4 gB and the analogous proteins of other herpesviruses is readily apparent from the dot matrix similarity analysis presented in Figure 4.14. This illustrates the very close relationship between EHV-4 gB and EHV-1 gB, the extensive similarity between EHV-4 gB and the gB-like proteins of other alphaherpesviruses, and the more limited yet significant similarity with the gB proteins of the betaherpesvirus HCMV and the gammaherpesvirus EBV. The overall percentage identity for pairwise comparisons of the herpesvirus gB-like proteins is summarised in Table 4.5. It is concluded that the alphaherpesvirus gB-like proteins show a similar degree

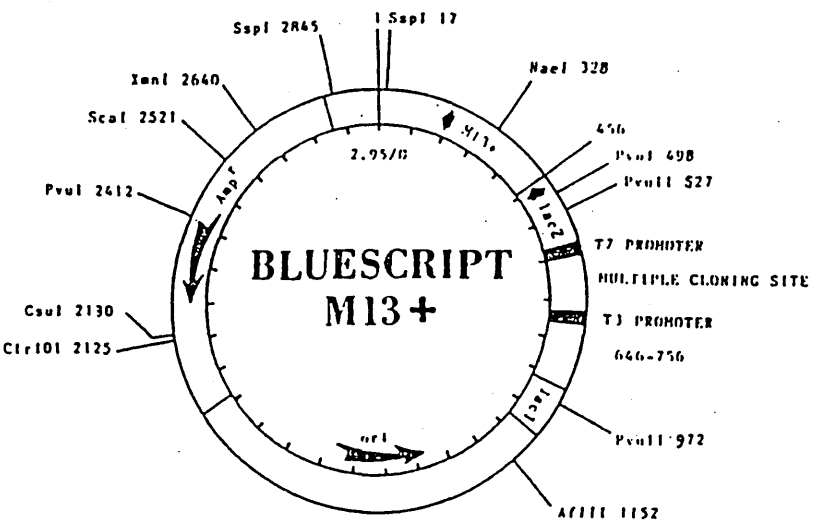
of homology to each other (about 50%) but significantly less homology to HCMV gB (about 30%) and EBV gB (about 30%). Furthermore, HCMV gB and EBV gB are as dissimilar from each other as they are from the alphaherpesvirus gB-like proteins (31% identity), which suggests that each family of herpesviruses may have undergone considerable evolution from a common ancestral virus.

Comparison of the Carboxyl-Terminal Amino Acids of the Gene Products of Eight Herpesviruses Analogous to HSV-1 ICP18.5

An alignment of the carboxyl-terminal amino acids of the ICP18.5-like gene products of eight herpesviruses is shown in Figure 4.15. The amino acid homology in this region is close to that observed for the gB proteins of these viruses. HSV-1 ICP18.5 has been shown to demonstrate an identity of 22% with the gene product of EBV BALF3 (Pellett et al., 1986) and 46% with the gene product of VZV gene 30 (McGeoch et al., 1988a). ICP18.5 is also a well conserved protein of the herpesviruses which can be expected to have a similar function in the transport of viral glycoproteins in each virus.

FIGURE 4.1

Physical Map and Polylinker of the Plasmid Bluescript



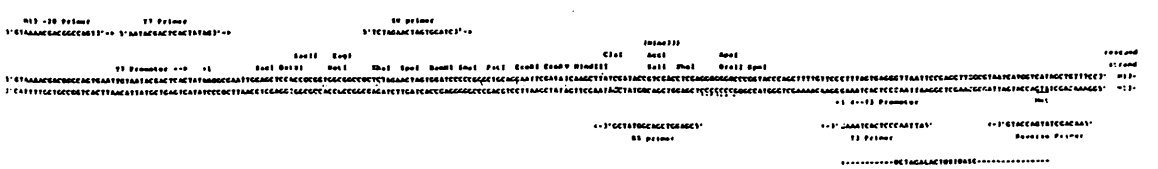


Figure 4.1 Physical map and polylinker of the plasmid Bluescript M13+. Primer recognition sites are indicated.

F I G U R E 4.2

Physical Map of Plasmid pBSgB

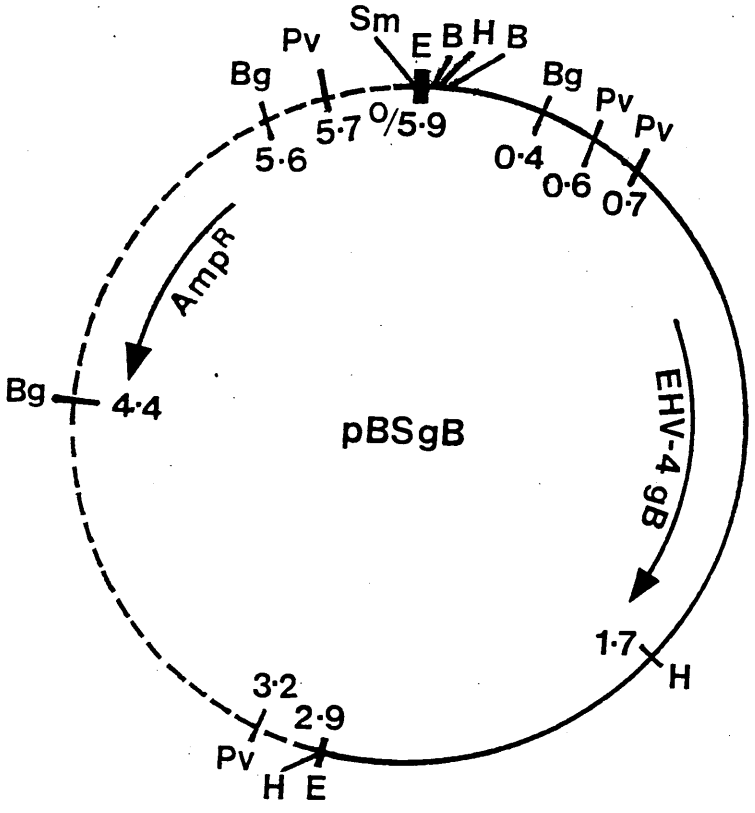


Figure 4.2 Physical map of plasmid pBSgB. The broken line represents Bluescript DNA and the solid line represents EHV-4 gB DNA. Sizes are shown in kilobases. The BamHI (B), BglI (Bg), EcoRI (E), HindIII (H), PvuII (Pv) and SmaI (Sm) sites are shown. The plasmid contains a small contaminating BamHI fragment excised from BamHI-C during the cloning process, and this fragment contains an additional HindIII site. The transcriptional orientation of the gB gene is denoted by an arrow.

F I G U R E 4.3

Restriction Endonuclease Analysis of Plasmid pBSgB

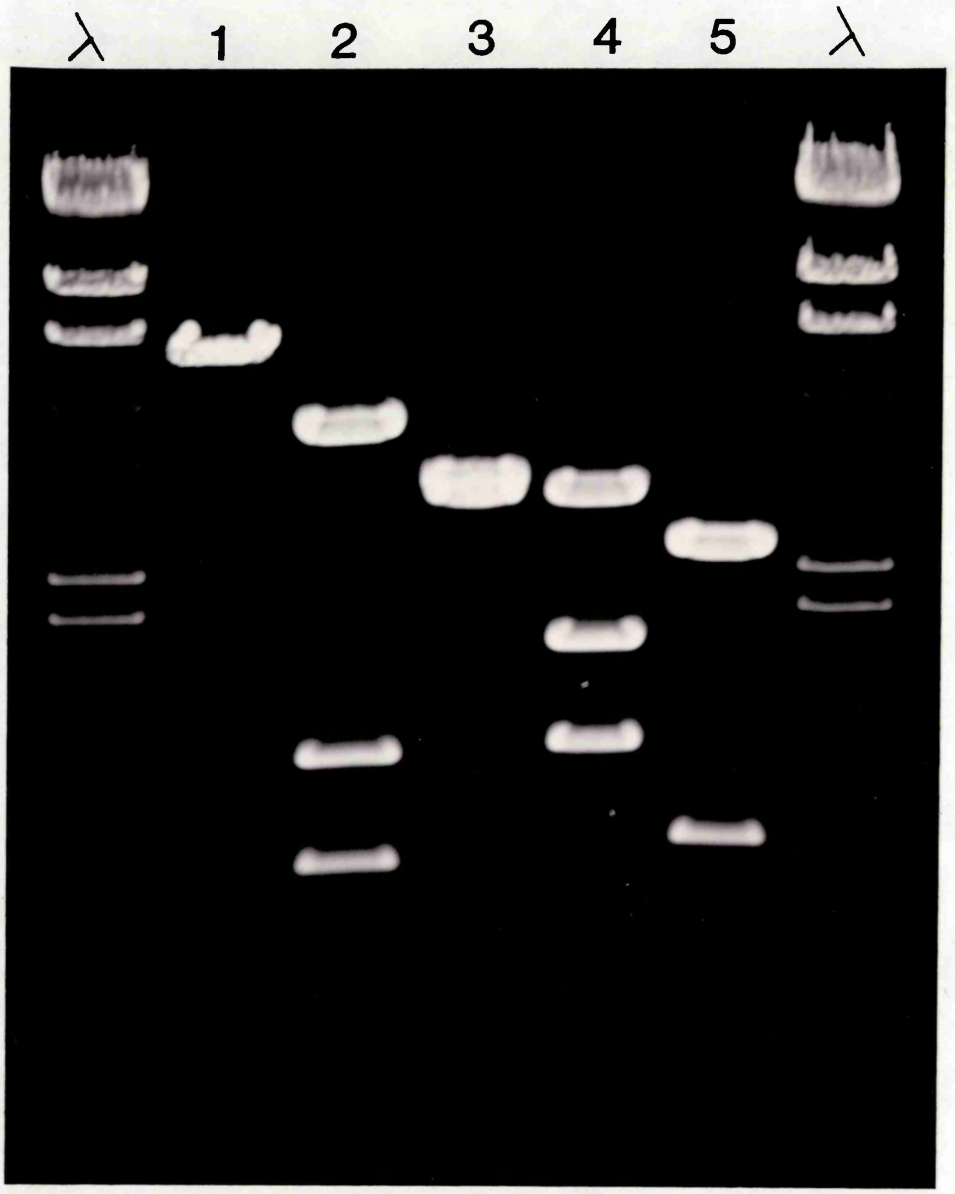


Figure 4.3 Restriction endonuclease analysis of plasmid pBSgB. The plasmid was digested with BamHI (lane 1), BglI (lane 2), EcoRI (lane 3), HindIII (lane 4) and PvuII (lane 5). HindIII digested λ DNA fragments were used as size markers.

F I G U R E 4.4

Physical Map of Plasmid pUC9/EHV-4 BamHI-M

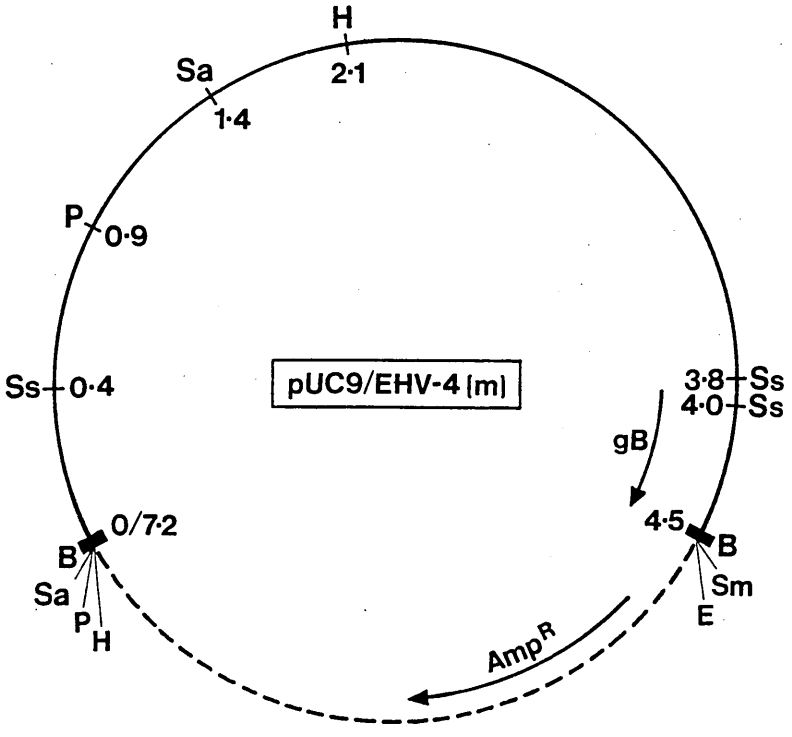


Figure 4.4 Physical map of plasmid pUC9/EHV-4 BamHI-M. The broken line represents pUC9 DNA and the solid line represents EHV-4 BamHI-M DNA. Sizes are shown in kilobases. The BamHI (B), EcoRI (E), HindIII (H), PstI (P), SalI (Sa), SmaI (Sm) and SstI (Ss) sites are shown. The start of the EHV-4 gB gene at the right-hand end of BamHI-M is indicated by an arrow.

F I G U R E 4.5

Determination of the DNA Sequence of the EHV-4 gB Gene

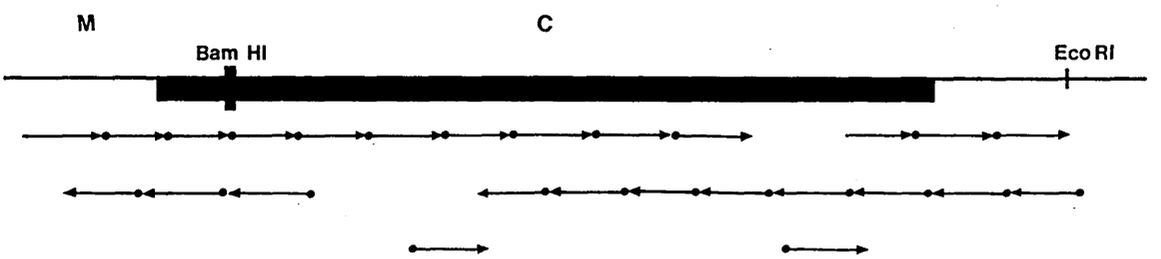


Figure 4.5 Strategy for DNA sequence determination of the EHV-4 gB gene. Sequence data were generated with custom made oligonucleotide primers. Dots represent the 5′ end and arrowheads the 3′ end of each portion of the sequence. The EHV-4 gB ORF is denoted by a thick bar.

Figure 4.6 Autoradiograph of a typical sequencing gel obtained in determining the DNA sequence of the EHV-4 gB gene. The custom made primers used were MR10 on the upper strand (5'-TCCATTTCGTCCACGTT-3', corresponding to nucleotides 1503 to 1519 of the EHV-4 gB DNA sequence shown in Figure 4.7) and MR11 on the lower strand (5'-TGTCTTCAAGCTCAGCT-3', corresponding to the complementary sequence of nucleotides 2568 to 2552 of the EHV-4 gB DNA sequence shown in Figure 4.7). These primers were annealed to single-stranded DNA template generated from plasmid pBSgB and sequencing reactions then carried out. The samples shown on the gel are as follows:-
primer MR10 (long run)- A track (lane 1), C track (lane 2), G track (lane 3) and T track (lane 4); primer MR11 (long run)- A track (lane 5), C track (lane 6), G track (lane 7) and T track (lane 8); primer MR10 (short run)- A track (lane 9), C track (lane 10), G track (lane 11) and T track (lane 12); primer MR11 (short run)- A track (lane 13), C track (lane 14), G track (lane 15) and T track (lane 16).

F I G U R E 4.6

Autoradiograph of a Typical Sequencing Gel Obtained in Determining the DNA Sequence of the EHV-4 gB Gene

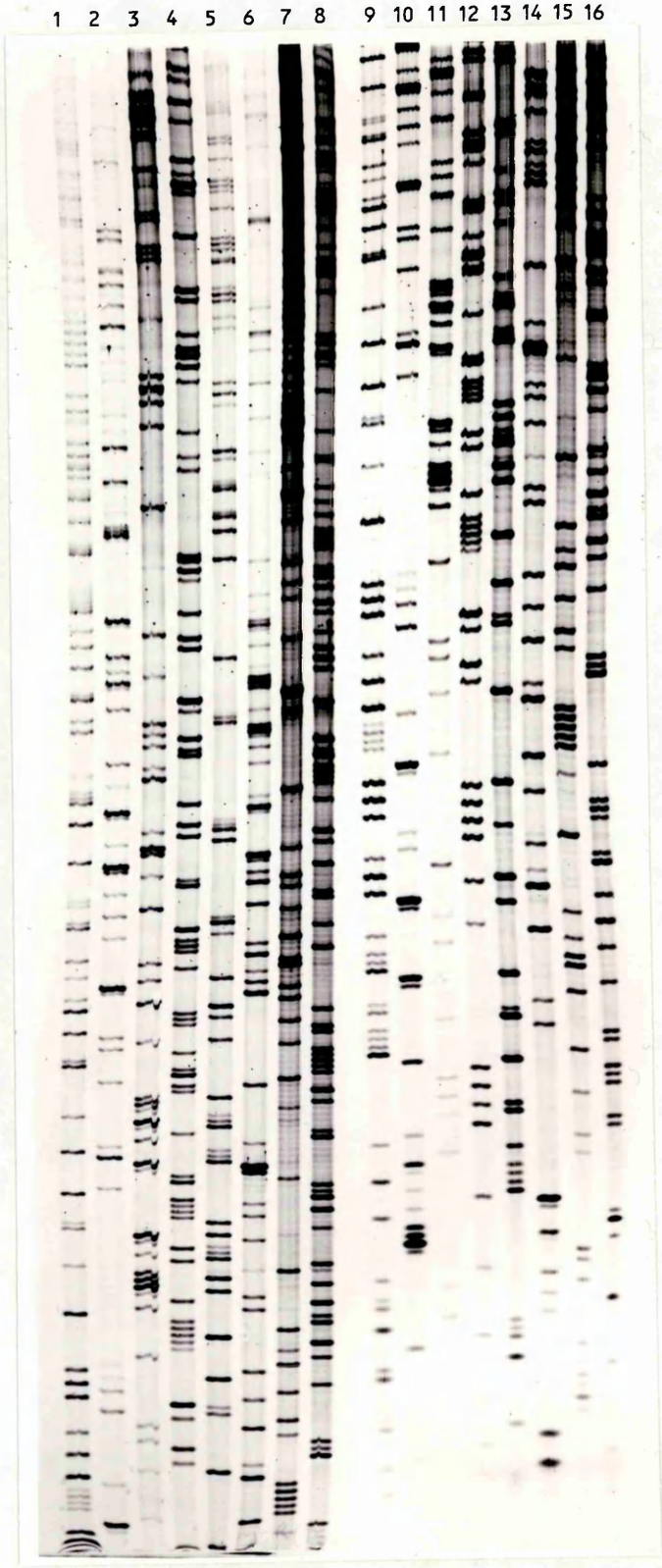


Figure 4.7 DNA sequence of the right-hand end of EHV-4 BamHI-M and the 2.9kb BamHI/EcoRI left-terminal subfragment of EHV-4 BamHI-C. The predicted amino acid sequences of the EHV-4 gB protein and the carboxyl-terminal region of the upstream overlapping EHV-4 gene product analogous to HSV-1 ICP18.5 are shown above the DNA sequence. The putative TATA box (TATA), CAT box (CAT), mRNA initiation site (mRNA init) and polyadenylation signal (poly A) of the gB gene are underlined. The signal sequence, surface, transmembrane and cytoplasmic domains of the gB protein are indicated.

FIGURE 4.7

DNA Sequence of the EHV-4 gB Gene

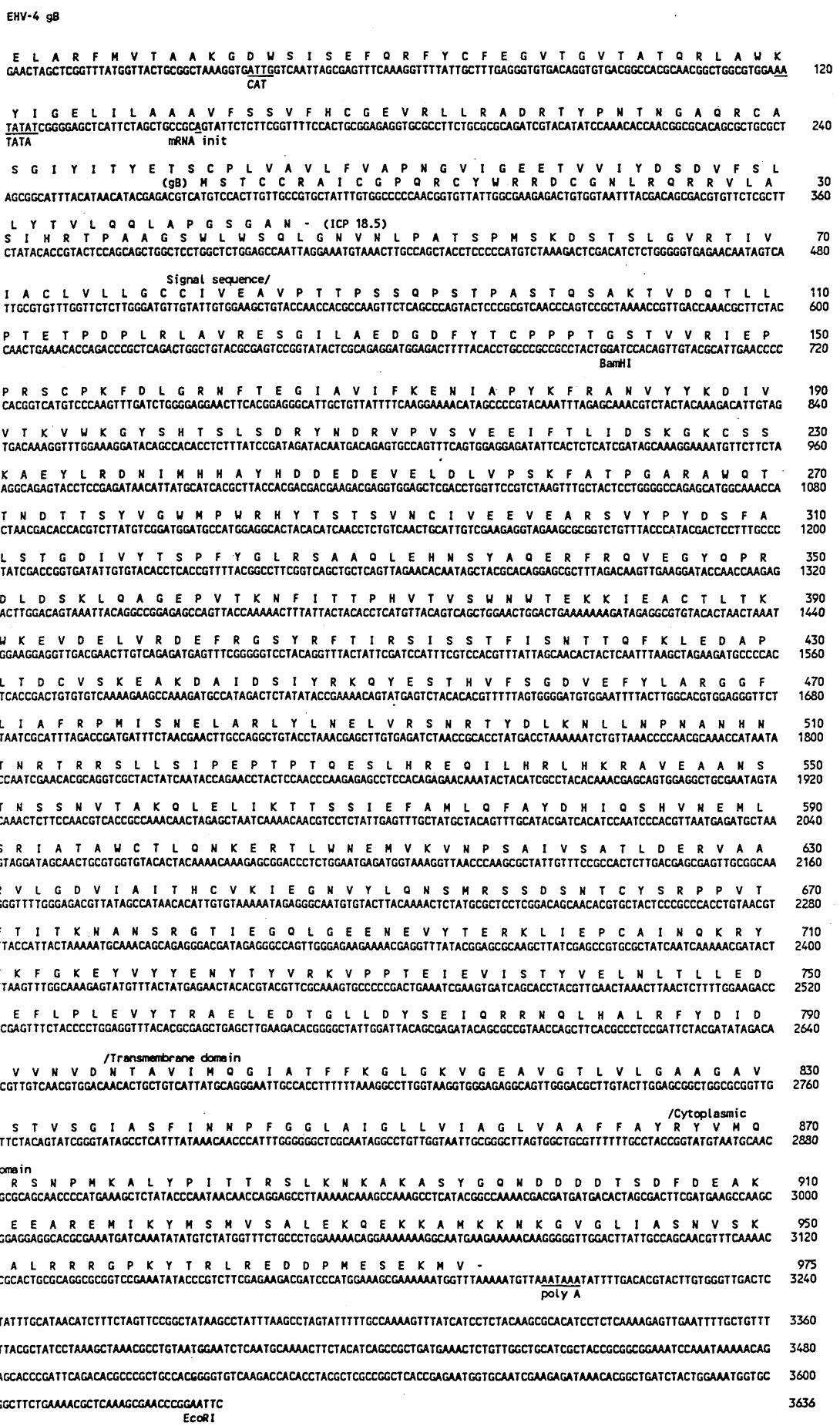


FIGURE 4.8

Alignment of the EHV-4 gB and HSV-1 gB DNA Sequences

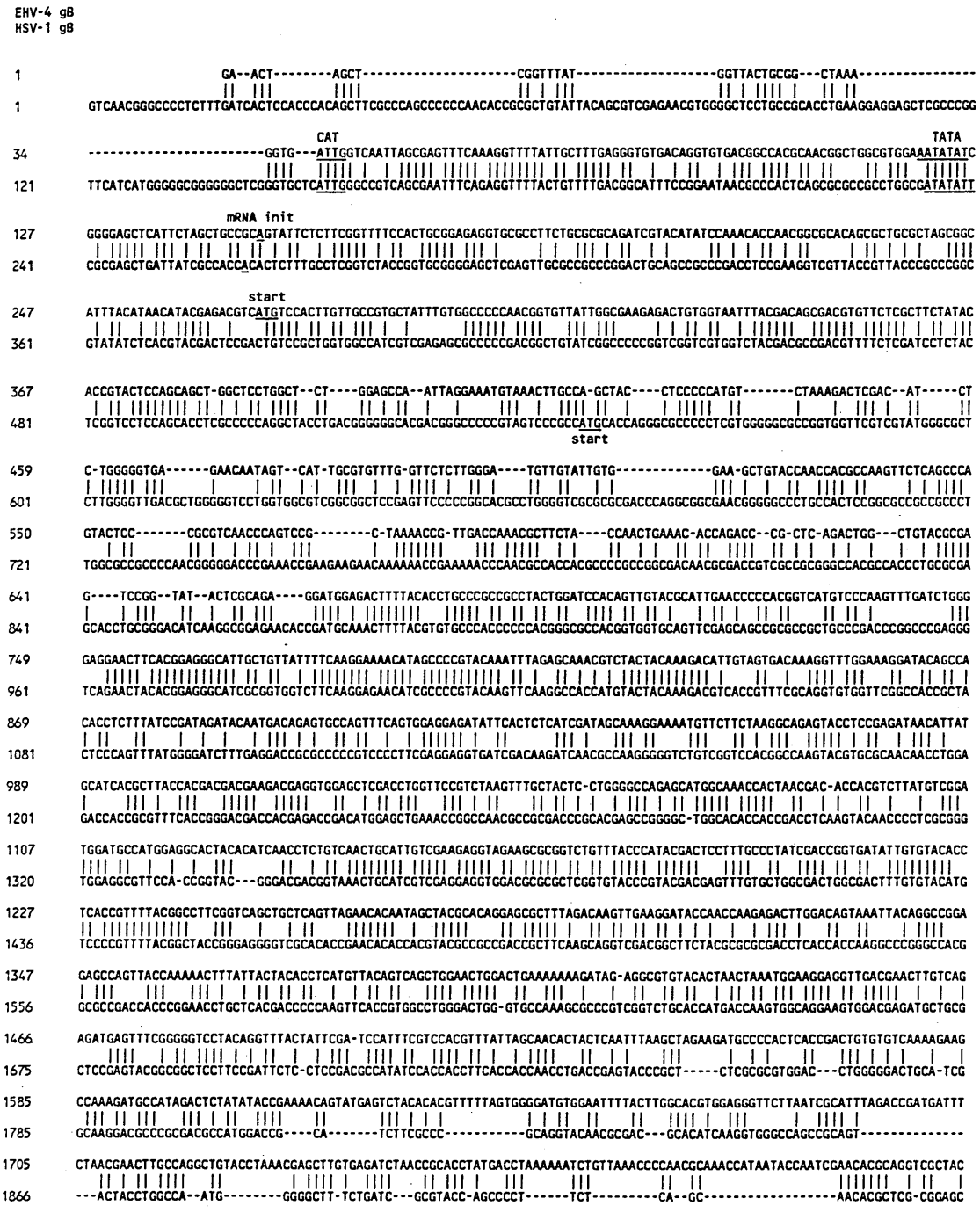


Figure 4.8- continued

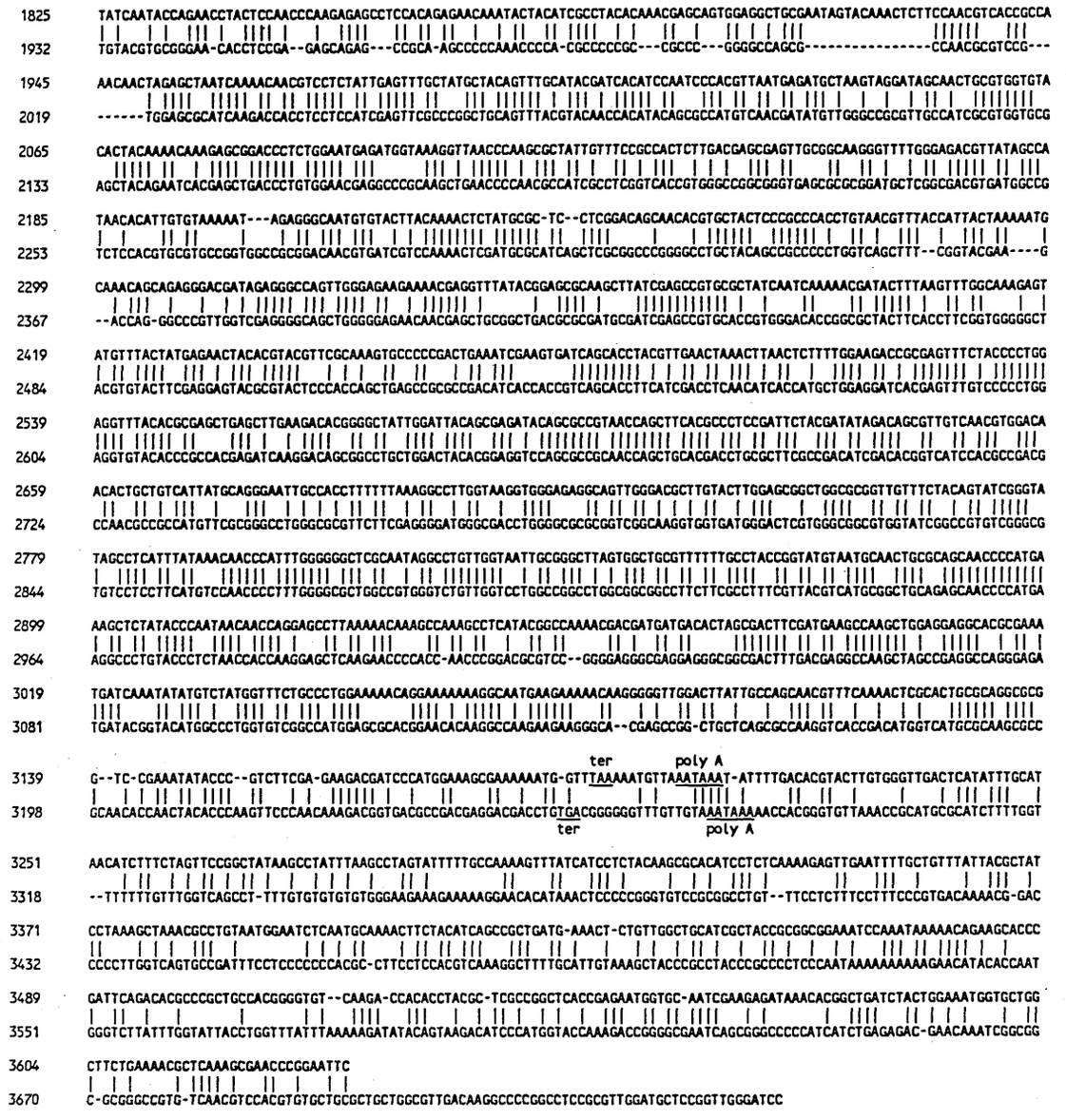


Figure 4.8 Alignment of the EHV-4 gB and HSV-1 (KOS) gB DNA sequences. The upper sequence is that of EHV-4 gB, and the lower sequence that of HSV-1 gB. Identical residues are indicated with a vertical bar, and dashes denote spaces introduced to maximise homology between the two sequences. Salient features (TATA box, CAT box, mRNA initiation site, termination codon [ter] and polyadenylation signal) are indicated for each sequence. The HSV-1 (KOS) gB DNA sequence is from Bzik et al. (1984a).

FIGURE 4.9

Alignment of the EHV-4 gB and EHV-1 gB DNA Sequences

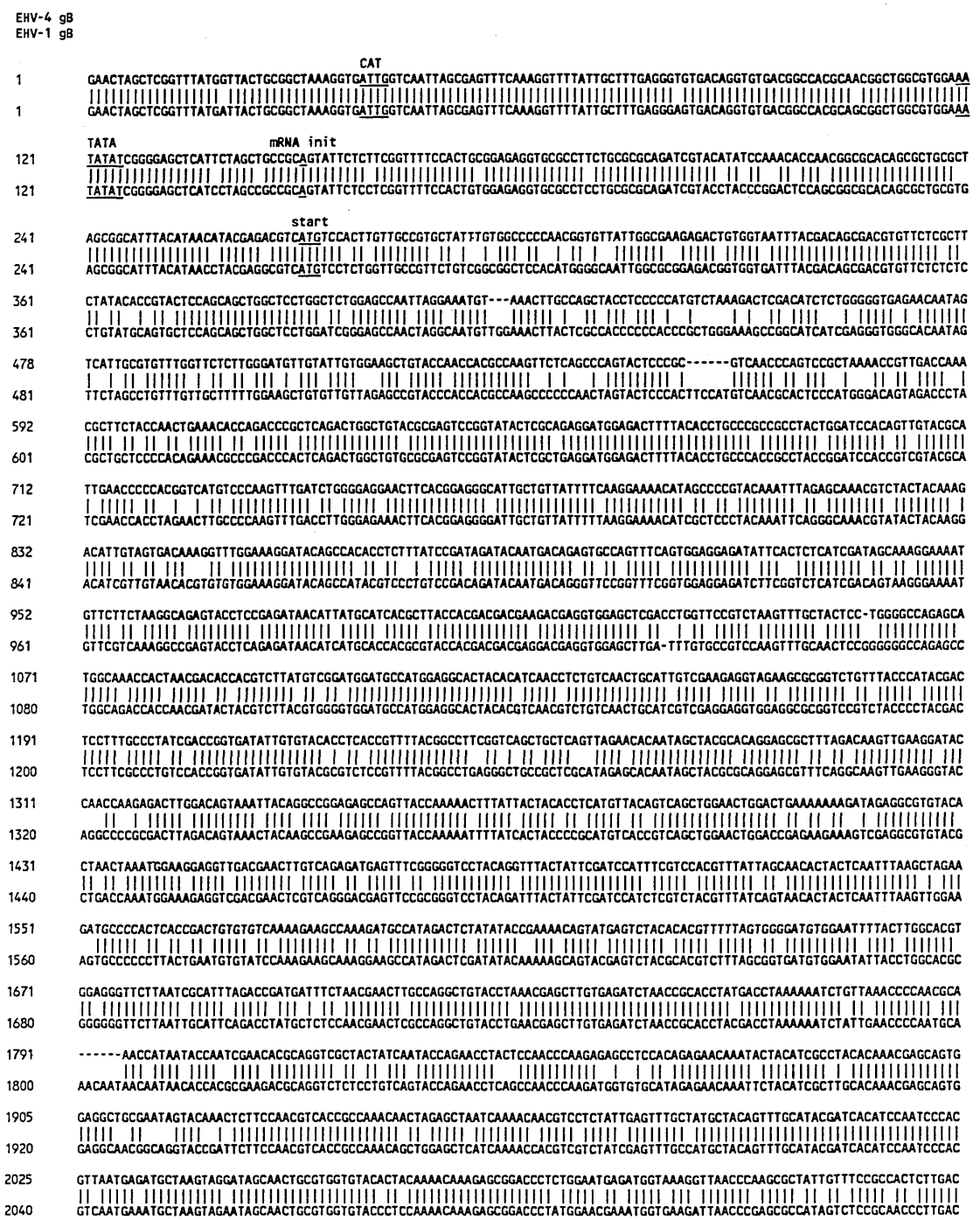


Figure 4.9- continued

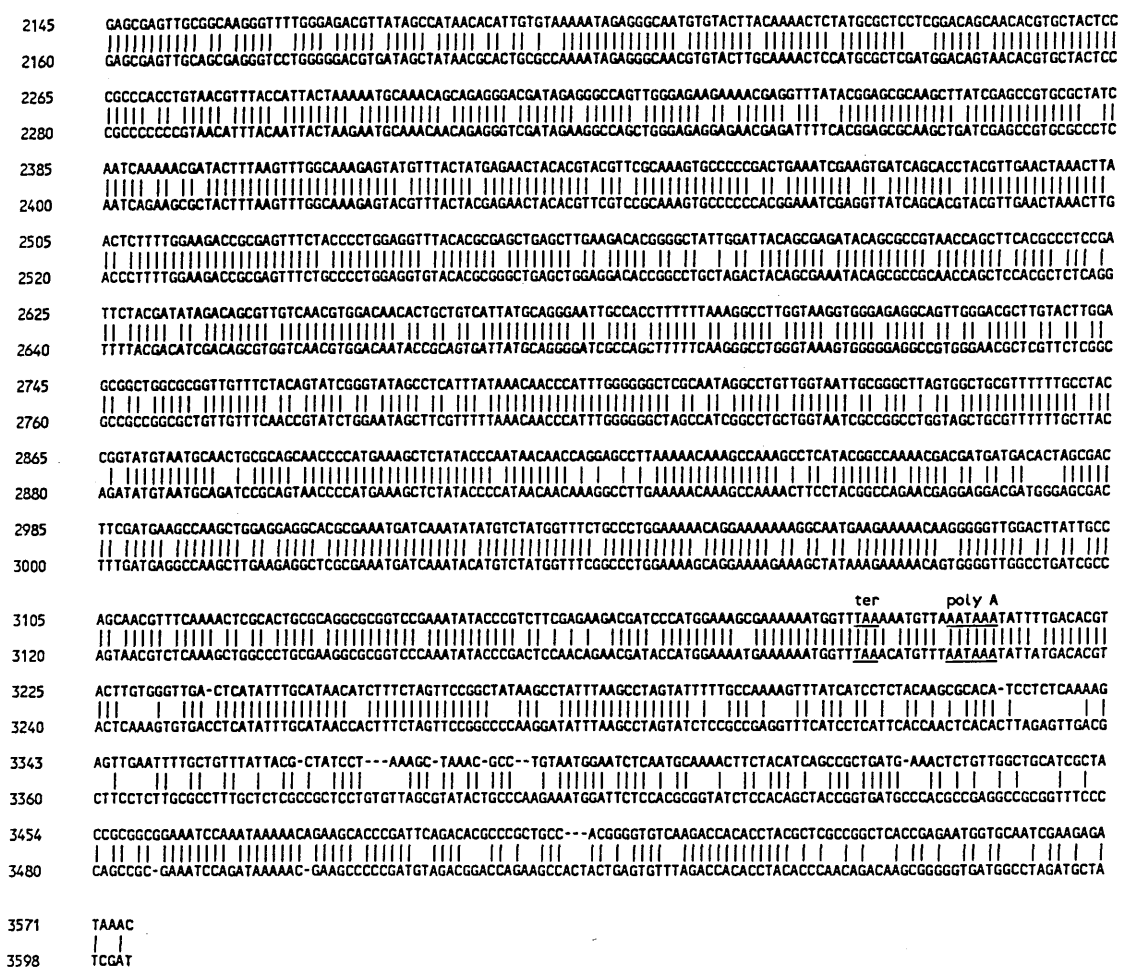


Figure 4.9 Alignment of the EHV-4 gB and EHV-1 gB DNA sequences. The upper sequence is that of EHV-4 gB, and the lower sequence that of EHV-1 gB. Identical residues are indicated with a vertical bar, and dashes denote spaces introduced to maximise homology between the two sequences. Salient features (TATA box, CAT box, mRNA initiation site, termination codon and polyadenylation signal) are indicated for each sequence. The EHV-1 gB DNA sequence is from Whalley et al. (1989).

FIGURE 4.10

Alignment of the EHV-4 gB and HSV-1 gB Amino Acid Sequences

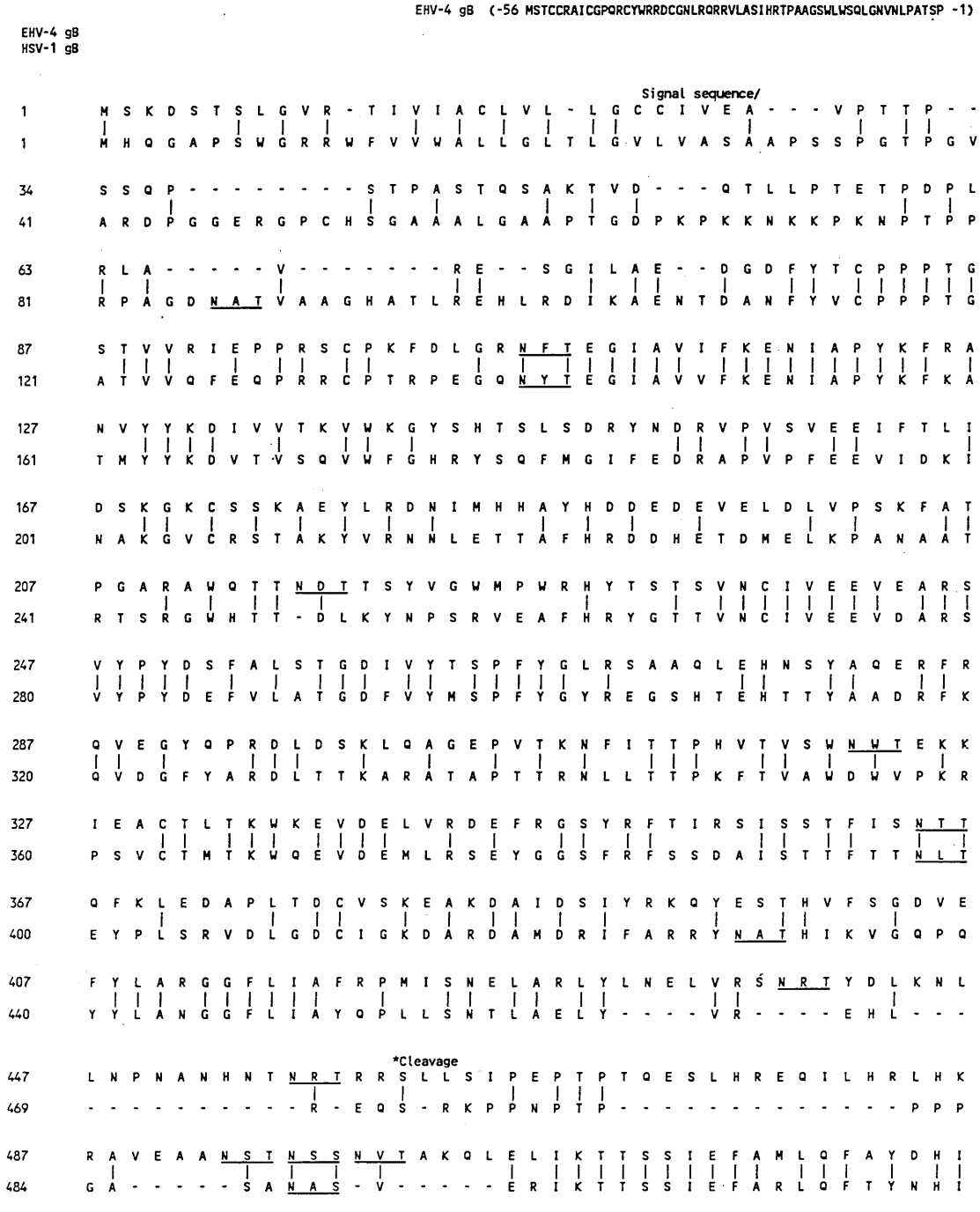


Figure 4.10- continued

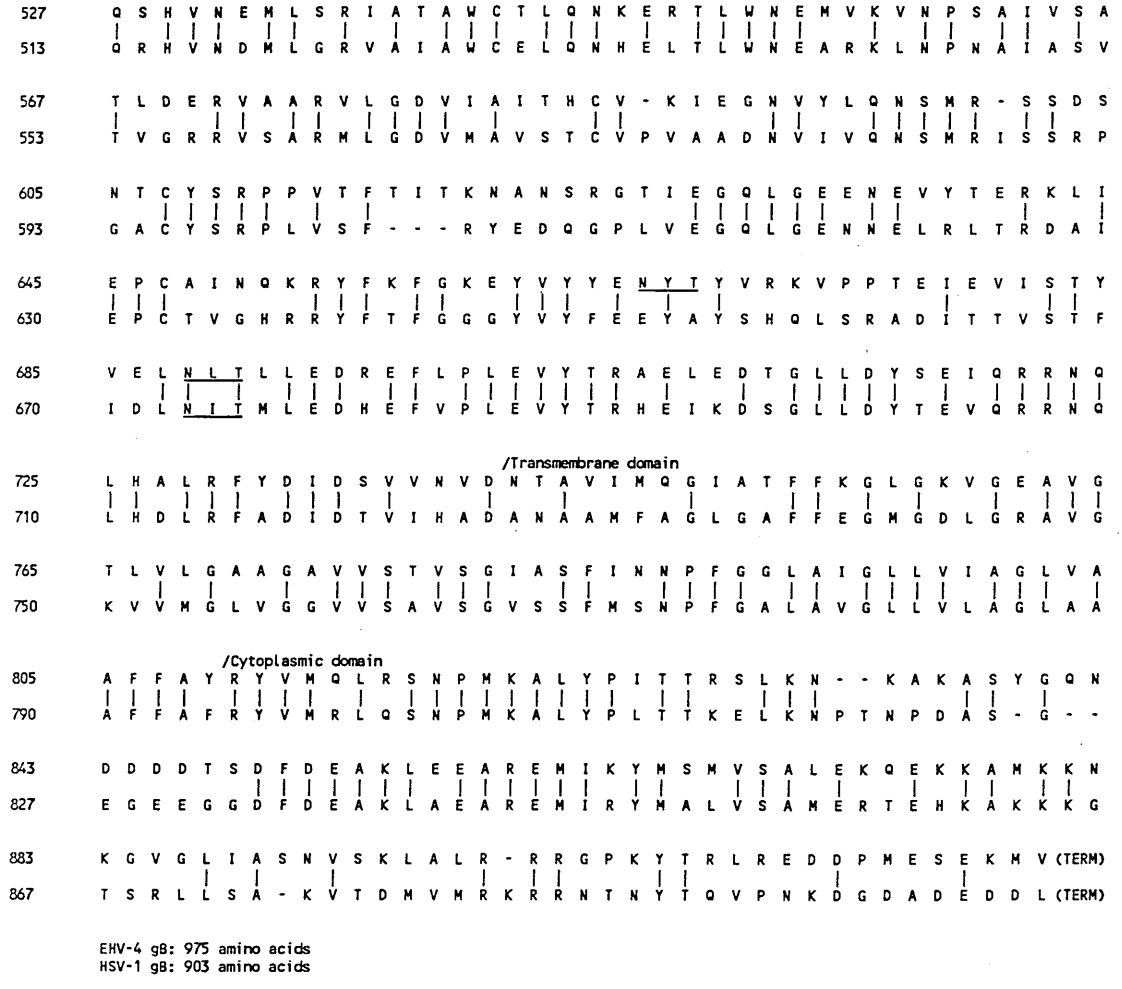


Figure 4.10 Alignment of the predicted amino acid sequences of the EHV-4 gB and HSV-1 (KOS) gB proteins. The upper sequence is that of EHV-4 gB, and the lower sequence that of HSV-1 gB. Identical residues are indicated with a vertical bar, and dashes denote spaces introduced to maximise homology between the two sequences. The signal sequence, surface, transmembrane and cytoplasmic domains of the proteins are indicated. Potential N-linked glycosylation sites are underlined. The predicted internal proteolytic cleavage site of the EHV-4 gB protein (RR/S), which is absent in HSV-1 gB, is indicated by an asterisk. The additional 56 amino acids that are hydrophilic in nature and which form the start of an unusually long 84 amino acid signal sequence for EHV-4 gB, and which are absent in HSV-1 gB, are indicated in brackets above the alignment (residues -56 to -1).

Figure 4.11 Comparison of the predicted amino acid sequences of the EHV-4 gB and HSV-1 gB proteins. (a) Hydropathic plots determined by the method of Kyte and Doolittle (1982). Points above the horizontal line represent regions of above average hydrophilicity. In each plot, the hydrophobic signal sequence and transmembrane domains are indicated by shaded boxes. The additional hydrophilic amino acids prior to the true EHV-4 gB signal sequence are indicated by an unshaded box. (b) Plots of surface probability determined by the method of Emini et al. (1985). (c) Plots of chain flexibility determined by the Karplus-Schulz method. (d) Plots of antigenic index determined by the method of Jameson and Wolf (1988). Antigenic index is a measure of the probability that a region is antigenic and is calculated by the summation of several weighted measures of secondary structure such as hydrophilicity, surface probability and chain flexibility. (e) Secondary structure analysis by the method of Chou and Fasman (1978) (CF) showing predicted turns (i), alpha helices (ii) and beta sheets (iii). (f) Secondary structure analysis by the method of Garnier et al. (1978) (GOR) showing predicted turns (i), alpha helices (ii) and beta sheets (iii). (g) Potential N-linked glycosylation sites. Height of the vertical bar represents the probability of glycosylation.
All analyses were carried out using PEPTIDESTRUCTURE and PLOTSTRUCTURE from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984).

FIGURE 4.11

Comparison of the Predicted Secondary Structure of the EHV-4 gB and HSV-1 gB Proteins

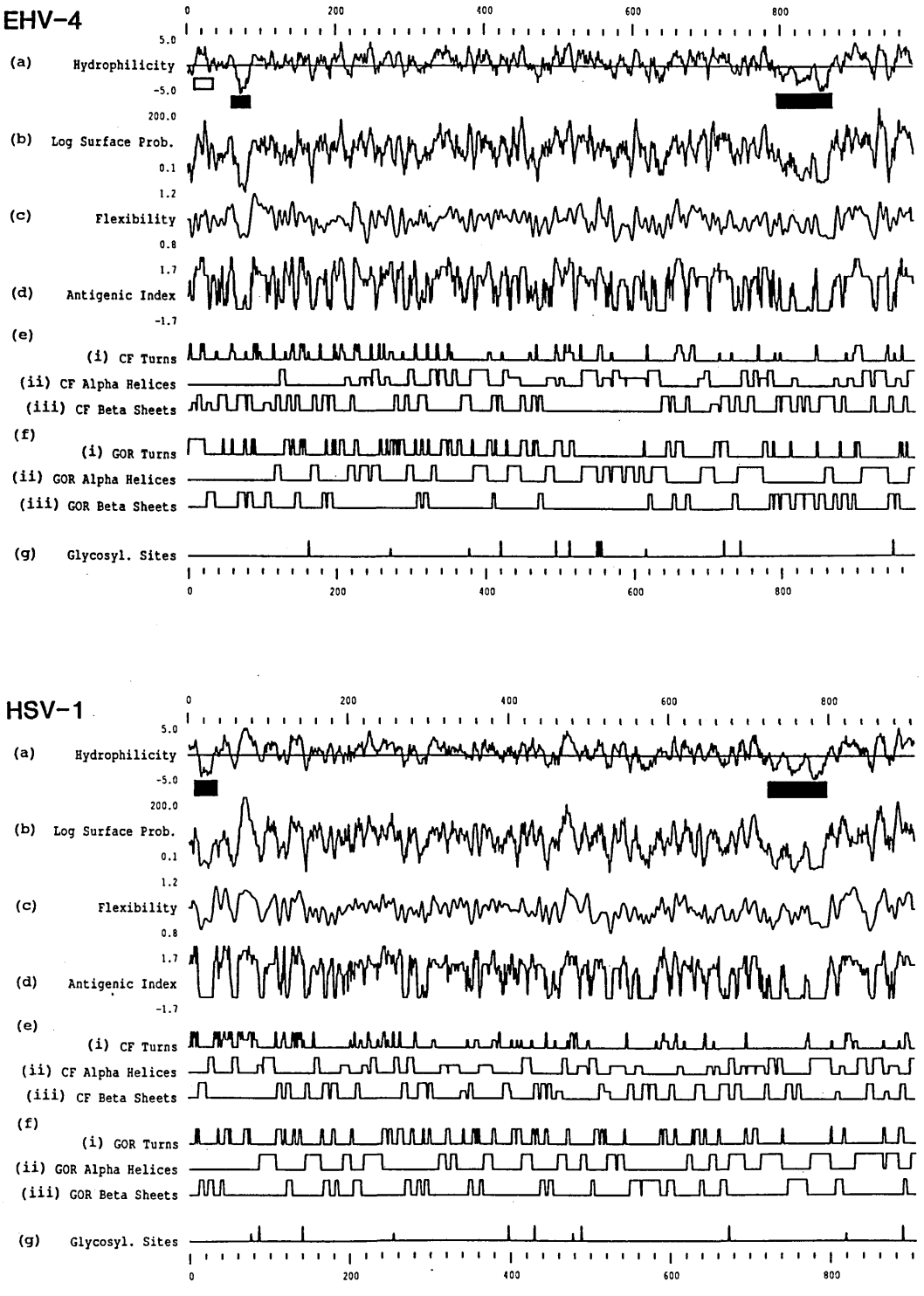


FIGURE 4.12

Alignment of the EHV-4 gB and EHV-1 gB Amino Acid Sequences

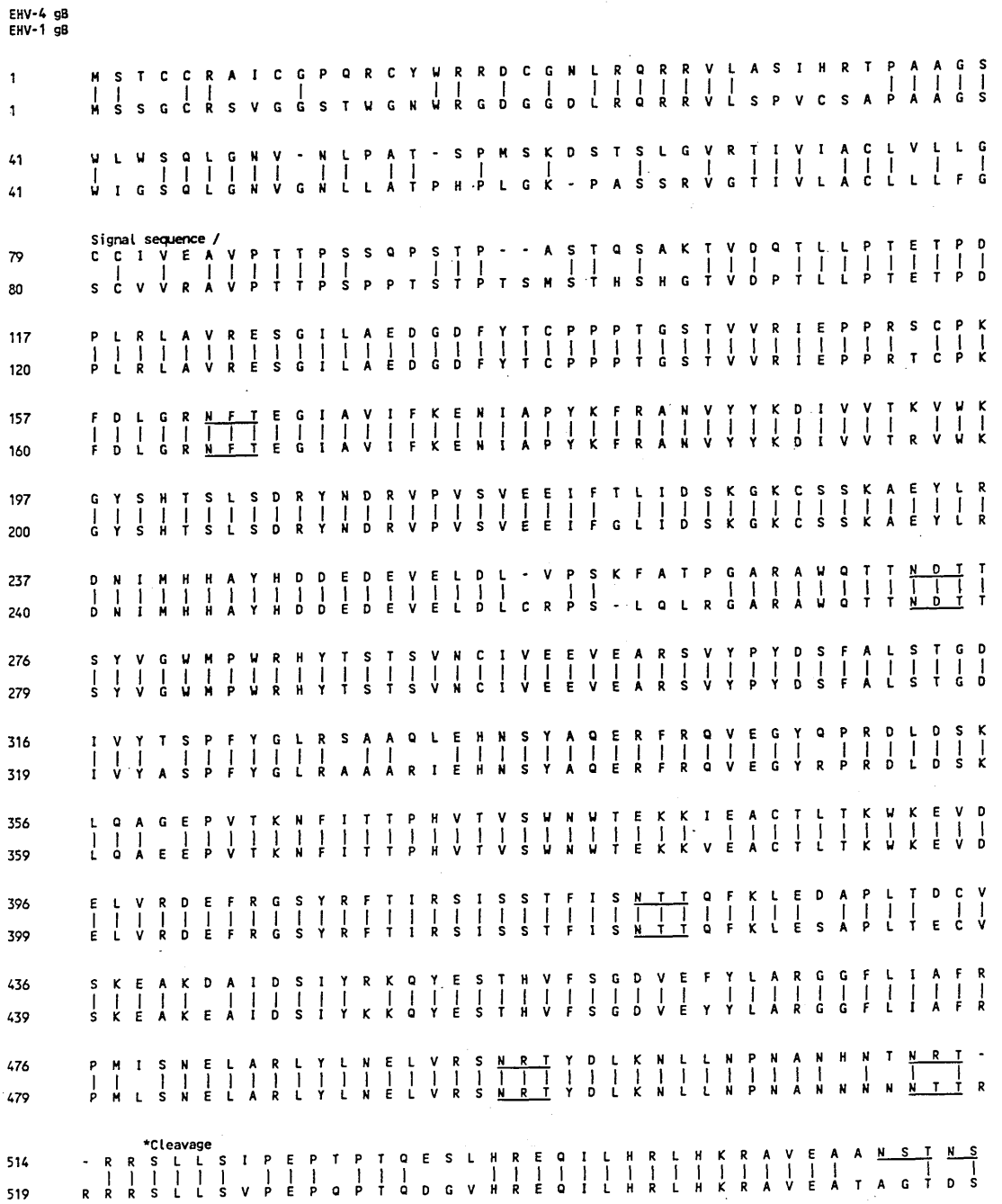


Figure 4.12- continued

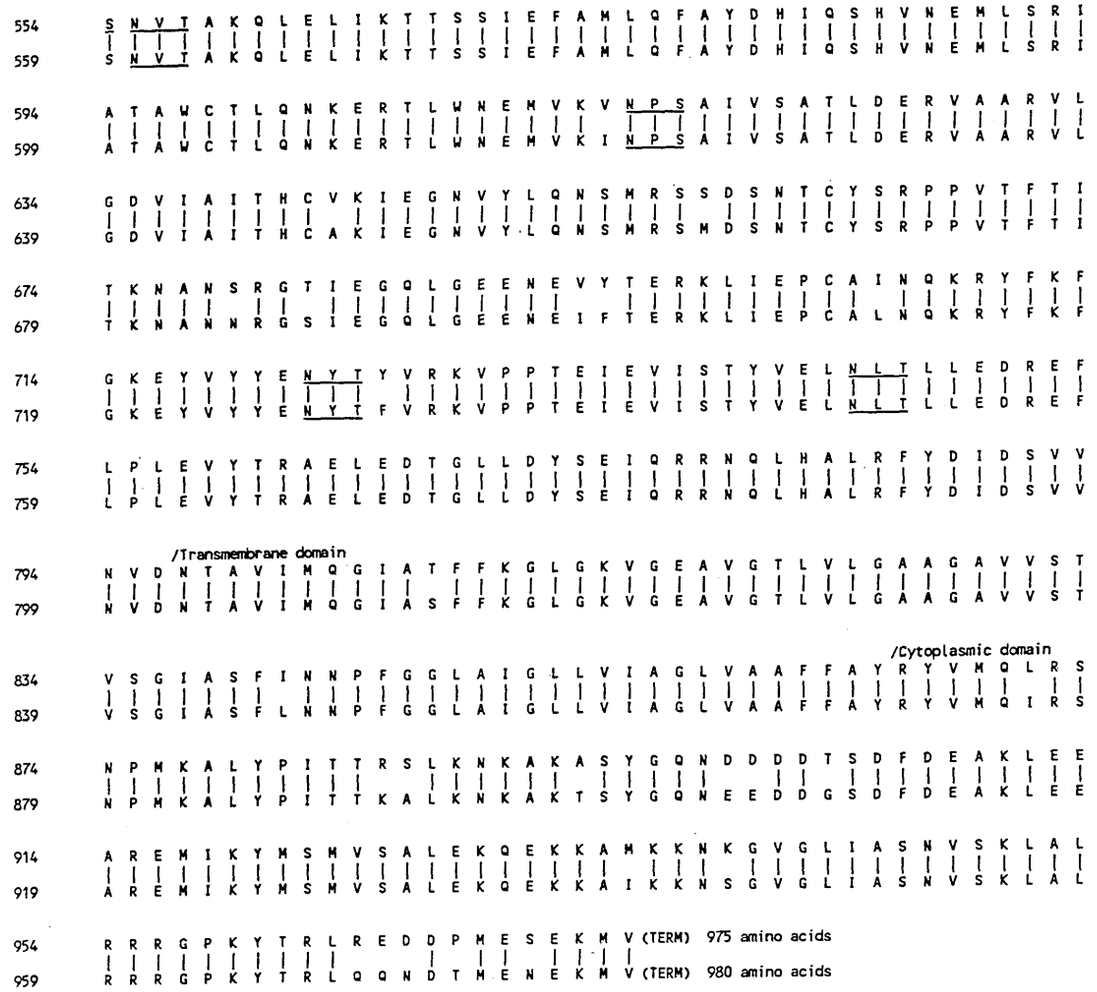


Figure 4.12 Alignment of the predicted amino acid sequences of the EHV-4 gB and EHV-1 gB proteins. The upper sequence is that of EHV-4 gB, and the lower sequence that of EHV-1 gB. Identical residues are indicated with a vertical bar, and dashes denote spaces introduced to maximise homology between the two sequences. The signal sequence, surface, transmembrane and cytoplasmic domains of the proteins are indicated. Potential N-linked glycosylation sites are underlined. The predicted internal proteolytic cleavage site of the proteins (RR/S) is indicated by an asterisk.

T A B L E 4.3

Composition of the EHV-4 gB Protein

Codon (amino acid)	Frequency	% of total	Codon (amino acid)	Frequency	% of total
TTT (Phe)	27	2.8	TAT (Tyr)	10	1.0
TTC (Phe)	6	0.6	TAC (Tyr)	33	3.4
TTA (Leu)	10	1.0	TAA (End)	0	0.0
TTG (Leu)	10	1.0	TAG (End)	0	0.0
CTT (Leu)	16	1.6	CAT (His)	5	0.5
CTC (Leu)	14	1.4	CAC (His)	12	1.2
CTA (Leu)	19	1.9	CAA (Gln)	16	1.6
CTG (Leu)	12	1.2	CAG (Gln)	13	1.3
ATT (Ile)	22	2.3	AAT (Asn)	13	1.3
ATC (Ile)	9	0.9	AAC (Asn)	36	3.7
ATA (Ile)	21	2.2	AAA (Lys)	36	3.7
ATG (Met)	18	1.8	AAG (Lys)	16	1.6
GTT (Val)	33	3.4	GAT (Asp)	17	1.7
GTC (Val)	10	1.0	GAC (Asp)	30	3.1
GTA (Val)	13	1.3	GAA (Glu)	31	3.2
GTG (Val)	17	1.7	GAG (Glu)	38	3.9
TCT (Ser)	21	2.2	TGT (Cys)	13	1.3
TCC (Ser)	17	1.7	TGC (Cys)	5	0.5
TCA (Ser)	11	1.1	TGA (End)	0	0.0
TCG (Ser)	6	0.6	TGG (Trp)	12	1.2
CCT (Pro)	5	0.5	CGT (Arg)	6	0.6
CCC (Pro)	11	1.1	CGC (Arg)	16	1.6
CCA (Pro)	19	1.9	CGA (Arg)	13	1.3
CCG (Pro)	10	1.0	CGG (Arg)	7	0.7
ACT (Thr)	22	2.3	AGT (Ser)	6	0.6
ACC (Thr)	23	2.4	AGC (Ser)	18	1.8
ACA (Thr)	17	1.7	AGA (Arg)	14	1.4
ACG (Thr)	14	1.4	AGG (Arg)	9	0.9
GCT (Ala)	21	2.2	GGT (Gly)	6	0.6
GCC (Ala)	20	2.1	GGC (Gly)	12	1.2
GCA (Ala)	19	1.9	GGA (Gly)	16	1.6
GCG (Ala)	11	1.1	GGG (Gly)	12	1.2

Table 4.3 Codon usage and predicted amino acid composition of EHV-4 gB (975 amino acids).

FIGURE 4.13

Alignment of the Amino Acid Sequences of the gB-Like Proteins of Eight Herpesviruses

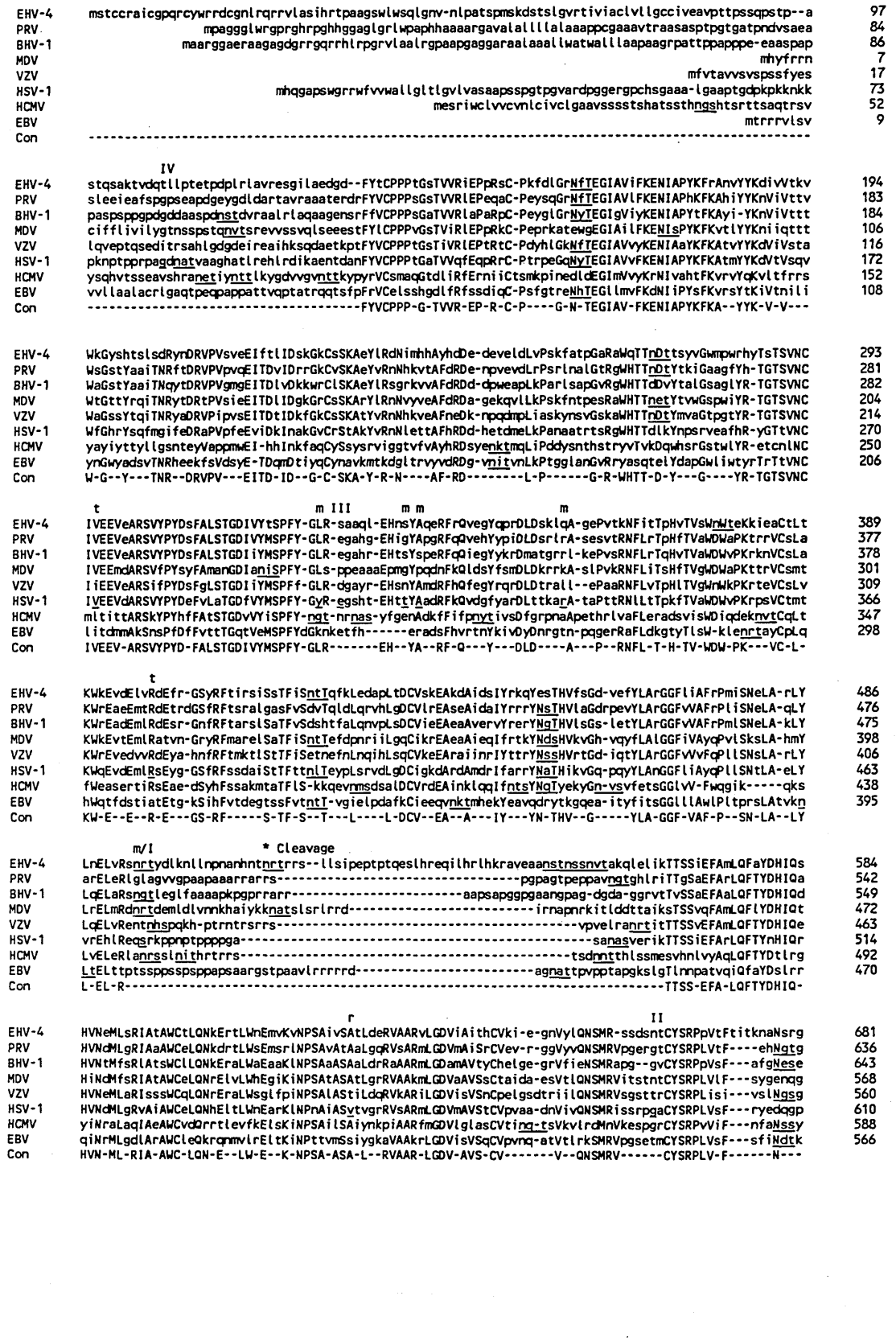


Figure 4.13- continued

Figure 4.13 Alignment of the amino acid sequences of the gB-like proteins of EHV-4, PRV, BHV-1, MDV, VZV, HSV-1, HCMV and EBV. Dashes denote spaces introduced to maximise homology between the sequences. The consensus sequence (Con) denotes residues that are identical in at least five of the eight proteins, and such residues are shown in capital letters. Potential N-linked glycosylation sites are underlined. Spans 1, 2 and 3 of the HSV-1 gB hydrophobic transmembrane domain are indicated. An asterisk denotes the internal proteolytic cleavage site of the VZV protein. HSV-1 gB mutants are indicated: rate of entry (r) (Bzik et al., 1984b), syncytial (s) (Bzik et al., 1984b), temperature-sensitive (t) (Bzik et al., 1984a) and monoclonal antibody-resistant mutants I, II, III, IV (Highlander et al., 1989) and (m) (Pellett et al., 1985b). The sources of the sequences were as follows: PRV (Robbins et al., 1987), BHV-1 (Lawrence et al., 1988), MDV (Ross et al., 1989), VZV (Keller et al., 1986), HSV-1 (Bzik et al., 1984a), HCMV (Cranage et al., 1986), EBV (Pellett et al., 1985a).

TABLE 4.4

Main Features of the Herpesvirus gB-Like Proteins

Herpesvirus gB homologue	Total size (a.a)	Signal sequence (a.a)	Mature form (a.a)	Number of NGS	Internal cleavage
EHV-4	975	84	891	11	Yes
EHV-1	980	85	895	9	Yes
PRV	913	53	860	6	Yes
BHV-1	932	67	865	6	Yes
MDV	865	21	844	9	Yes
VZV	868	8	860	7	Yes
HSV-1	903	30*	873*	6	No
HCMV	906	24	882	18	Yes
EBV	857	22	835	9	No

Table 4.4 Sources of the gB sequences are as in Figure 4.13. *The signal sequence of HSV-1 (strain F) gB (Pellett et al., 1985b) is 29 amino acids, yielding a mature form of 874 amino acids. NGS, N-linked glycosylation sites (in external domain); a.a., amino acids.

F I G U R E 4.14

Dot Matrix Similarity Analysis of the EHV-4 gB Amino Acid Sequence Against Those of Other Herpesvirus gB-Like Proteins

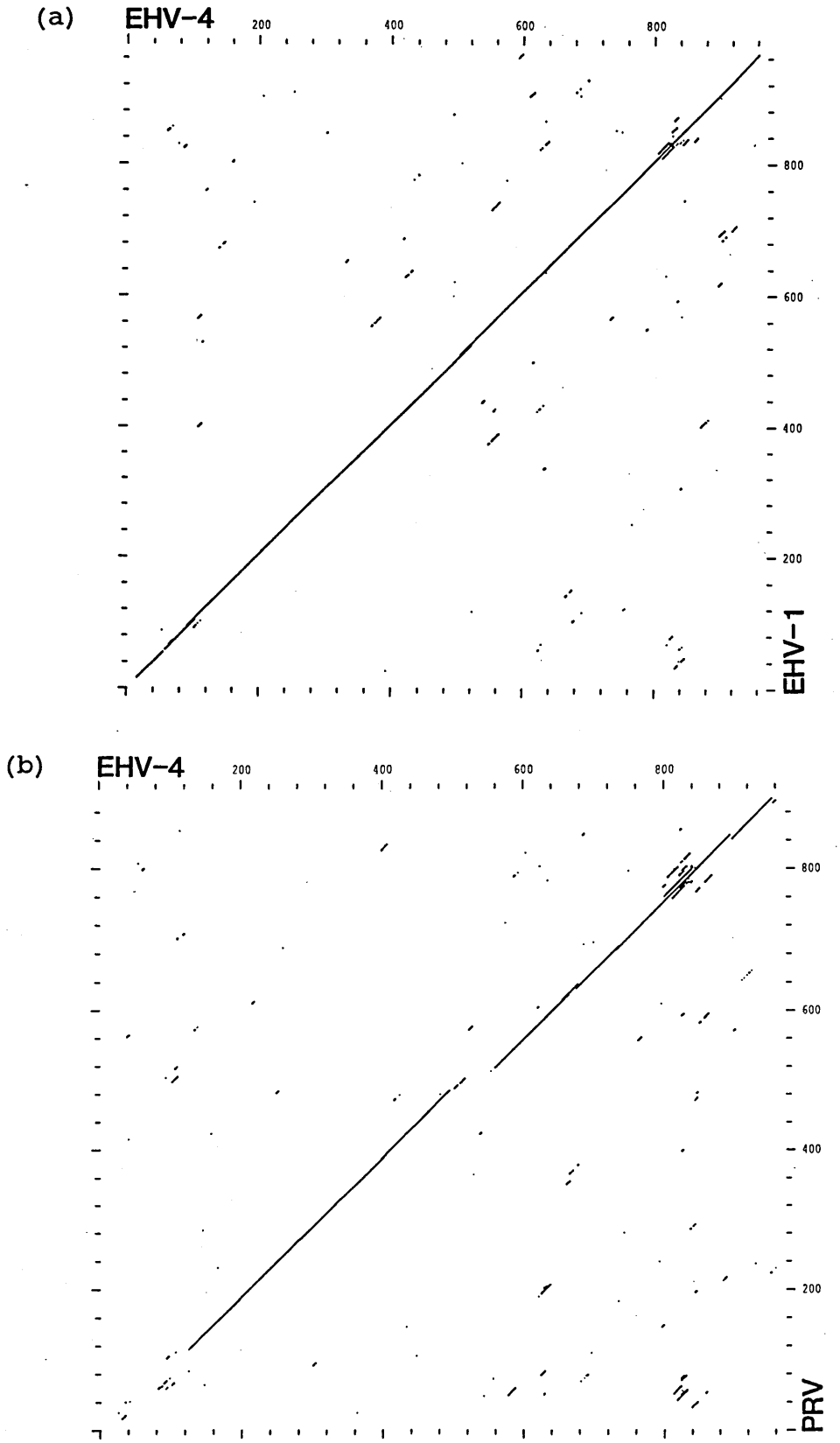
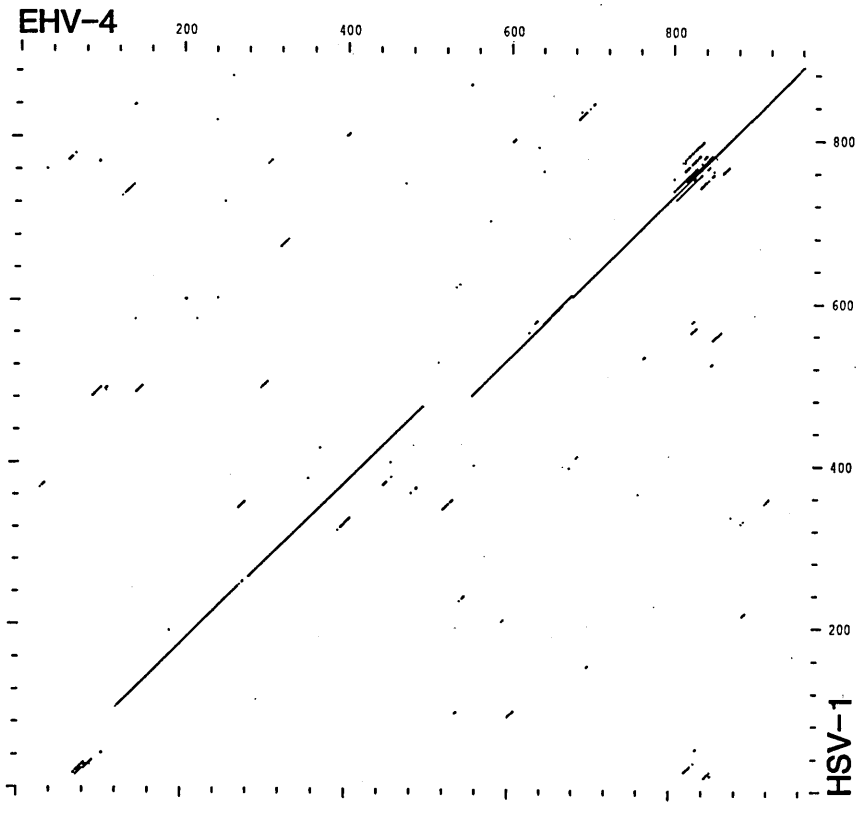


Figure 4.14- continued

(c)



(d)

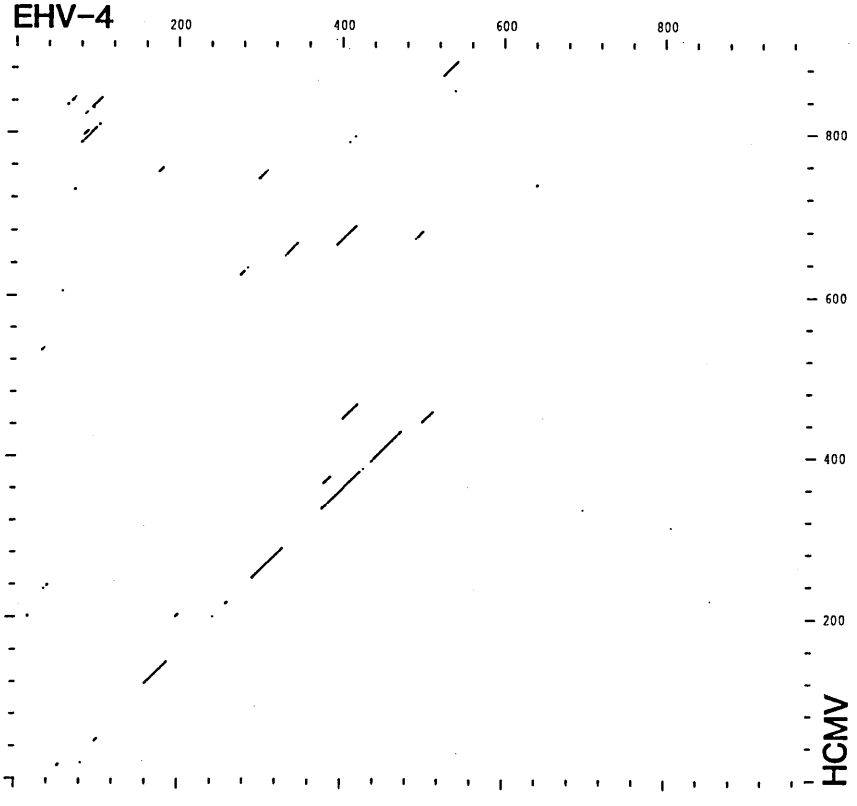


Figure 4.14- continued

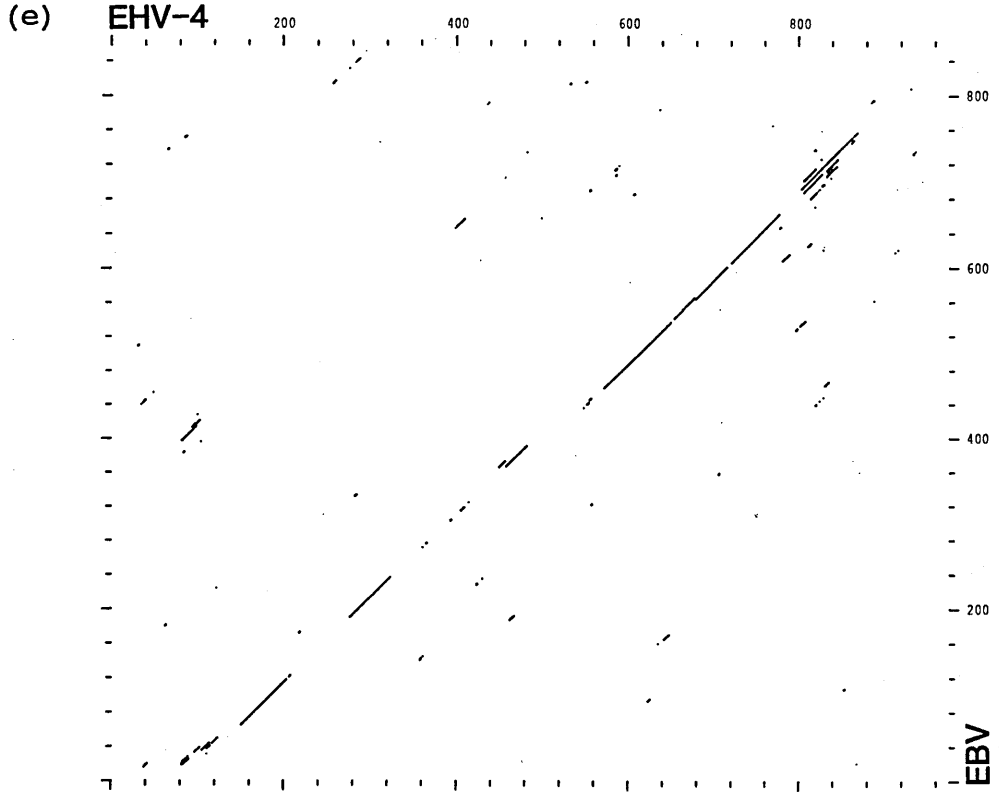


Figure 4.14 Dot matrix similarity analysis of the EHV-4 gB amino acid sequence against those of the gB-like proteins of five other herpesviruses. Analysis was carried out using COMPARE and DOTPLOT from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984). In each plot, the horizontal axis is the EHV-4 gB amino acid sequence numbered as in Figures 4.12 and 4.13. Vertical axes represent the amino acid sequences of the gB-like proteins of EHV-1 (a), PRV (b), HSV-1 (c), HCMV (d) and EBV (e). Points are plotted when at least 15 amino acids in a moving window of 30 are identical. Large regions of similarity are indicated by uninterrupted diagonal lines from lower left to upper right.

TABLE 4.5

Homology Between the Herpesvirus gB-Like Proteins

	EHV-4	EHV-1	PRV	BHV-1	MDV	VZV	HSV-1	HCMV	EBV
EHV-4	-	88.6	53.1	53.0	46.8	49.7	46.0	28.2	30.1
EHV-1		-	53.4	52.7	45.8	49.7	46.2	28.0	29.5
PRV			-	60.7	48.0	50.9	50.7	29.3	30.5
BHV-1				-	46.6	51.6	48.8	28.3	29.4
MDV					-	48.7	46.4	29.3	29.5
VZV						-	46.1	29.2	28.5
HSV-1							-	29.5	30.3
HCMV								-	31.4
EBV									-

Table 4.5 Percentage amino acid homologies for pairwise comparisons of the herpesvirus gB-like proteins.

FIGURE 4.15

Alignment of the Carboxyl-Terminal Amino Acids of the ICP18.5-Like Proteins of Eight Herpesviruses

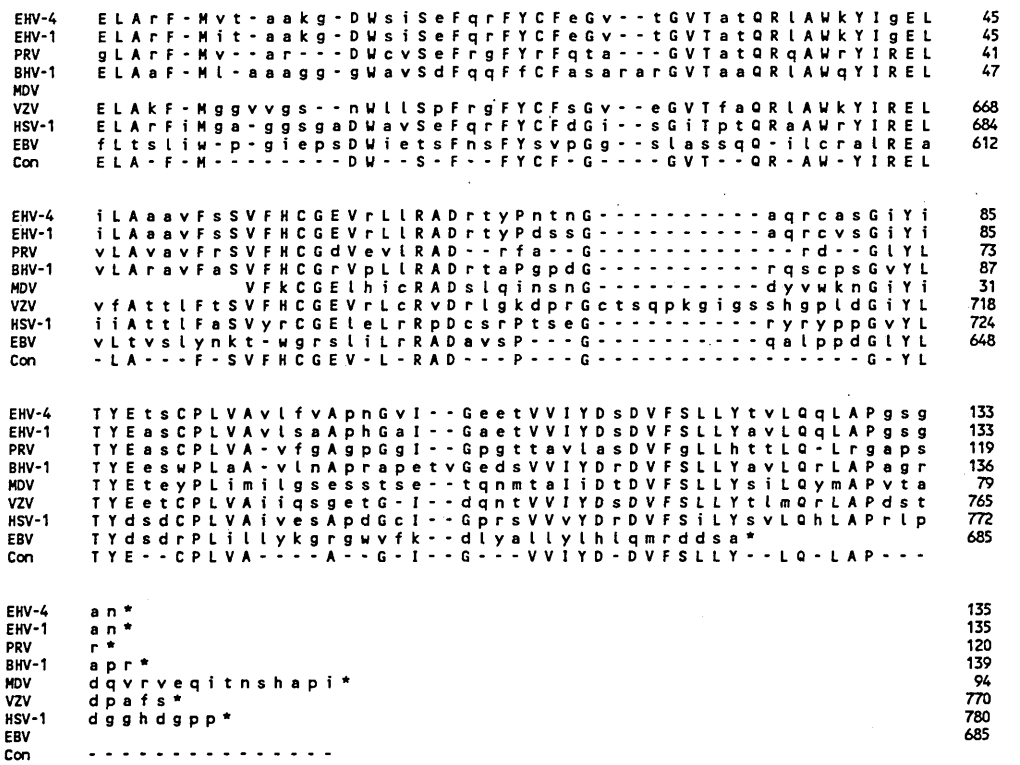


Figure 4.15 Alignment of the carboxyl-terminal amino acids of the ICP18.5-like proteins of EHV-4, EHV-1, PRV, BHV-1, MDV, VZV, HSV-1 and EBV. Dashes denote spaces introduced to maximise homology between the sequences. The consensus sequence (Con) denotes residues that are identical in at least five of the eight proteins, and such residues are shown in capital letters. Residue 1 of the EHV-4 protein is aligned with residue 1 of the EHV-1, PRV, BHV-1 and MDV proteins for which complete sequence data are not available, and with residues 624, 638 and 568 of the VZV, HSV-1 and EBV proteins, respectively, for which complete sequence data are available. The sources of the sequences were as in Figure 4.13 except for VZV (Davison and Scott, 1986a), HSV-1 (Pellett et al., 1986) and EBV (Pellett et al., 1986). The EHV-1 sequence is from Whalley et al. (1989).

D I S C U S S I O N

The DNA sequence of the gB gene of EHV-4 has been determined and its gene product compared with the gB-like proteins of several other herpesviruses. Alignment of these proteins identified gB as a highly conserved species across the herpesviruses with regions of particularly homologous amino acids which may be of functional importance. The conservation of all ten cysteine residues and several N-linked glycosylation sites in the external domain of the molecules indicates that they probably share very similar secondary and tertiary structures. Taken together with their remarkably similar hydropathic profiles, the gB proteins probably adopt an orientation in membranes similar to that predicted for HSV-1 gB (Pellett et al., 1985b). This model predicts a cleavable signal sequence, a hydrophilic surface domain oriented to the extracellular environment, a membrane-spanning domain that traverses the membrane three times and a short cytoplasmic anchor domain that projects into the cytoplasm or virion envelope interior. This generally accepted model was supported by the studies of Claesson-Welsh and Spear (1987). By comparing the amino acid sequence at the amino-terminus of gB purified from cells infected with HSV-1 strains KOS and F with that deduced from DNA sequence analysis they confirmed that gB was synthesised with a cleavable signal sequence of 29 (strain F) or 30 (strain KOS) amino acids. HSV-1 gB synthesised from in vitro translation of gB mRNA in the presence of membranes was shown to be inserted into membranes and glycosylated cotranslationally but not able to be inserted after most of the protein had been

synthesised. A large fragment of gB synthesised in this way, which carried all the N-linked glycosylation sites of intact gB, was protected from proteolysis by membranes. Since the deduced amino acid sequence of HSV-1 gB indicated that all the N-linked glycosylation sites were on the amino-terminal side of the proposed transmembrane domain, these findings supported the orientation of gB in membranes as previously proposed. The pattern of fragments produced by trypsin cleavage of gB supported the theory that gB traverses the membrane three times. The conservation of orientation in membranes for other gB-like proteins was reinforced by analysis and comparison of the secondary structure of the HSV-1 gB and EBV gB proteins (Pellett et al., 1985a). The surface, transmembrane and, in particular, cytoplasmic domains were near identical in structure despite their relatively extensive divergence of amino acid sequence. Further detailed analysis of EHV-4 gB and other herpesvirus gB-like proteins should confirm these predictions.

The prediction of an internal proteolytic cleavage site for EHV-1 gB and EHV-4 gB from sequence data was confirmed by Meredith et al. (1989) who were the first workers to unequivocally identify EHV-1 gB and EHV-4 gB as disulphide-linked heterodimers. Using one- and two-dimensional gel electrophoresis techniques to compare the major structural proteins of EHV-1 strain Ab1 and EHV-4 strain MD, in addition to Western blotting and immunoprecipitation with monoclonal antibodies to EHV-1, they identified EHV-1 gB as migrating as a 108kDa glycoprotein (140kDa under non-reducing conditions) that was cleaved to give two glycoprotein species of 76kDa and 58kDa held together by disulphide bonds. EHV-4 gB was demonstrated to

exist as a 112kDa glycoprotein that was cleaved into 74kDa and 61kDa species linked by disulphide bonds. Internal proteolytic cleavage at the site predicted on the basis of sequence data would yield two unglycosylated protein species of a similar size (50kDa and 51kDa) for EHV-4 gB, identical in size to those predicted for EHV-1 gB (Whalley et al., 1989). Since the majority of N-linked glycosylation sites reside in the amino-terminal half of the gB molecules, this region probably corresponds to the higher molecular mass species and the carboxyl-terminal half to the lower molecular mass species of the disulphide-linked complexes. A separate study confirmed the existence of EHV-1 gB and EHV-4 gB as disulphide-linked complexes (Sullivan et al., 1989). A particularly interesting observation made by Meredith et al. (1989) was that one EHV-1 monoclonal antibody (9∝C2) reacted with the 58kDa and 108kDa, but not the 76kDa, species of EHV-1 in Western blotting or immunoprecipitations, and cross-reacted with only the 112kDa species of EHV-4. This suggested that 9∝C2 reacted with an epitope present on both the 58kDa and 108kDa species of EHV-1, which is therefore conserved during proteolytic cleavage of the 108kDa species but destroyed during cleavage of the 112kDa EHV-4 species. This led the authors to suggest that the proteolytic cleavage sites of EHV-1 gB and EHV-4 gB may be in different locations. However, alignment of the EHV-1 gB and EHV-4 gB amino acid sequences demonstrated that the proposed cleavage sites are in identical positions (Figure 4.12). An interesting finding was that EHV-1 gB contains an additional two consecutive arginine residues immediately prior to the proposed cleavage site which are absent in EHV-4 gB. It could be possible that EHV-1 gB is

cleaved after these two additional arginine residues and not at the proposed R-R/S sequence which remains the only possible cleavage site for EHV-4 gB. This may represent the differential cleavage of the EHV-1 gB and EHV-4 gB proteins suggested by Meredith et al. (1989) which could account for the reactivity pattern of 9∝C2 and, if proved correct, the epitope for 9∝C2 would be expected to encompass the R-R/S sequence. The exact location at which EHV-1 gB and EHV-4 gB are cleaved awaits determination by amino-terminal sequencing of the purified components of the disulphide-linked complexes, a procedure that would also identify the signal peptidase cleavage site of the gB proteins.

The fast-entry phenotype of HSV-1 mutant tsB5 relative to wild-type KOS was shown to be the result of a Val-to-Ala substitution at position 552 of gB (Bzik et al., 1984b). It may be of significance that the amino acid in the corresponding position of the gB homologues of EHV-4, PRV, BHV-1, MDV, HCMV and EBV is also an Ala (Figure 4.13). Interestingly, this Val residue is also replaced by an Ala residue in the gB protein of HSV-1 strain F. The rate of entry mutation lies within a domain that is well conserved across the herpesvirus gB-like proteins, which accentuates the probable important conserved role played by this domain in the entry of virus into infected cells.

The HSV-1 mutants tsJ12 and tsJ20 which are temperature-sensitive for the accumulation of gB and viral growth are associated with amino acid changes at positions 376 (Arg-to-Cys) and 272 (Val-to-Ile), relative to wild-type KOS, respectively (Bzik et al., 1984a). Inspection of the amino acid sequences of herpesvirus gB-like proteins revealed that an Arg residue is

also present in a position equivalent to HSV-1 gB residue 376 in all cases apart from EBV. A Val residue is also present in a position aligning with HSV-1 gB residue 272 for EHV-4, PRV, BHV-1 and MDV; VZV and EBV contain an Ile in this position, the substituting amino acid which confers the temperature-sensitive phenotype to HSV-1 tsJ20. Since the sites of these temperature-sensitive mutations lie within well conserved regions of the HSV-1 gB protein, it seems likely that temperature-sensitive mutations for other herpesviruses may also map to this region.

It has been proposed that HSV-1 gB is part of a multiprotein complex which determines the social behaviour of infected cells (Ruyechan et al., 1979). The cytoplasmic domain of gB is thought to interact with the virion tegument proteins and other membrane proteins to affect the social behaviour of infected cells (Pellett et al., 1985a), since a syncytial mutation has been mapped to this domain in HSV-1 gB (Bzik et al., 1984b). A single amino acid substitution at position 857 (Arg-to-His) in mutant tsB5, relative to wild-type KOS, is associated with the syncytial phenotype, characterised by extensive cell fusion (Bzik et al., 1984b). An Arg residue was also found to be present in this position in the gB-like proteins of BHV-1, MDV and VZV (Figure 4.13). It has been predicted that residues 841 to 858 of HSV-1 gB are amphipathic, adopting both a hydrophilic and a hydrophobic face, and that this region may interact with other proteins (Pellett et al., 1985a). The highly conserved nature of this region between all the alphaherpesvirus gB homologues indicates a common, conserved function.

Cai et al. (1988) identified two syncytial mutations in the

cytoplasmic domain of HSV-1 (KOS) gB, one of which was that previously described in tsB5. The second site which could be altered to induce extensive cell fusion was located between residues 816 (leucine) and 817 (lysine). Mutations in gB that decreased cell fusion activity were shown to be located both inside and outside the cytoplasmic domain, suggesting that gB may contain separate functional regions responsible for the induction and inhibition of cell fusion. It is of interest that residues 816 and 817 of HSV-1 gB are aligned with identical residues in all the alphaherpesvirus gB-like proteins (Figure 4.13).

Huff et al. (1988) demonstrated that the carboxyl-terminal 41 amino acids of HSV-1 gB are not essential for the production of infectious virus particles and for gB function. It can be seen from Figure 4.13 that the 40 or so amino acids at the carboxyl-terminal of all the herpesvirus gB-like proteins are poorly conserved and, as such, may also be dispensable for protein function.

Truncation of the 194 carboxyl-terminal amino acids of HSV-1 gB resulted in secretion of the product from transfected cells due to deletion of the transmembrane and cytoplasmic domains which contain sequences essential for a membrane anchoring function (Pachl et al., 1987). Analysis of the EHV-1 gB and HSV-1 gB amino acid sequences led Whalley et al. (1989) to speculate that a hydrophobic stretch of amino acids (EHV-1 gB residues 853 to 868 and EHV-4 gB residues 848 to 863, Figure 4.12) could serve to anchor the protein into the membrane in accordance with the criteria defined by Engelman et al. (1986). The general conservation of amino acid sequence in this region

of all herpesvirus gB-like proteins suggests a common anchoring function.

Domains within HSV-1 (strain KOS-321) gB that are involved in virus penetration of cells have been studied using monoclonal antibody-resistant (mar) mutants. Five distinct epitopes for monoclonal antibodies on gB (antigenic sites I to V) have been identified using virus neutralising monoclonal antibodies and mar mutants (Marlin et al., 1986). Mutations affecting either site II or site III caused temperature-sensitive defects in gB glycosylation at the non-permissive temperature (39^{o}C), whereas variants altered in both sites produced no mature gB and showed reduced titres when grown at 39^{o}C. Since other HSV-1 strains carrying temperature-sensitive mutations in the gB gene produced a gB precursor at 39^{o}C that was not recognised by antibodies to sites II and III, it was suggested that sites II and III are linked with gB structures that are antigenic and essential for the processing of gB and its function in virus infectivity (Marlin et al., 1986). Highlander et al. (1988) demonstrated that gB-specific monoclonal antibodies recognised two major antigenic sites located in separate regions of the surface domain. The residues critical to recognition by monoclonal antibodies to sites I, III and IV, which neutralised virus infectivity by inhibiting penetration, were mapped to the centre of the surface domain (residues 241 to 441). Antibodies specific for site II and which had no effect on viral penetration recognised residues near the transmembrane domain (residues 596 to 737). Only the antibody to site III could neutralise infectivity in the absence of complement. In the absence of complement, antibodies to sites I and IV slowed the rate of

virus entry into cells. Antibodies that bind to gB were therefore shown to inhibit viral penetration by binding to a domain that is directly involved in this process. The authors concluded that sites I, III and IV comprise a major antigenic site associated with structures which contribute to virus infectivity. This analysis of the antigenic structure of HSV-1 gB was further extended by the studies of Highlander et al. (1989). Mutations affecting antibody neutralisation at sites I to IV were identified as single amino acid changes at residues 473 (Ser-to-Asn), 594 (Gly-to-Arg), 305 (Glu-to-Lys) and 85 (Gly-to-Asp), respectively. Additionally, two distinct site II antibodies selected mar mutants which demonstrated a decreased rate of entry into host cells (residue 594, Gly-to-Arg), the mar and rate of entry phenotypes thus being due to a single mutation. Insertion of amino acids throughout the surface domain of gB produced proteins that were not recognised by antibodies to sites II and III but which were still recognised by antibodies to sites I and IV. Since mar mutations in sites II or III could cause temperature-sensitive defects in gB glycosylation and variants in both sites were temperature-sensitive for virus production (Marlin et al., 1986), these new data confirmed that sites II and III contain structures important for gB function in virus infectivity. Since antibodies to site II did not inhibit penetration but selected mutants showing altered rate of entry kinetics, it was suggested that this site does not contribute to gB function to the same extent as site III but still remains important in the infectious process (Highlander et al., 1989).

Pellett et al. (1985b) also reported several mutations that

result in resistance to neutralisation by monoclonal antibodies in HSV-1 (strain F) gB at residues 302 (Tyr-to-Asn), 312 (Ser-to-Thr), 314 (Ala-to-Thr), 334 (Arg-to-Gln) and 472 (Ser-to-Asn). Most of these mutations were close to the site III mutation described previously. All these mutations are indicated in Figure 4.13. It can be seen that the site I mutation is located within a very poorly conserved region of the gB molecule and, as such, little conservation of function can be expected. This site I mutation is located in a part of the molecule which closely aligns with the predicted proteolytic cleavage site of the other herpesvirus gB-like proteins. The very divergent nature of this region suggests that epitopes in this area are likely to show no cross-reactivity. This site I mutation is located close to the region where a putative epitope for the EHV-1 monoclonal antibody 9∝C2 may reside, as discussed earlier. HSV-1 gB antigenic site IV is located near the amino-terminus in a region displaying little amino acid conservation with other herpesvirus gB-like proteins, and represents an epitope unique to HSV-1; the Gly residue at position 85 is type-specific, being replaced by an Arg in HSV-2 gB. An epitope for the EHV-1 gB monoclonal antibody 3F6 (Allen and Yeargan, 1987) has been identified as the sequence ETPDPLR at EHV-1 gB residues 116 to 122 (G. Allen, Equine Virology Research Foundation Grantholders Meeting, Newmarket, October 1989), which is conserved with EHV-4 gB residues 113 to 119 (Figure 4.12), representing a gB type-common epitope. This epitope aligns with a region adjacent to the HSV-1 gB antigenic site IV mutation (Figure 4.13). The HSV-1 gB antigenic site II mutation maps near a region that is highly conserved between the herpesvirus

gB-like proteins, suggesting conservation of high order structure and function. This HSV-1 gB epitope may share cross-reacting determinants with other gB-like proteins. The region in which the HSV-1 gB antigenic site III mutation maps is highly conserved with the other gB-like proteins, with a predicted conservation of secondary as well as primary structure and a predicted similar important function in the gB-like proteins. That this region can affect gB processing and viral infectivity was supported by studies on the mutants tsJ12 and tsJ20, which contain mutations close to the site III mutation as described earlier.

Further detailed studies on EHV-4 gB and the gB-like proteins of other herpesviruses will provide a more accurate overview of the precise functions and antigenic structure of each protein which, on the basis of sequence data, can be expected to be very similar.

CHAPTER 5

Expression of the EHV-4 Glycoprotein gB Gene in Prokaryotic Vector Systems and Evaluation of the Immunogenicity of the Purified Recombinant Proteins and of Peptides Derived From the EHV-4 gB Amino Acid Sequence

INTRODUCTION

Two commercial vaccines are currently available in the U.K. which offer protection against EHV-induced abortigenic and respiratory diseases. Rhinomune contains a live attenuated EHV-1 strain and is licensed to protect against respiratory disease. Pneumabort-K is a formalin-inactivated EHV-1 Army 183 strain and is licensed to protect against abortion. Initial challenge studies with the latter vaccine in both young horses and pregnant mares demonstrated that it could protect against abortion. Two vaccinations, three weeks apart, protected 11 pregnant mares following challenge eight weeks later with virulent EHV-1 (Bryans, 1978). In other trials, two vaccinations 62 days apart resulted in only two out of 11 mares aborting following challenge with EHV-1 Army 183 15 days after the second vaccination (Moore and Koonse, 1977). These studies demonstrated that the vaccine preparation was immunogenic in as much as it could induce virus neutralising antibody levels comparable to those resulting from infection with virulent virus. Use of the vaccine in Kentucky between 1977 and 1980 was reported to be associated with a significant reduction in the incidence of abortion due to EHV-1 from 6.8 per 1000 in an unvaccinated population to 1.8 per 1000 in a vaccinated population (Bryans, 1981; Bryans and Allen, 1982). Further use of the vaccine in Kentucky during the four year period following its commercial introduction in 1980 resulted in a decrease in the incidence of abortion induced by EHV-1 to the lowest level ever recorded in the breeding population (Allen and Bryans, 1986). Despite such initial promise, more recent trials

have placed doubt on the efficacy and safety of this vaccine. In studies to establish the nature and longevity of the immunity established by Pneumabort-K, it was demonstrated that the vaccine could not protect against an aerosol infection with EHV-1, even in ponies that had previously been exposed to EHV-1 on many occasions, although the incidence and duration of clinical signs and the amounts and duration of virus shedding were reduced (Burrows et al., 1984). Of particular concern was the inability to prevent the cell-associated viraemia caused by EHV-1 infection, and the probable subsequent infection of the foetus. However, protection was afforded against challenge with EHV-4 (Mumford and Bates, 1984).

Commercially available vaccines based on EHV-1 strains are still capable of stimulating the production of antibodies cross-reactive with EHV-4 by virtue of shared antigens. In recent years much emphasis has been placed on the development of more effective and safer vaccines to protect horses against abortigenic and respiratory diseases. Any such new vaccines must be capable of eliciting an effective immune response against the envelope glycoproteins, especially those directly involved in cell fusion and viral infectivity. The EHV-1 envelope glycoproteins have been specifically identified as the virion components to which antibodies that inhibit virus infectivity bind (Papp-Vid and Derbyshire, 1978, 1979). Although it has been suggested that monovalent vaccines based on EHV-1 (Edington and Bridges, 1990) and EHV-4 (Fitzpatrick and Studdert, 1984) could elicit antibody and cellular immune responses to both virus types, a bivalent vaccine incorporating the appropriate immunogenic antigens from both EHV-1 and EHV-4 should prove to

be most effective. By analogy with HSV-1, glycoproteins gB and gC of EHV-1 and EHV-4 would be the best antigens for development as novel vaccines. Indeed, the gp2, gp10, gp13 (gC) and gp14 (gB) glycoproteins of EHV-1 and EHV-4 have been shown to be the most immunogenic viral antigens, whilst gp2 and gp14 (gB) were the most cross-reactive antigens between the two viruses (Allen and Bryans, 1986). The recombinant DNA technology currently available greatly facilitates the development of more effective and safer novel vaccines. Strategies for vaccine development include the construction of recombinant viruses which express all the necessary immunogenic antigens of both EHV-1 and EHV-4 and the production of individual immunogenic glycoproteins by expression of their genes in bacterial expression systems for direct use as subunit vaccines. The bivalent vaccine approach and other strategies will be more fully discussed in Chapter 7 of this thesis. This chapter is concerned with the evaluation of EHV-4 gB as a subunit vaccine.

Knowledge of the DNA sequence of the EHV-4 gB gene (Chapter 4) permitted selected segments of the gene to be expressed as fusion proteins by cloning in-frame with bacterial coding sequences in bacterial expression vector systems. Many vectors have been constructed which simplify the expression and subsequent purification of foreign proteins expressed in *E.coli* (Marston, 1987). Probably the most widely used are those which direct the intracellular synthesis of proteins as soluble fusion products with β-galactosidase (Koenen *et al*., 1982; Ruther and Muller-Hill, 1983), which can then be directly purified from crude cell lysates by immunoaffinity chromatography or electroelution from polyacrylamide gels. Cloned DNA fragments

can also be expressed as fusion proteins with staphylococcal protein A and purified from crude cell lysates by immunoaffinity chromatography on IgG-sepharose (Uhlen et al., 1983; Nilsson et al., 1985). Alternatively, fusion proteins can be secreted into the periplasmic space or culture medium, leading to rapid purification (Abrahmsen et al., 1986; Kato et al., 1987; Marston, 1987).

Segments of EHV-4 gB were expressed intracellularly as β-galactosidase fusion proteins using the pUR vector system (Ruther and Muller-Hill, 1983) which were then purified by electroelution from polyacrylamide gels. The pUR series of plasmids contain four unique cloning sites at the 3' end of the lacZ coding region and since the positions of the inserted restriction sites have been shifted relative to the lacZ coding region in each of the six vectors, any foreign coding region can be inserted into one of the vectors in the same reading frame as lacZ to allow expression as a β-galactosidase fusion product. LacZ expression is repressed by maintaining the plasmids in the E.coli JM101 host, which overproduces the lac repressor due to the $lacI^q$ allele. Fusion protein synthesis is induced with isopropyl- β -D-thiogalactopyranoside (IPTG). Another, more efficient, expression system was also used which directed the intracellular synthesis of gB as a fusion product with the carboxyl-terminus of Sj26, a 26kDa glutathione-S-transferase (GST) encoded by the parasitic helminth Schistosoma japonicum, and fusion protein was rapidly purified by affinity chromatography on glutathione-agarose beads (Smith and Johnson, 1988). The pGEX series of plasmids contain a tac promoter followed by the complete coding sequence of GST in which the

normal termination codon has been replaced by a polylinker containing unique restriction sites for BamHI, SmaI and EcoRI, closely followed by a termination codon in all three reading frames. These plasmids contain a fragment of the *lac* operon carrying the overexpressed *lacI*^{q} allele of the *lac* repressor and a portion of *lacZ*. Under uninduced conditions the *lacI*^{q} allele efficiently inhibits transcription from the strong *tac* promoter, regardless of the *lacI* status of the host, until induction with IPTG.

The immunogenicity of purified gB fusion proteins was evaluated by analysing the nature of the immune response induced in hamsters, and by subsequent challenge studies on immunised animals. Additionally, the immunogenicity of several synthetic peptides derived from the primary amino acid sequence of EHV-4 gB was evaluated in a similar manner.

MATERIALS AND METHODS

Construction of Recombinant Plasmids

The plasmid pBSgB (Figure 4.2) was double-digested with BamHI and HindIII and the 1674bp fragment released directionally cloned between the BamHI and HindIII sites of the expression vector pUR288 to generate the recombinant plasmid pUR1.7gB. The 624bp fragment released by double-digestion of pBSgB with BglII and HindIII was directionally cloned between the BamHI and HindIII sites of pUR288 to generate the recombinant plasmid pUR0.6gB. Finally, the 1056bp fragment released by double-digestion of pBSgB with BamHI and BglII was cloned into the BamHI site of both pUR288 and the GST expression vector pGEX-2T to generate the recombinant plasmids pUR1.0gB and pGEX1.0gB, respectively. Each of these plasmids contained gB coding sequences cloned in-frame with β-galactosidase (pUR1.7gB, pUR1.0gB, pUR0.6gB) or GST (pGEX1.0gB) coding sequences. Insertion of the DNA insert in the correct orientation in plasmids pUR1.0gB and pGEX1.0gB was confirmed by restriction endonuclease analysis.

Induction of Fusion Protein Synthesis

E.coli strain JM101 carrying the recombinant plasmid was inoculated into 10ml of L broth containing ampicillin at 100ug/ml and grown at 37°C for 16 to 20hr with vigorous shaking in an orbital incubator. 1ml of this culture was diluted into 100ml of L broth containing 100ug/ml ampicillin and grown at 37°C with vigorous shaking until the OD reading at 600nm was approximately 0.5 (took 2.5 to 3hr). The culture was then

induced for fusion protein production by the addition of IPTG (Table 5.1) to a final concentration of 0.5mM and incubation at 37°C with vigorous shaking for a further 2hr. For each recombinant plasmid, a time-course study was initially carried out in order to determine the induction time with IPTG that gives optimum levels of fusion protein synthesis. In this case, bacterial cells were grown to an OD reading of 0.5 as above, and then individual aliquots of culture were induced with 0.5mM IPTG for varying lengths of time, typically 0hr, 0.5hr, 1hr, 1.5hr, 2hr, 2.5hr, 3hr, 3.5hr and 4hr.

Preparation of Cell Extracts (Small Scale)

2ml of each induced bacterial culture was spun in a benchtop microcentrifuge (13000rpm/1min), pelleted cells were washed by resuspending in 1ml of dH_2O and recovered by centrifugation once more. Cells were lysed in 200ul of SDS-PAGE sample buffer (Table 5.1) by vigorous pipetting and cell debris removed by centrifugation as above for 5min. 20ul of each supernatant was analysed for fusion protein production by SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell extracts were fractionated by SDS-PAGE as previously described (Laemmli, 1970). Gels contained 10% acrylamide with a 5% stacking gel. The composition of the gel mixes is shown in Table 5.1. SDS-PAGE was conducted on a vertical gel set (BRL) used according to the suppliers instructions. Following pouring of the running gel, leaving a 4cm space above for application of the stacking gel, 100ul of water-saturated butanol was applied

to the top of the gel to ensure a smooth surface and the gel left to polymerise for at least 30min. Following removal of the butanol by rinsing with dH_2O, the stacking gel was poured, the well-forming comb inserted and the gel left to polymerise for at least 30min. Samples were boiled in SDS-PAGE sample buffer for 3min and then loaded onto the gel. All gels were electrophoresed at a constant voltage of 200V for 3 to 4hr at room temperature in SDS-PAGE running buffer (Table 5.1). Gels were stained with Coomassie blue (Table 5.1) for 2hr with gentle agitation. Protein bands were visualised by gentle agitation in destaining solution (Table 5.1) for 4 x 45min. Finally, gels were dried under vacuum at 80°C for 2hr.

Purification of β-Galactosidase Fusion Proteins

Fusion proteins were isolated by electroelution (Maniatis et al., 1982) from preparative 3mm SDS-PAGE gels. The cell extract from 50ml of induced bacterial culture, prepared by lysis of cells in 5ml of SDS-PAGE sample buffer as previously described, was electrophoresed on each gel as previously described. Only a small strip of gel was stained with Coomassie blue and this was aligned with the unstained master gel so that the location of the fusion protein on the unstained gel could be located and excised. Excised gel slices were placed in sterile dialysis tubing (3cm wide) sealed at one end with a clip, 30ml of SDS-PAGE running buffer added, air bubbles removed and the tubing sealed with another clip. The dialysis bag was placed in a horizontal gel electrophoresis tank containing SDS-PAGE running buffer, ensuring that the bag was fully submerged. A constant voltage of 100V was applied for 14 to 16hr, then the

polarity was reversed for 10min to remove any protein from the side walls of the dialysis bag. The buffer was removed from the bag and protein precipitated by adding 5 volumes of ice cold acetone and incubation at -20°C for 2hr. Protein was recovered by centrifugation in a Beckman JS7.5 rotor (7000rpm/15min/4°C). The pellet was washed by resuspending in 20ml of dH_2O and the protein recovered by centrifugation. This washing step was repeated twice more. Finally, the pellet was resuspended in 1ml of PBS buffer (Table 5.1) and 10ul was analysed by SDS-PAGE.

Purification of GST Fusion Proteins

A 500ml induced bacterial culture was centrifuged (7000rpm/15min/4°C) in a Beckman JS7.5 rotor. Pelleted cells were resuspended in 10ml of MTPBS (Table 5.1) containing 1% Triton X-100 and lysed by mild sonication (6 x 30 second bursts) on ice. Lysed cells were centrifuged in a Beckman JA20 rotor (10000rpm/10min/4°C). Fusion protein was absorbed onto glutathione-agarose beads by mixing the supernatant with 2ml of 50% glutathione-agarose beads (sulphur linkage, Sigma) in a 20ml polypropylene tube with gentle agitation on a rotating platform for 10min. Beads were previously pre-swollen in MTPBS, washed twice in the same buffer and stored as a 50% suspension in MTPBS at 4°C. Following absorption, beads were collected by centrifugation at 1000rpm for 3min in a benchtop centrifuge and washed by resuspending in 50ml of MTPBS. Beads were collected by centrifugation and the washing procedure repeated twice more. Finally, fusion protein was eluted from the beads by competition with reduced glutathione using 2 x 10min washes with 1 bead volume (2ml) of 50mM TrisHCl (pH 8.0) containing freshly

prepared 20mM reduced glutathione. The eluates were combined and 20ul was analysed by SDS-PAGE.

Peptide Synthesis

Peptides corresponding to EHV-4 gB amino acid sequences QESLHREQILHRLHKRAVEAANS (peptide 24; residues 528 to 550, Figure 5.3), VELDLVPSKFATPGARA (peptide 25; residues 251 to 267, Figure 5.3) and HTSLSDRYNDRVPVSVEEIF (peptide 27; residues 200 to 219, Figure 5.3) were prepared using solid-phase synthetic methods as previously described (Stokes et al., 1990). An additional cysteine residue was added at the terminus of each peptide in order to promote the formation of cross-links between peptides and thus eliminating the requirement for a carrier protein. These peptides were selected on the basis of predicted immunogenicity from criteria such as hydrophilicity (Kyte and Doolittle, 1982) and predicted secondary structure (Chou and Fasman, 1978). Peptide 24 was predicted to be alpha-helical, peptide 25 to be strongly alpha-helical and hydrophilic and probably situated close to the surface of the gB molecule since it is located near a potential N-linked glycosylation site, whilst peptide 27 was predicted to be very hydrophilic. In addition, peptides 24 and 25 demonstrated some divergence of sequence with the corresponding regions of EHV-1 gB and, as such, may represent type-specific antigenic sites. All peptides showed divergence of sequence with HSV-1 gB.

In Vitro and In Vivo Immunogenicity Studies

Studies on the immunogenicity of EHV-4 gB fusion proteins and peptides were carried out by Dr. Anne Stokes, AFRC Institute

for Animal Health, Pirbright Laboratory, Pirbright, Surrey. All procedures with experimental animals, ELISA assays, virus neutralisation assays, immunisation of hamsters with peptides and challenge experiments were carried out as previously described (Stokes et al., 1989, 1990). For immunisation with fusion proteins, 5ug of purified fusion protein emulsified in an equal volume of complete Freunds adjuvant was administered to each hamster. Further immunisations with 5ug of fusion protein in incomplete Freunds adjuvant were given on days 14 and 28 and hamsters were challenged with EHV-1 12 days after the final immunisation.

TABLE 5.1

Stock Solutions, SDS-PAGE Buffers and Gel Mixes

Stock Acrylamide

acrylamide	29g
methylene-bisacrylamide	1g
dH_2O	to 100ml

SDS-PAGE Running Gel

stock acrylamide	10.0ml
1M TrisHCl (pH 8.8)	11.2ml
dH_2O	8.5ml
10% SDS	0.3ml
10% APS	75ul
TEMED	20ul

SDS-PAGE Stacking Gel

stock acrylamide	1.7ml
1M TrisHCl (pH 6.8)	1.2ml
dH_2O	7.0ml
10% SDS	0.1ml
10% APS	75ul
TEMED	8ul

SDS-PAGE Running Buffer

glycine	14.4g
Tris base	3.1g
SDS	1.0g
dH_2O	to 1 litre

SDS-PAGE Sample Buffer

2.5% SDS
2.5% β-mercaptoethanol
0.1M TrisHCl (pH 6.8)
10% glycerol
0.01% bromophenol blue

Coomassie Blue Staining Solution

Coomassie blue	0.5g
methanol	300ml
glacial acetic acid	100ml
dH_2O	to 1 litre

Destaining Solution

methanol	300ml
glacial acetic acid	100ml
dH_2O	600ml

IPTG

IPTG at 100mM in dH_2O. Store at $-20^{o}C$. Use at final concentration of 0.5mM.

PBS

Dissolve PBS tablet in 100ml dH_2O. Sterilise by autoclaving.

MTPBS

5M NaCl	30ml
1M Na_2HPO_4	16ml
1M NaH_2PO_4	4ml
dH_2O	to 1 litre

Sterilise by autoclaving.

RESULTS

Construction, Structure and Restriction Endonuclease Analysis of Recombinant Plasmids

The construction, structure and restriction endonuclease analysis of the recombinant plasmids used to express segments of the EHV-4 gB protein as fusions with β-galactosidase are shown in Figure 5.1. The structure of the pUR series of plasmids is shown in Figure 5.1a,b. The fortuitous location of restriction endonuclease cleavage sites in the EHV-4 gB gene permitted expression of segments of this gene in-frame with *lacZ* in a single vector, pUR288. Three segments of EHV-4 gB DNA were separately expressed in pUR288 (Figure 5.1c). The 1674bp BamHI/HindIII fragment corresponds to amino acid residues 142 to 699, the 1056bp BamHI/BglIIfragment to residues 142 to 493 and the 624bp BglII/HindIII fragment to residues 492 to 699, of the EHV-4 gB protein (Figure 5.3). The structure of these recombinant plasmids is shown in Figure 5.1d and these were confirmed by restriction endonuclease analysis (Figure 5.1e).

The construction, structure and restriction endonuclease analysis of recombinant plasmid pGEX1.0gB is shown in Figure 5.2. The structure of the pGEX series of plasmids is shown in Figure 5.2a,b. The 1056bp BamHI/BglII fragment described above (Figure 5.2c) was cloned in-frame with GST coding sequences to generate the plasmid pGEX1.0gB (Figure 5.2d). Insertion of the EHV-4 gB DNA in the correct orientation was confirmed by restriction endonuclease analysis (Figure 5.2e).

As can be seen from Figure 5.3, the regions of EHV-4 gB expressed represent 558 amino acids from the hydrophilic surface

domain and correspond to 78% of the total amino acids in this domain.

Expression and Purification of Fusion Proteins

The fusion product of pUR1.7gB was expected to be 184kDa, comprising 116kDa β-galactosidase and 68kDa gB moieties. For each of four clones expressing pUR1.7gB, the level of expression of fusion protein was very low (Figure 5.4a). To determine whether this low yield was due to the induction time with IPTG being too long or too short, a time-course study was carried out (Figure 5.4b). This confirmed that there was a constant low level of fusion protein accumulation, probably due to degradation of the protein soon after its synthesis. Although the fusion of a protein to β-galactosidase greatly increases its stability and prevents it being recognised as foreign and degraded, there is obviously a limit to the size of the exogenous protein that can be expressed. As has been demonstrated here, the fusion protein is degraded soon after its synthesis. Protein stability, rather than the strength of the promoter driving protein expression, is the most important consideration when expressing proteins as fusion products. However, high levels of fusion protein expression were obtained with pUR1.0gB and pUR0.6gB. The fusion product of pUR1.0gB was predicted to be 159kDa (β-galactosidase, 116kDa; gB, 43kDa). As can be seen from Figure 5.5a, three separate clones successfully expressed pUR1.0gB to a level representing 2 to 5% of total cellular protein (lanes 4, 6 and 8). In contrast, a clone containing gB DNA inserted in the opposite, incorrect orientation expressed only the β-galactosidase product (lane

10). A time-course study indicated that fusion protein accumulation reaches its highest level between 2 and 3hr after induction prior to decreasing once more (Figure 5.5b). The fusion product of pUR0.6gB was predicted to be 141kDa (β-galactosidase, 116kDa; gB, 25kDa). Four separate clones were demonstrated to successfully express pUR0.6gB to high levels (Figure 5.6a; lanes 4, 6, 8 and 10). In addition to full length fusion protein, small amounts of another product slightly larger than β-galactosidase were also synthesised by each induced clone. This truncated product was probably a result of in vivo cleavage by E.coli at the proposed internal proteolytic cleavage site of EHV-4 gB, RR/S, which occurs 25 amino acids from the amino-terminus of the gB segment expressed (Figure 5.3). The fusion product following proteolytic cleavage at this site would contain β-galactosidase fused to 25 amino acids from gB and be 119kDa. This is consistent with the observed size of the truncated product. A time-course study demonstrated that maximum accumulation of pUR0.6gB occurs between 2.5 and 3hr after induction prior to levels decreasing once more- the levels of the truncated product also increased and decreased in direct parallel with the full length product (Figure 5.6b).

The pGEX1.0gB fusion product was predicted to be 70.5kDa (GST, 27.5kDa; gB, 43kDa). Three separate clones were demonstrated to successfully express pGEX1.0gB to high levels, and certainly equal to or better than those achieved for the β-galactosidase fusion proteins (Figure 5.7a; lanes 4, 6 and 8). Induction of pGEX-2T alone produced a similar amount of native GST (lane 2). A time-course study demonstrated that the induction time which gave maximum levels of fusion protein

accumulation was 2hr, after which fusion protein levels decreased once more (Figure 5.7b).

The purity of purified fusion proteins was determined by SDS-PAGE. An aliquot of pUR0.6gB purified by electroelution from gels is shown in Figure 5.8a. Generally, fusion protein purified in this manner was consistently over 95% pure, with typical yields of 200 to 300ug/100ml of culture. The results of purification of pGEX1.0gB by affinity chromatography on glutathione-agarose beads are shown in Figure 5.8b. They demonstrate that the fusion protein readily bound to the beads and was efficiently eluted from beads with reduced glutathione. Eluted material contained two protein species present in equal amounts, the larger species corresponding to the full length pGEX1.0gB fusion product. Despite several separate purifications of fusion protein, the same two protein species were consistently purified. How the smaller species arose is unclear but several explanations exist. These include partial degradation of the full length product or premature termination of translation during protein synthesis.

Evaluation of the Immunogenicity of EHV-4 gB Fusion Proteins and Synthetic Peptides

The immunogenicity of fusion proteins pUR0.6gB and pGEX1.0gB, and peptides 24, 25 and 27 from the EHV-4 gB sequence, was evaluated in hamsters. As can be seen from Figure 5.3, pUR0.6gB contains the sequence of peptide 24 whilst pGEX1.0gB encompasses the sequences of both peptides 25 and 27. The results of *in vitro* and *in vivo* studies with EHV-4 gB fusion proteins and peptides are summarised in Table 5.2. Antibodies

raised against each peptide *in vivo* reacted well with that peptide *in vitro*. Similarly, antibodies raised against peptide 24 also reacted with pUR0.6gB and antibodies raised against peptides 25 and 27 both reacted with pGEX1.0gB, demonstrating the integrity of the purified fusion proteins. Antibodies raised against each fusion protein *in vivo* reacted with that fusion protein *in vitro*. A monoclonal antibody to EHV-1 gp17/18 reacted strongly with peptide 24, which is consistent with the fact that peptide 24 is predicted, on the basis of sequence data, to reside on the small subunit of gB, gp18. Somewhat surprisingly, a monoclonal antibody specific for EHV-1 gp2 demonstrated strong reactivity with peptides 24 and 27. Serum from a horse vaccinated with Pneumabort-K reacted with all three peptides whereas serum from a hamster taken at 10 days p.i. only reacted with peptide 24. The most crucial findings of these studies were as follows. Firstly, no peptide could stimulate the production of virus neutralising antibodies or confer protective immunity in hamsters, although antibodies against peptide 25 reacted against whole EHV-4 virus in an ELISA assay. Secondly, no fusion protein could stimulate the production of virus neutralising antibodies in hamsters, although 1 out of 3 hamsters immunised with pUR0.6gB was protected from a lethal EHV-1 challenge. Thirdly, an additional observation not shown in Table 5.2 was that immunisation of hamsters with all three peptides induced lymphocytes to proliferate in response to whole virus antigen *in vitro*.

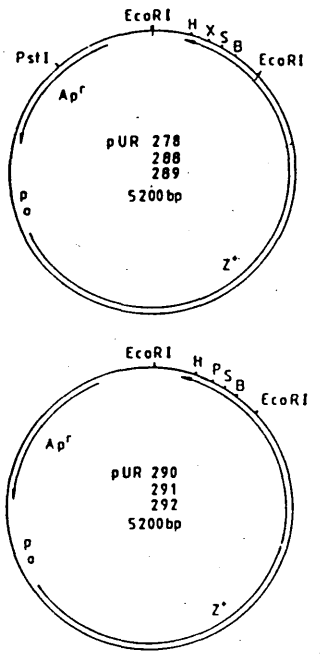
Figure 5.1 Construction, structure and restriction endonuclease analysis of pUR recombinant plasmids. (a) Physical map of the pUR series of plasmid expression vectors. (b) Polylinkers of the pUR vectors. (c) EHV-4 gB fragments expressed in pUR288. (d) Physical maps of the plasmids pUR1.7gB, pUR1.0gB and pUR0.6gB. The broken lines represent pUR288 DNA and the solid lines represent EHV-4 gB DNA. Sizes are shown in kilobases. The sites for BamHI (B), EcoRI (E), HindIII (H), PstI (P), SalI (Sa), SstI (Ss) and XbaI (Xb) are shown. (*) indicates a destroyed BamHI site as a result of insertion of a BglII end. The direction of transcription of the EHV-4 gB gene is denoted by an arrow. (e) Restriction endonuclease analysis of plasmids pUR1.7gB, pUR1.0gB and pUR0.6gB. Plasmid pUR1.7gB was digested with EcoRI (lane 1), HindIII (lane 2) and SstI (lane 3), plasmid pUR1.0gB was digested with EcoRI (lane 4), HindIII (lane 5) and SstI (lane 6), and plasmid pUR0.6gB was digested with EcoRI (lane 7), HindIII (lane 8) and SstI (lane 9). HindIII digested λ DNA fragments were used as size markers.

Diagrams (a) and (b) are taken from Ruther and Muller-Hill (1983).

FIGURE 5.1

Construction, Structure and Restriction Endonuclease Analysis of pUR Recombinant Plasmids

(a)



(b)

Glu Phe Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln Leu Val Trp Cys Gln Lys

Lac Z 1010 1020

EcoRI HaeIII

GAA TTC CAG CTG AGC GCC GGT CGC TAC CAT TAC CAG TTG GTC TGG TGT CAA AAA TAA TAA TAA CCGGGCAGGCCATGTCTGCCCG.....

1020 BamHI SalI XbaI HindIII ClaI

EcoRI.....TGG TGT CAA AAA GGG GAT CCG TCG ACT CTA GAA AGC TTA TCG ATG ATA ..... pUR 278

EcoRI.....TGG TGT C GG GGA TCC GTC GAC TCT AGA AAG CTT ATC GAT GAT ..... pUR 288

EcoRI.....TGG TGT CA G GGG ATC CGT CGA CTC TAG AAA GCT TAT CGA TGA ..... pUR 289

BamHI SalI PstI HindIII ClaI

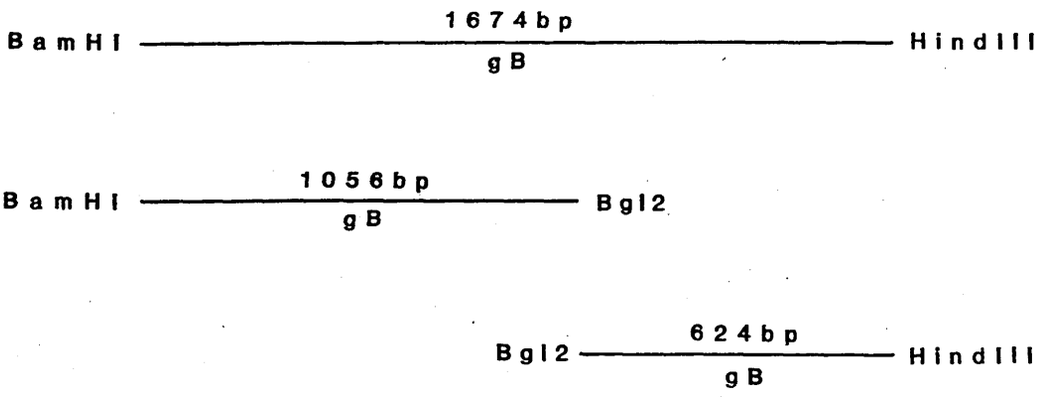
EcoRI.....TGG TGT CAA AAA GGG GAT CCG TCG ACC TGC AGC CAA GCT TAT CGA TGA ..... pUR 290

EcoRI.....TGG TGT C GG GGA TCC GTC GAC CTG CAG CCA AGC TTA TCG ATG ..... pUR 291

EcoRI.....TGG TGT CA G GGG ATC CGT CGA CCT GCA GCC AAG CTT ATC GAT ..... pUR 292

Figure 5.1- continued

(c)



(d)

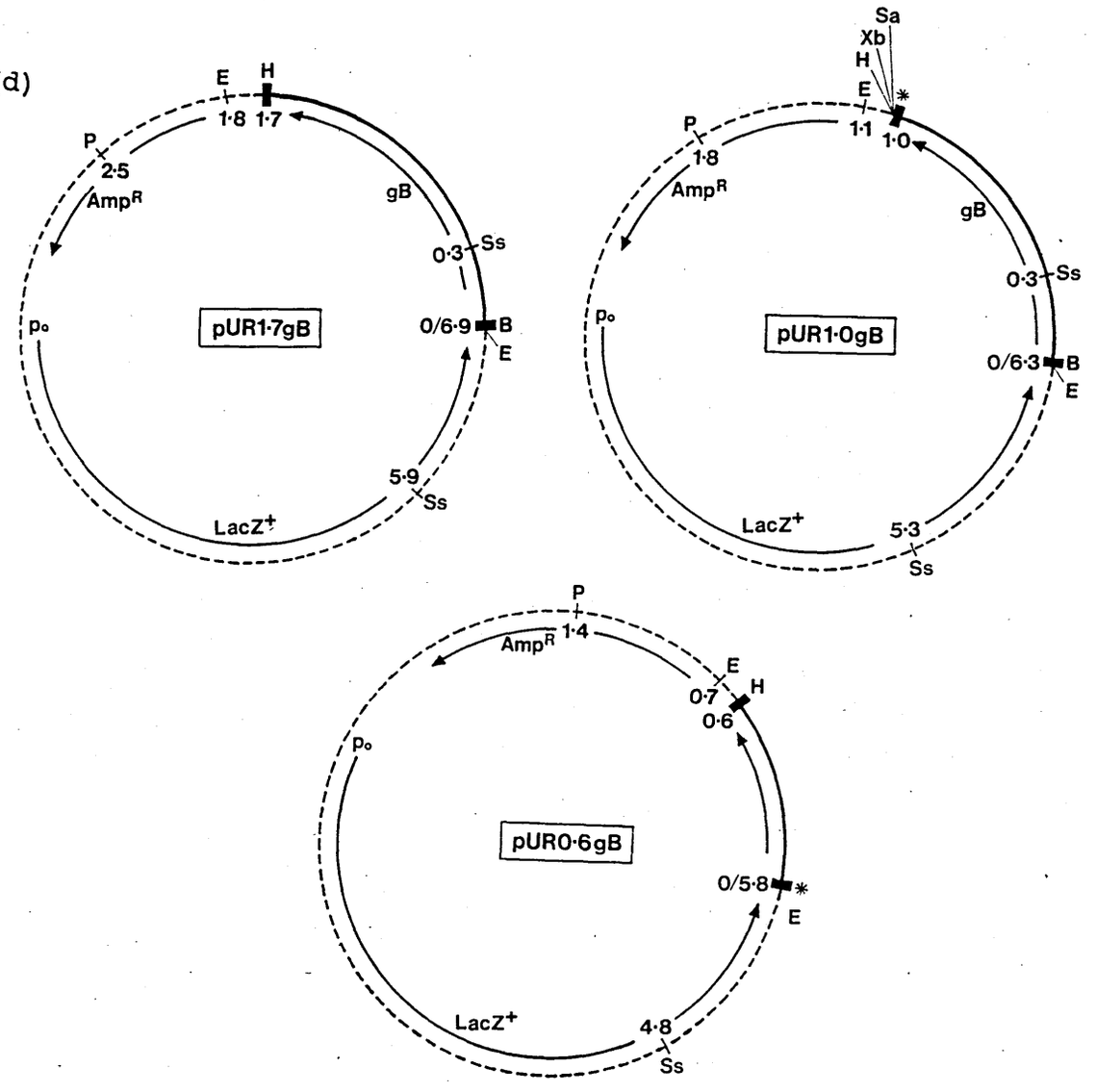


Figure 5.1- continued

(e)

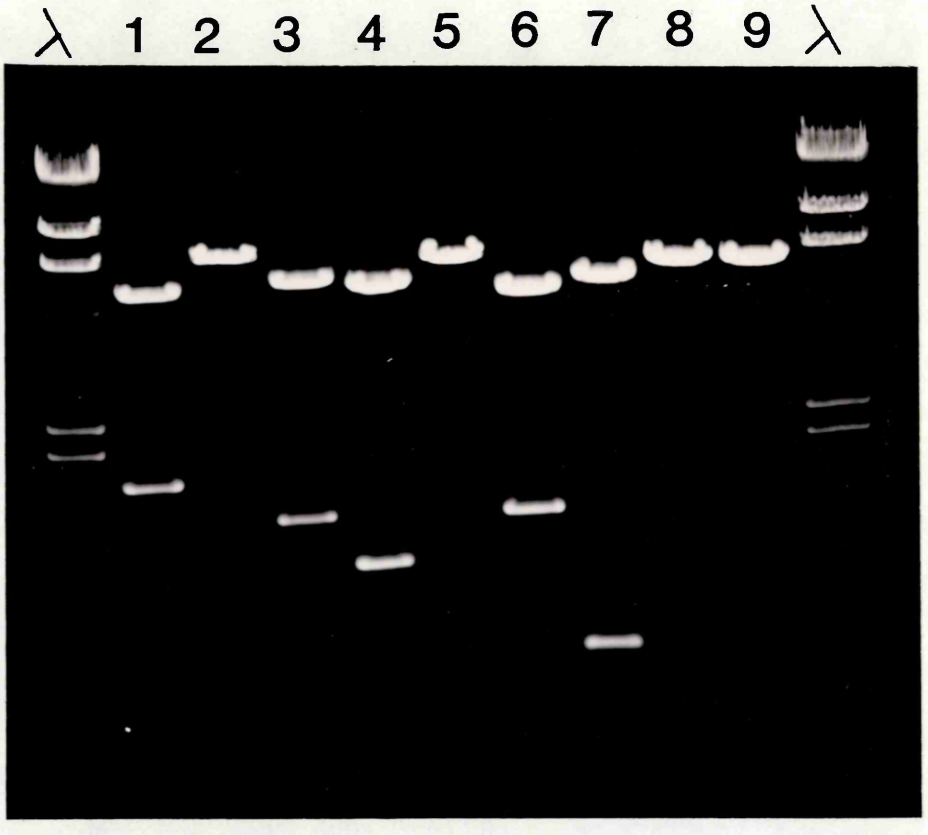


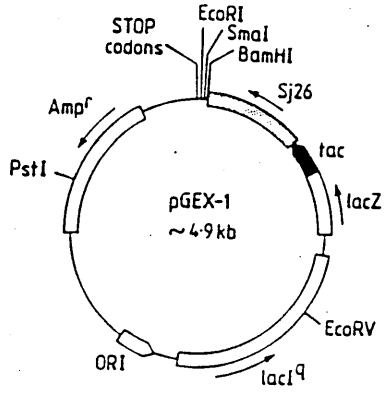
Figure 5.2 Construction, structure and restriction endonuclease analysis of plasmid pGEX1.0gB. (a) Physical map of the pGEX plasmids. (b) Polylinker of the pGEX plasmids. Thrombin and factor X cleavage sites are indicated. (c) EHV-4 gB fragment expressed in pGEX-2T. (d) Physical map of plasmid pGEX1.0gB. The broken line represents pGEX-2T DNA and the solid line represents EHV-4 gB DNA. Sizes are shown in kilobases. The sites for BamHI (B), EcoRI (E), SmaI (Sm) and SstI (Ss) are shown. (*) indicates a destroyed BamHI site as a result of insertion of a BglII end. The direction of transcription of the EHV-4 gB gene is denoted by an arrow. (e) Restriction endonuclease analysis of plasmid pGEX1.0gB. The plasmid was digested with BamHI (lane 1), BamHI/EcoRI (lane 2) and SstI (lane 3). HindIII digested λ DNA fragments were used as size markers.

Diagrams (a) and (b) are taken from Smith and Johnson (1988).

FIGURE 5.2

Construction, Structure and Restriction Endonuclease Analysis of Plasmid pGEX1.0gB

(a)



(b)

```
                Pro  Lys  Ser  Asp  Pro  Arg  Glu  Phe  Ile  Val  Thr  Asp  ***
pGEX-1          CCA  AAA  TCG  GAT  CCC  CGG  GAA  TTC  ATC  GTG  ACT  GAC  TGA  CGA  TCT  G
                                   BamHI     SmaI      EcoRI

                                        Thrombin
                Pro  Lys  Ser  Asp  Leu  Val  Pro  Arg↓ Gly  Ser  Pro  Gly  Ile  His  Arg  Asp  ***
pGEX-2T         CCA  AAA  TCG  GAT  CTG  GTT  CCG  CGT  GGA  TCC  CCG  GGA  ATT  CAT  CGT  GAC  TGA  CTC  ACG  ATC  TG
                                                  BamHI     SmaI      EcoRI

                                   Factor X
                Pro  Lys  Ser  Asp  Leu  Ile  Glu  Gly  Arg↓ Gly  Ile  Pro  Gly  Asn  Ser  Ser  ***
pGEX-3X         CCA  AAA  TCG  GAT  CTG  ATC  GAA  GGT  CGT  GGG  ATC  CCC  GGG  AAT  TCA  TCG  TGA  CTG  ACT  GAC  GAT  CTG
                                             BamHI     SmaI      EcoRI
```

(c)

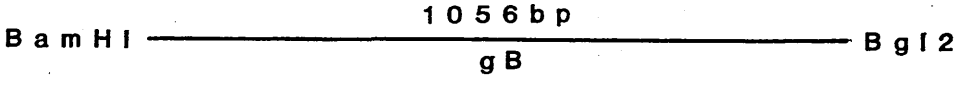
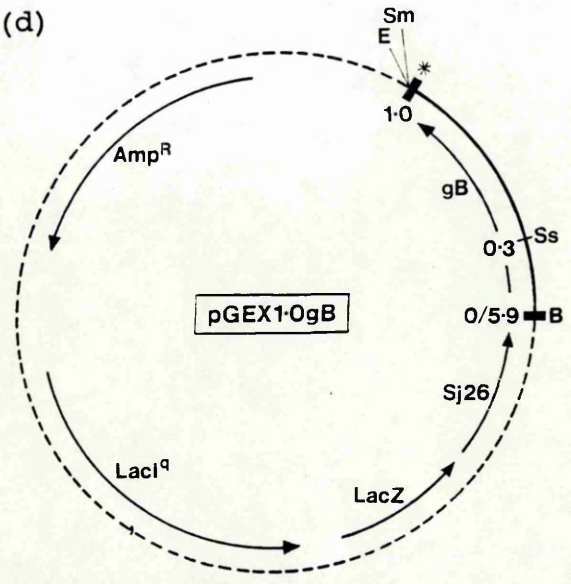


Figure 5.2- continued

(d)



(e)

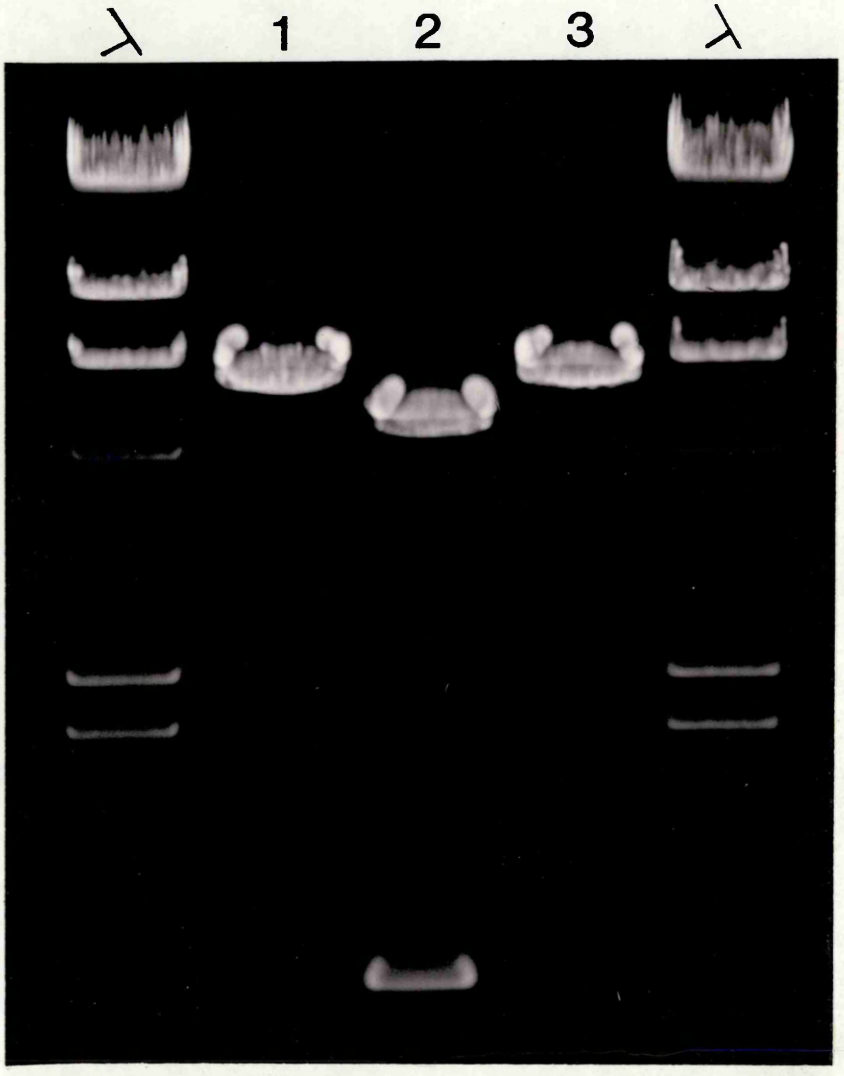


Figure 5.3 Summary of the regions of the EHV-4 gB protein expressed as fusion products and from which peptides were prepared. The DNA sequence of the EHV-4 gB gene and the predicted amino acid sequence of its gene product are shown. Potential N-linked glycosylation sites are boxed and the internal proteolytic cleavage site is indicated by an arrow. The restriction endonuclease cleavage sites for BamHI, BglII and HindIII, which were utilised in cloning segments of the gene into expression vectors, are indicated. The three EHV-4 gB peptides whose immunogenicity was evaluated in hamsters (peptides 24, 25 and 27) are underlined.

FIGURE 5.3

Derivation of EHV-4 gB Fusion Proteins and Peptides

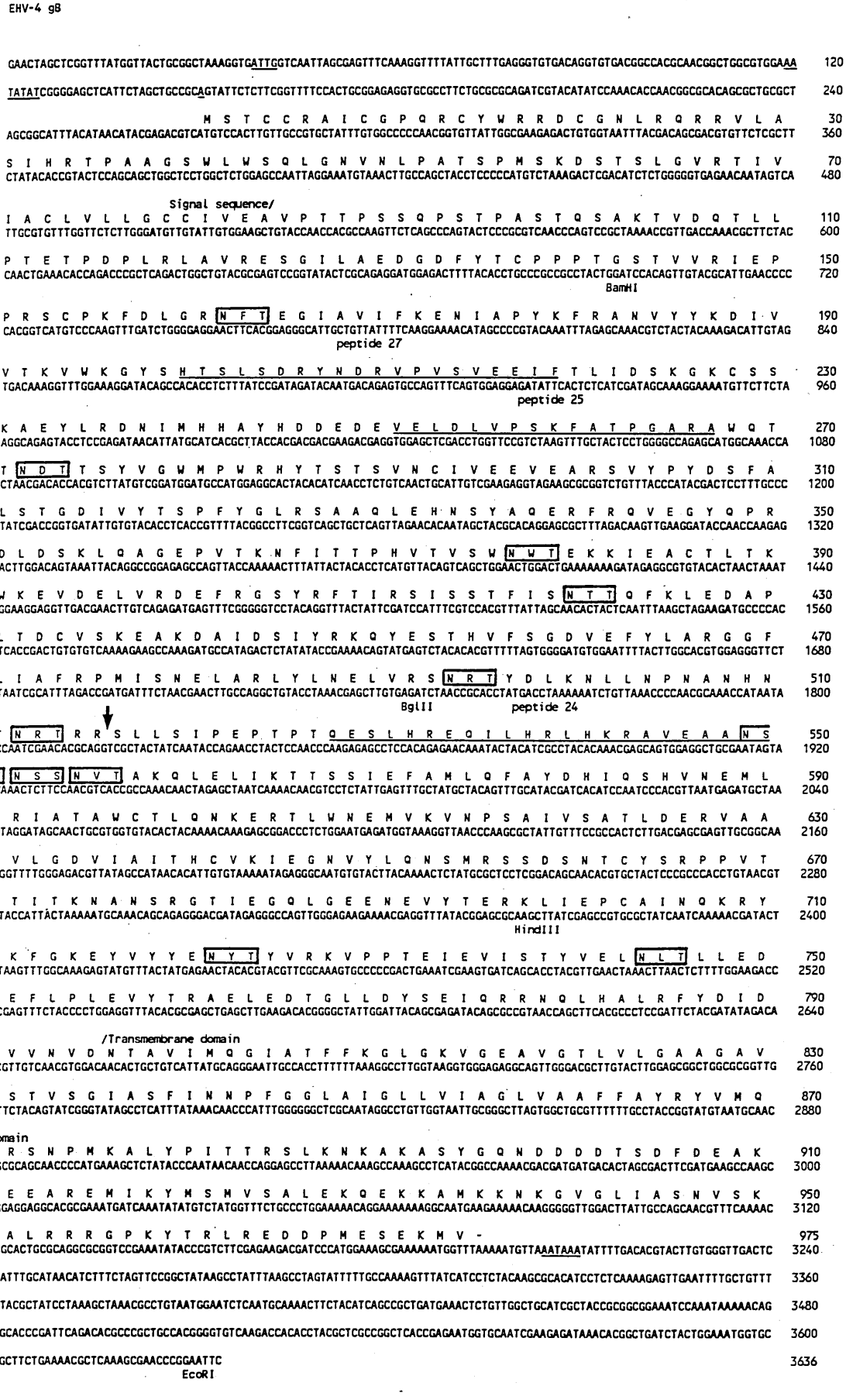


Figure 5.4 Analysis of expression of the fusion protein pUR1.7gB by SDS-PAGE.
(a) Four clones (1 to 4) containing the 1674bp BamHI/HindIII EHV-4 gB DNA fragment cloned in plasmid pUR288 were analysed for expression of the pUR1.7gB β-galactosidase/EHV-4 gB fusion protein following induction with IPTG for 2hr. The samples shown on the gel are as follows:-
clone 1, uninduced (lane 1) and induced (lane 2); clone 2, uninduced (lane 3) and induced (lane 4); clone 3, uninduced (lane 5) and induced (lane 6); clone 4, uninduced (lane 7) and induced (lane 8).
(b) Time-course study to determine induction time required for optimum yield of fusion protein. The samples shown on the gel are as follows:-
pUR288, uninduced (lane 1) and induced (lane 2); pUR1.7gB, uninduced (lane 3) and induced for 0.5hr (lane 4), 1hr (lane 5), 1.5hr (lane 6), 2hr (lane 7), 2.5hr (lane 8), 3hr (lane 9), 3.5hr (lane 10) and 4hr (lane 11).

For each gel, an arrow indicates the position of the induced 184kDa fusion protein. Sizes of the protein markers (M) are shown in kDa.

FIGURE 5.4

Expression of the Fusion Protein pUR1.7gB

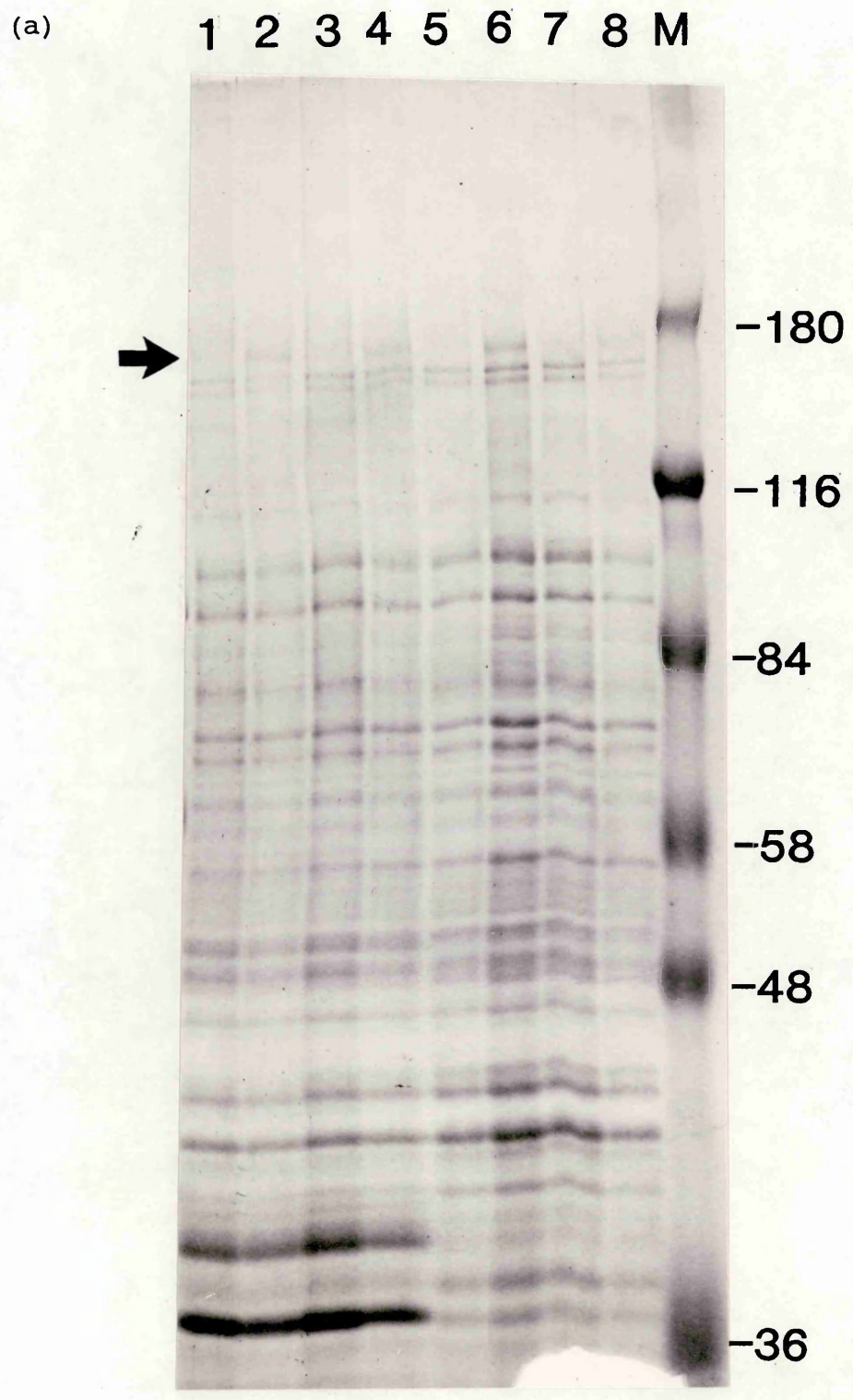


Figure 5.4- continued

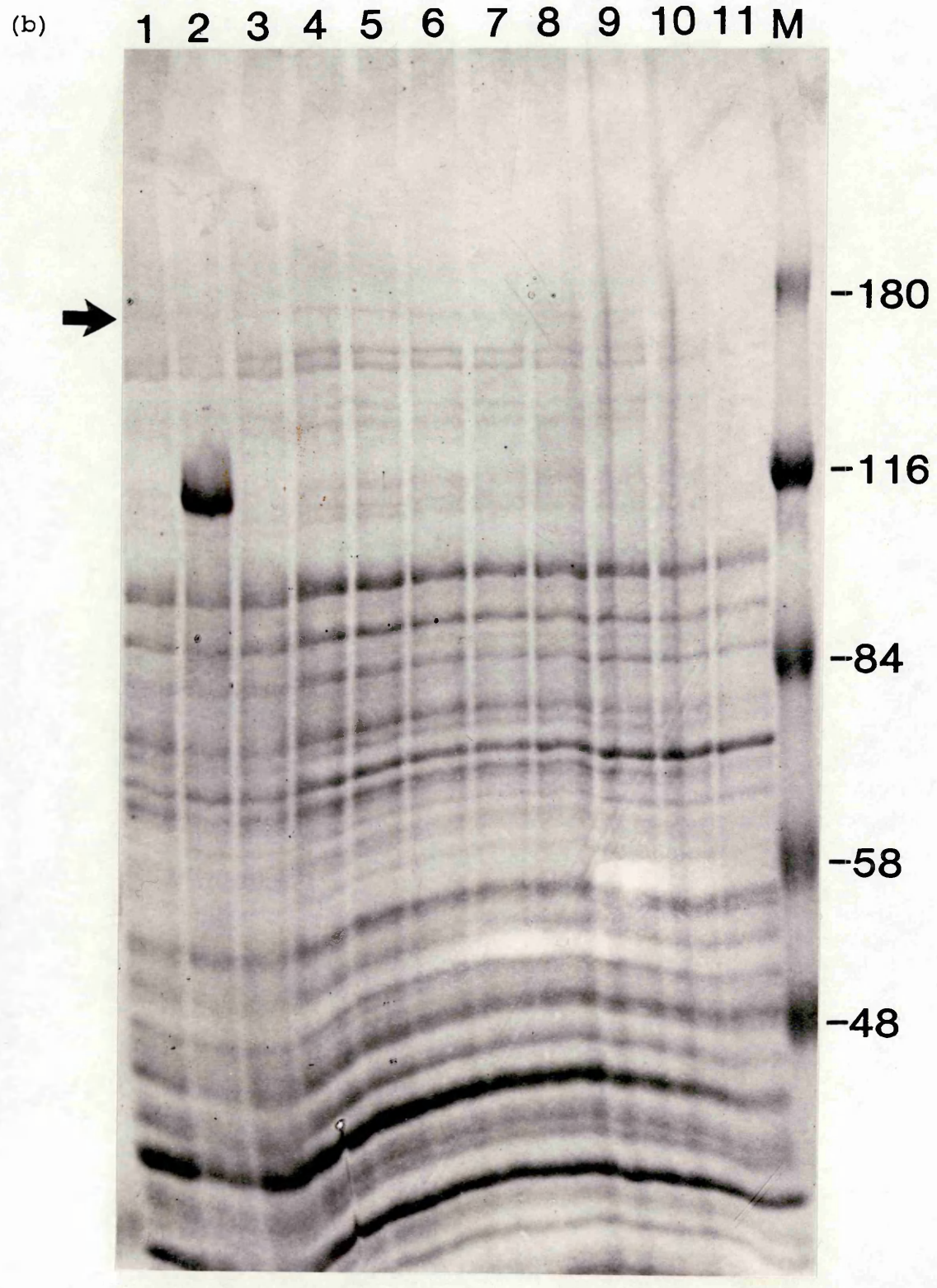


Figure 5.5 Analysis of expression of the fusion protein pUR1.0gB by SDS-PAGE.
(a) Three clones (1 to 3) containing the 1056bp BamHI/BglII EHV-4 gB DNA fragment cloned in the correct orientation in pUR288 were analysed for expression of the pUR1.0gB β-galactosidase/EHV-4 gB fusion protein following induction with IPTG for 2hr. Clone 4, which contains the EHV-4 gB DNA inserted in the incorrect orientation in pUR288 was used as a negative control. The samples shown on the gel are as follows:-
pUR288, uninduced (lane 1) and induced (lane 2); clone 1, uninduced (lane 3) and induced (lane 4); clone 2, uninduced (lane 5) and induced (lane 6); clone 3, uninduced (lane 7) and induced (lane 8); clone 4, uninduced (lane 9) and induced (lane 10).
(b) Time-course study to determine induction time required for optimum yield of fusion protein. The samples shown on the gel are as follows:-
pUR288, uninduced (lane 1) and induced (lane 2); pUR1.0gB, uninduced (lane 3) and induced for 0.5hr (lane 4), 1hr (lane 5), 1.5hr (lane 6), 2hr (lane 7), 2.5hr (lane 8), 3hr (lane 9), 3.5hr (lane 10) and 4hr (lane 11).

For each gel, an arrow indicates the position of the induced 159kDa fusion protein. Sizes of the protein markers (M) are shown in kDa.

FIGURE 5.5

Expression of the Fusion Protein pUR1.0gB

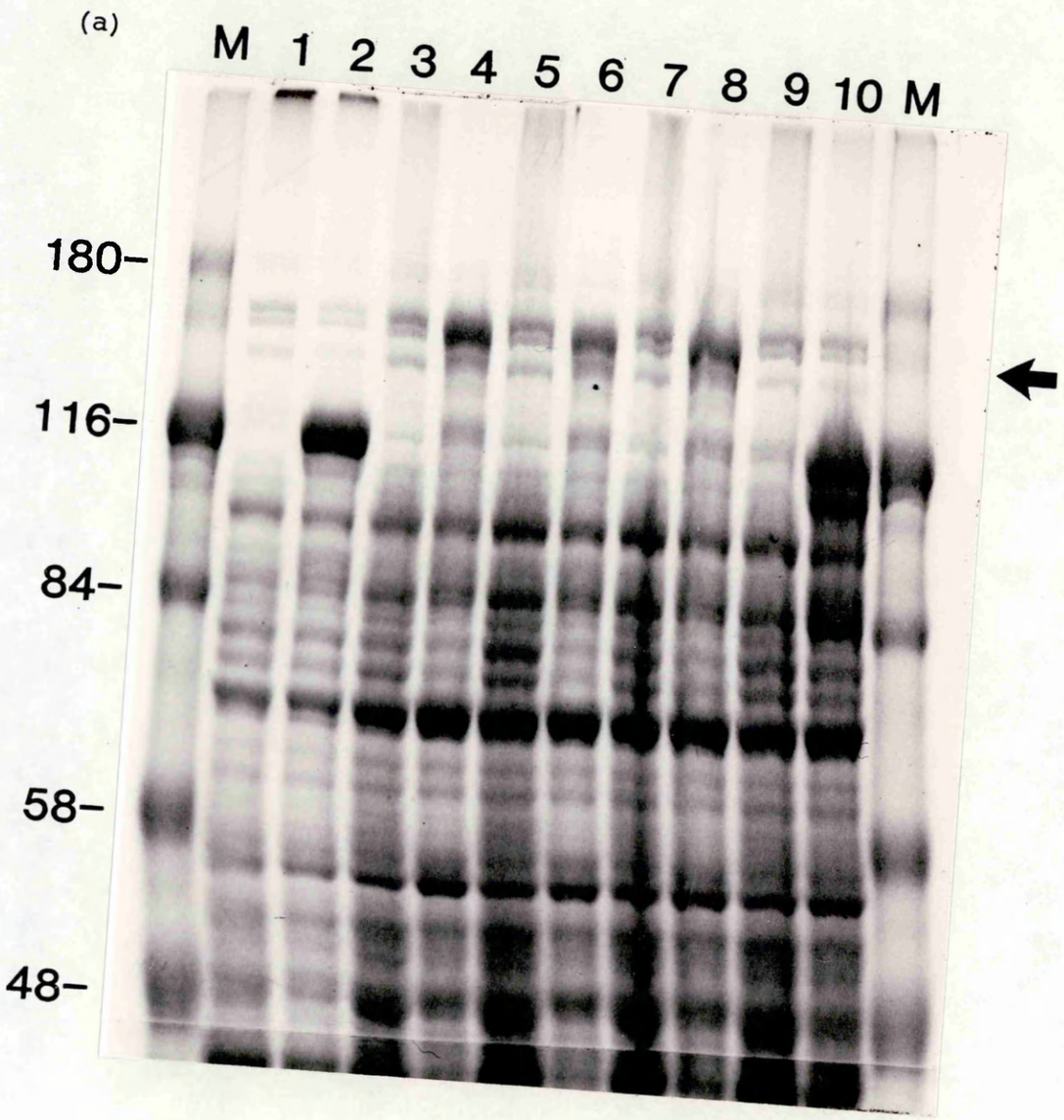


Figure 5.5- continued

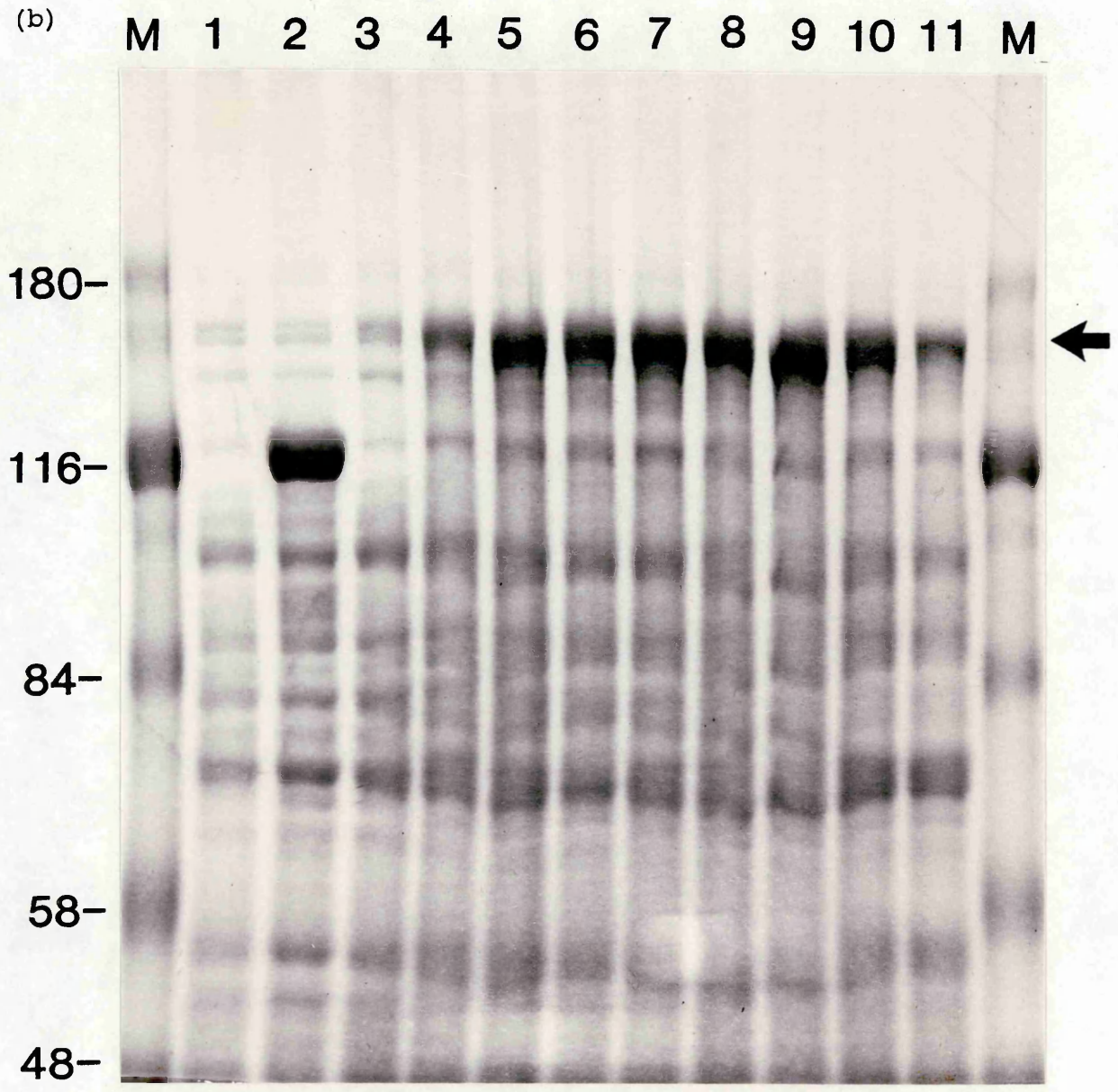


Figure 5.6 Analysis of expression of the fusion protein pUR0.6gB by SDS-PAGE.
(a) Four clones (1 to 4) containing the 624bp BglII/HindIII EHV-4 gB DNA fragment cloned in pUR288 were analysed for expression of the pUR0.6gB β-galactosidase/EHV-4 gB fusion protein following induction with IPTG for 2hr. The samples shown on the gel are as follows:-
pUR288, uninduced (lane 1) and induced (lane 2); clone 1, uninduced (lane 3) and induced (lane 4); clone 2, uninduced (lane 5) and induced (lane 6); clone 3, uninduced (lane 7) and induced (lane 8); clone 4, uninduced (lane 9) and induced (lane 10).
(b) Time-course study to determine induction time required for optimum yield of fusion protein. The samples shown on the gel are as follows:-
pUR288, uninduced (lane 1) and induced (lane 2); pUR0.6gB, uninduced (lane 3) and induced for 0.5hr (lane 4), 1hr (lane 5), 1.5hr (lane 6), 2hr (lane 7), 2.5hr (lane 8), 3hr (lane 9), 3.5hr (lane 10) and 4hr (lane 11).

For each gel, an arrow indicates the position of the induced 141kDa fusion protein. Sizes of the protein markers (M) are shown in kDa.

F I G U R E 5.6

Expression of the Fusion Protein pUR0.6gB

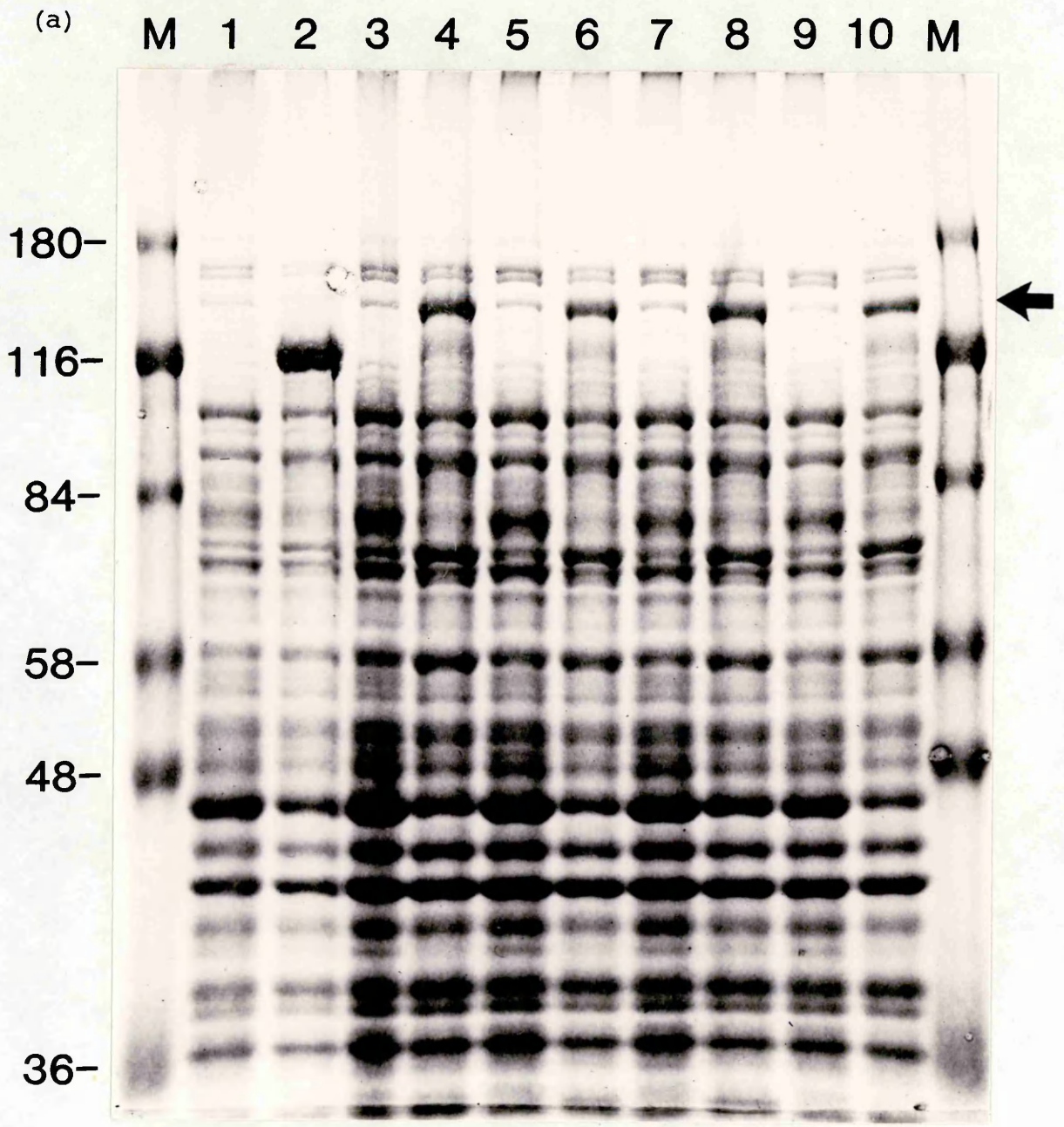


Figure 5.6- continued

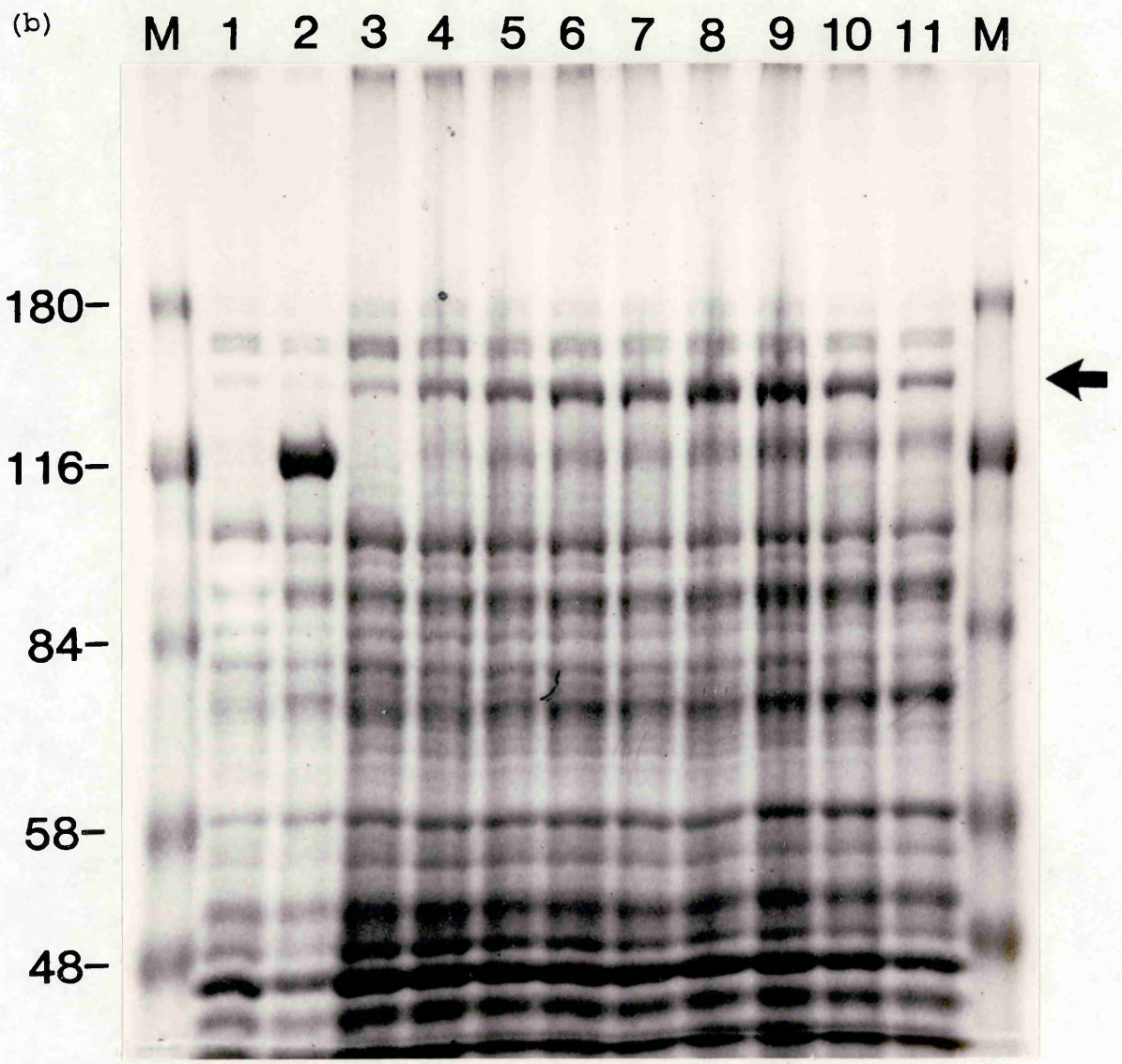


Figure 5.7 Analysis of expression of the fusion protein pGEX1.0gB by SDS-PAGE.
(a) Three clones (1 to 3) containing the 1056bp BamHI/BglII EHV-4 gB DNA fragment cloned in the correct orientation in pGEX-2T were analysed for expression of the pGEX1.0gB glutathione-S-transferase/EHV-4 gB fusion protein following induction with IPTG for 2hr. The samples shown on the gel are as follows:-
pGEX-2T, uninduced (lane 1) and induced (lane 2); clone 1, uninduced (lane 3) and induced (lane 4); clone 2, uninduced (lane 5) and induced (lane 6); clone 3, uninduced (lane 7) and induced (lane 8).
(b) Time-course study to determine induction time required for optimum yield of fusion protein. The samples shown on the gel are as follows:-
pGEX1.0gB, uninduced (lane 1) and induced for 0.5hr (lane 2), 1hr (lane 3), 1.5hr (lane 4), 2hr (lane 5), 2.5hr (lane 6), 3hr (lane 7) and 4hr (lane 8).

For each gel, an arrow indicates the position of the induced 70.5kDa fusion protein. Sizes of the protein markers (M) are shown in kDa.

F I G U R E 5.7

Expression of the Fusion Protein pGEX1.0gB

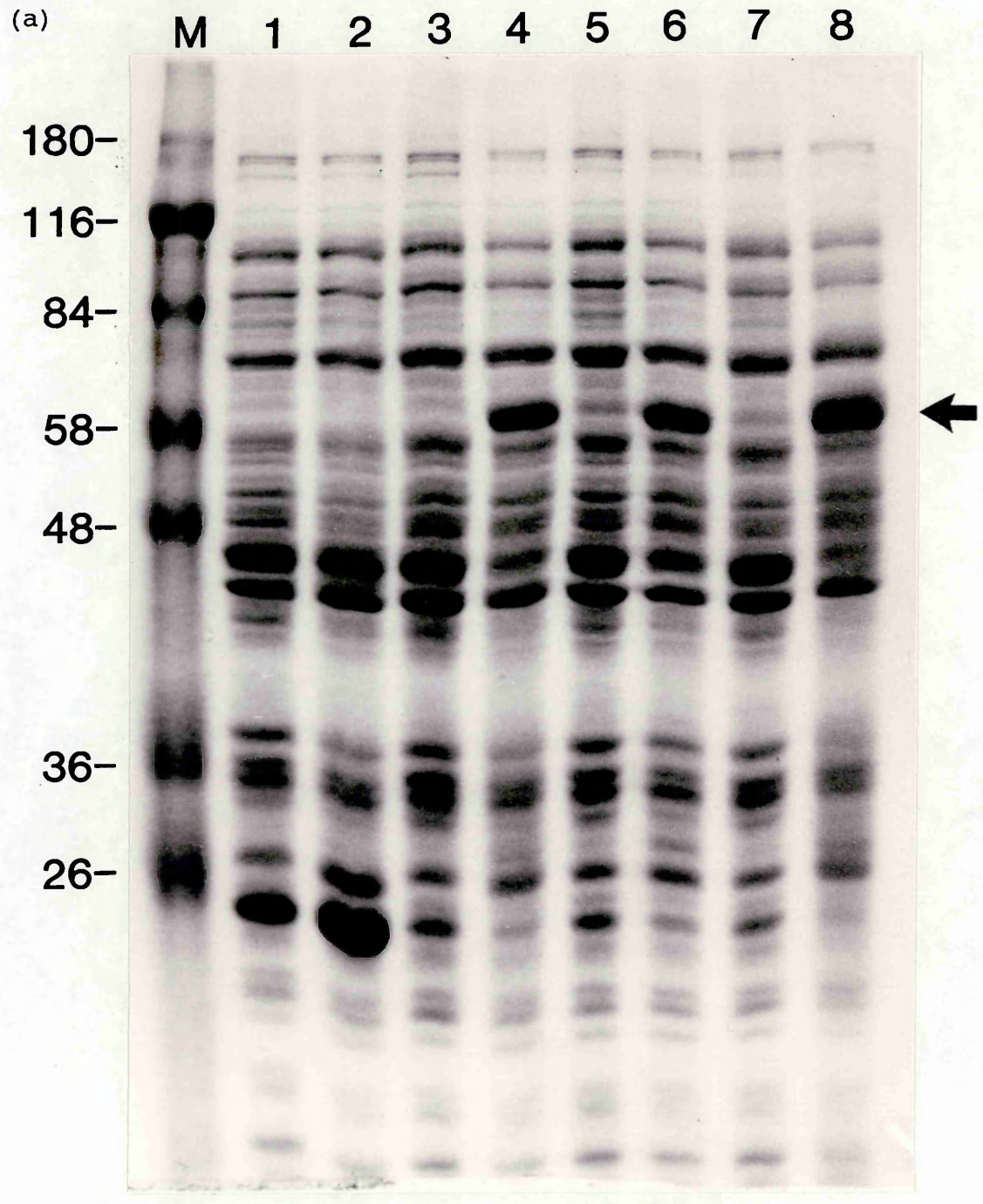


Figure 5.7- continued

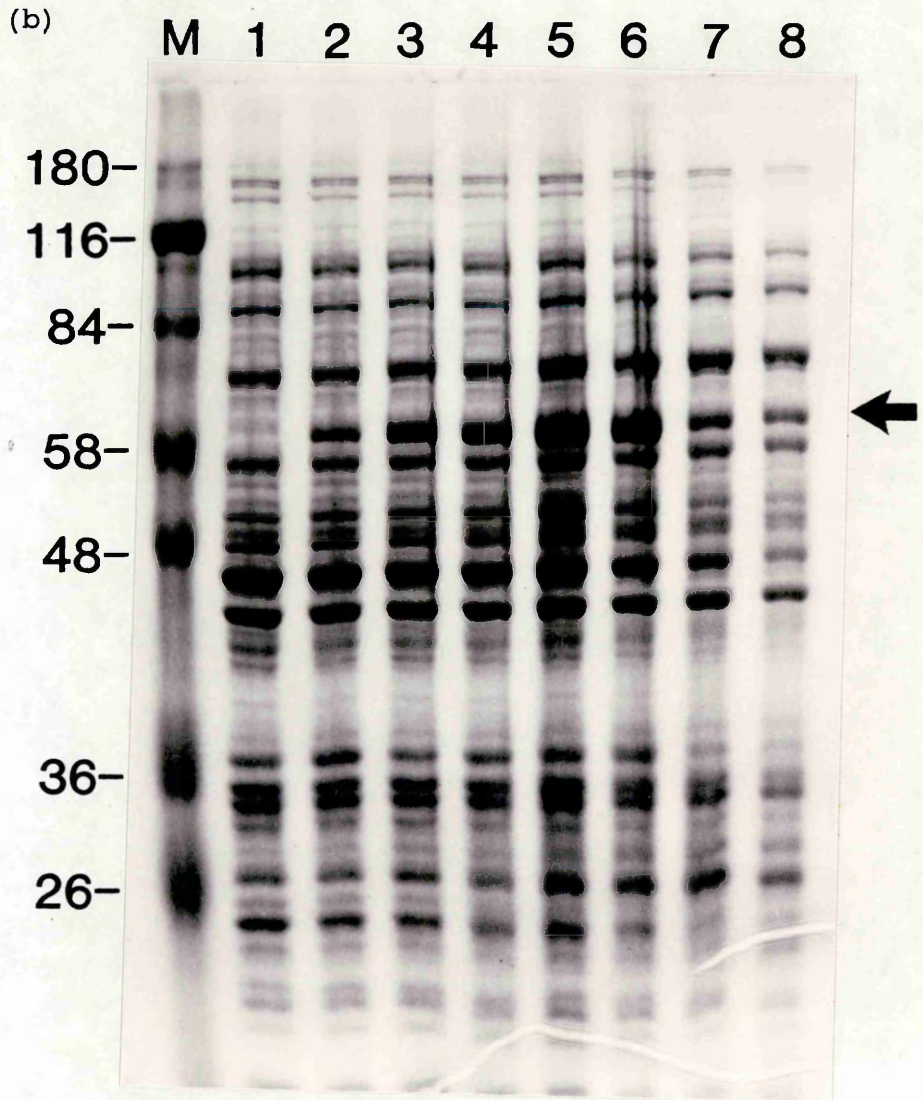
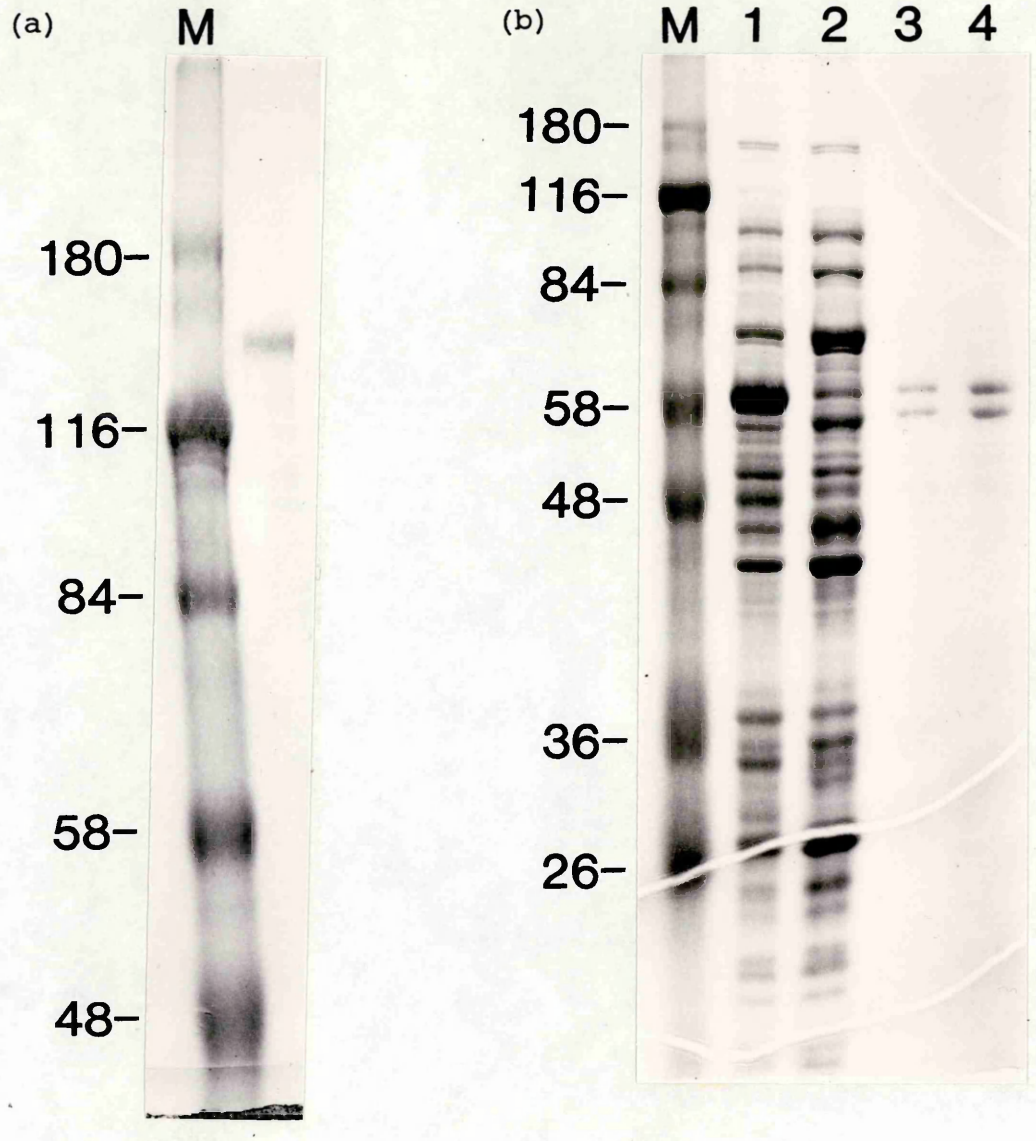


Figure 5.8 Analysis of purified fusion proteins by SDS-PAGE.
(a) pUR0.6gB fusion protein purified by electroelution from gels.
(b) Purification of pGEX1.0gB fusion protein by immunoaffinity chromatography on glutathione-agarose beads.
The samples shown on the gel are as follows:-
whole induced cell extract (lane 1); unbound material after incubation of supernatant from a lysed, induced cell extract with glutathione-agarose beads (lane 2); purified material eluted from beads (lanes 3 and 4).

The sizes of the protein markers (M) are shown in kDa.

F I G U R E 5.8

Purification of Fusion Proteins



T A B L E 5.2

Summary of In Vitro and In Vivo Studies With EHV-4 gB Fusion Proteins and Peptides

ANTIBODY	ANTIGEN							
	Virus (EHV-1/4)		gB Fusions		Peptides			Protection
	Elisa	Neut	pUR0.6	pGEX1.0	24	25	27	in hamsters
24	-	-	+	-	+	-	-	-
25	+	-	-	+	-	+	-	-
27	-	-	-	+	-	-	+	-
pUR0.6gB	-	-	+	-	+	-	-	1/3
pGEX1.0gB	-	-	-	+	-	+	+	-
MAb 17/18	+	-	ND	ND	++	-	-	+
MAb 14(gB)	+	-	ND	ND	-	-	-	+
MAb 21/22a	+	-	ND	ND	-	-	-	-
MAb 13(gC)	+	-	ND	ND	-	-	-	+
MAb 2	+	-	ND	ND	++	-	++	-
MAb 10	+	-	ND	ND	-	-	-	-
Horse Serum	+	+	ND	ND	+	++	++	NA
Hamster (10 days p.i.)	+	+	ND	ND	+	-	-	NA

Table 5.2 Summary of in vitro and in vivo studies with EHV-4 gB fusion proteins and peptides. The key to this table is shown below.
Elisa:- reactivity of antibodies against whole virus.
Neut:- denotes if antibodies have virus neutralising activity.
Horse Serum:- from horses vaccinated with Pneumabort-K.
Hamster (10 days p.i.):- serum from hamster 10 days after infection with EHV-1.
MAb:- monoclonal antibodies against EHV-1 glycoproteins (Allen and Yeargan, 1987).
ND:- not determined.
NA:- not applicable.

DISCUSSION

The glycoprotein gB of EHV-4 has been evaluated as a potential candidate for vaccine development. Studies carried out in hamsters seeked to identify the nature of the immune response elicited to purified preparations of EHV-4 gB fusion proteins and to several synthetic peptides derived from the primary amino acid sequence of this glycoprotein. Hamsters immunised with purified fusion proteins seroconverted but failed to produce antibodies capable of neutralising virus infectivity *in vitro*. Identical results were obtained using synthetic peptides. Generally, hamsters immunised with purified fusion proteins or peptides were not protected from a lethal challenge with virulent EHV-1, although one out of three hamsters immunised with pUR0.6gB survived a lethal virus challenge.

The general failure of recombinant EHV-4 gB and synthetic peptides to stimulate the production of virus neutralising antibodies and to confer protective immunity in hamsters is somewhat at odds with similar studies using recombinant gB-like proteins produced by several methods for other herpesviruses. Expression of HCMV gB in vaccinia virus and immunisation of rabbits with the live recombinant vaccinia virus elicited the production of antibodies which neutralised virus infectivity *in vitro* (Cranage *et al.*, 1986). Cattle immunised with BHV-1 gI, purified using a monoclonal antibody to gI, produced antibodies which neutralised virus infectivity *in vitro* and participated in ADCC of BHV-1-infected cells, and were also protected from challenge with virulent BHV-1 (Babiuk *et al.*, 1987). Similarly, immunisation of cattle with a recombinant vaccinia virus

expressing BHV-1 gI elicited virus neutralising antibody production (van Drunen Littel-van den Hurk et al., 1989) and immunisation of mice with transfected cells expressing gI also stimulated the production of BHV-1-specific virus neutralising antibodies (Fitzpatrick et al., 1988). The use of these transfected cells as targets in immune-mediated cytotoxicity assays demonstrated that BHV-1 gI was recognised by murine antibody and CTLs. There is a wide body of literature concerned with the expression of, and immune response to, HSV-1 gB. Mice immunised with a recombinant vaccinia virus expressing HSV-1 gB produced virus neutralising antibodies and were protected from a lethal HSV-1 challenge (Cantin et al., 1987). The construction of a recombinant adenovirus expressing HSV-1 gB to high levels has been reported (Johnson et al., 1988b). Immunisation of mice with this recombinant adenovirus produced neutralising antibodies to HSV-1 and HSV-2 and protected mice from a subsequent lethal challenge with HSV-2 (McDermott et al., 1989). Secreted recombinant HSV-1 gB expressed in human cells was demonstrated to induce neutralising antibodies specific for HSV-1 and HSV-2 in mice, and protected animals against a lethal HSV-1 challenge (Manservigi et al., 1990). In addition, a recombinant vaccinia virus expressing HSV-1 gB was shown to induce gB-specific CTLs in mice (McLaughlin-Taylor et al., 1988). In a separate study, a mouse L cell line expressing HSV-1 gB was shown to induce CTLs in mice and virus-specific CTLs also recognised the gB expressed by this cell line (Blacklaws et al., 1987). Furthermore, immunisation of mice with this cell line provided protection from a lethal HSV-1 challenge.

The most extensive characterisation of EHV-1 gB as an

antigen for stimulating antibody production in the horse was carried out by George Allen (Equine Virology Research Foundation Grantholders Meeting, Newmarket, October 1989). His studies demonstrated that the vast majority of the antibody response in the serum of foals experimentally vaccinated with EHV-1 or EHV-4 was directed against gB. Conversely, the antibody response was directed predominantly against gC in foals vaccinated with an inactivated EHV-1 vaccine although a significant antibody response to all the other major glycoproteins (gp2, gp10 and gp22a) was also observed in these animals. During these studies a panel of 37 monoclonal antibodies to EHV-1 gB were generated, of which nine were directed against epitopes on the large subunit of the gB dimer (gp14) and 18 against epitopes on the smaller gp18 subunit. The type-common nature of the gB epitopes was demonstrated by the ability of 33 out of 37 monoclonal antibodies to recognise epitopes present on EHV-4 gB. However, only two monoclonal antibodies possessed virus neutralising activity and only two were directed against linear (continuous) epitopes. The fact that only two out of a panel of 37 monoclonal antibodies specific for EHV-1 gB could neutralise virus infectivity, taken together with the results presented in this chapter, suggests that EHV-1 gB and EHV-4 gB may not be such important targets for virus neutralising antibodies as are the gB-like proteins of other herpesviruses. It therefore seems likely that EHV-1 gB and EHV-4 gB may play more important roles in stimulating immune mechanisms other than virus neutralisation. Further support for such a role in immunity was provided by the observation that a recombinant vaccinia virus expressing EHV-1 gB was unable to stimulate the production of

virus neutralising antibodies following immunisation of mice (Bell et al., 1990). However, in a separate study EHV-1 gB expressed in vaccinia virus was successful in stimulating the production of virus neutralising antibodies in guinea pigs and in protecting hamsters from a lethal EHV-1 challenge (Guo et al., 1990). CDL of infected cells has been proposed to be important for protection in the hamster model of EHV-1 infection, since the appearance of virus-specific antibodies which could lyse infected cells in vitro in the presence of complement following EHV-1 infection resulted in the clearance of virus (Stokes et al., 1989). Such antibodies were apparent 10 days after infection of the horse with EHV-1 and correlated with the time at which virus was beginning to be cleared from the system (Stokes and Wardley, 1988). The spleen of hamsters infected with EHV-1 contained virus-specific CTLs at 6 days p.i. which could lyse EHV-1-infected cells (Stokes et al., 1989). Such a CTL response has also been observed following infection of horses with EHV-4 (Bridges and Edington, 1987). CTLs have often been regarded as forming the first line of defence following infection. A role for EHV-1 gB and EHV-4 gB in stimulating ADCC, CDL and CTL responses is consistent with similar roles reported for HSV-1 gB and BHV-1 gI. Furthermore, gB has been shown to be one of several glycoproteins recognised by equine lymphocytes following an EHV-4 infection (Bridges et al., 1988). It seems likely that an immune mechanism such as CTL activity may account for the protection afforded one hamster in the absence of virus neutralising antibodies following immunisation with pUR0.6gB (Table 5.2). The role of EHV-4 gB in cell-mediated immunity is further reinforced by the finding

that immunisation of hamsters with three EHV-4 gB synthetic peptides could stimulate the proliferation of lymphocytes in response to whole virus antigen *in vitro*.

Antigenic determinants of proteins have been classified as continuous (linear) or discontinuous (conformation-dependent). Continuous epitopes comprise local amino acids whereas discontinuous epitopes are composed of amino acids from different parts of the molecule which are brought together to form the epitope by folding of the protein into its native structure (Barlow *et al.*, 1986). One of the two continuous epitopes of EHV-1 gB recognised by EHV-1 gB-specific monoclonal antibodies has been characterised (G. Allen, EVRF Grantholders Meeting, Newmarket, October 1989). The epitope recognised by the non-neutralising monoclonal antibody 3F6 was initially localised to a 60 amino acid sequence near the amino-terminus of the molecule by determination of the sequence of the EHV-1 DNA inserts contained within a library of λgt11 recombinants that expressed the 3F6 epitope (Allen and Yeargan, 1987). By testing the ability of truncated peptides from this region to bind 3F6, the 3F6 epitope was identified as the amino acid sequence ETPDPLR between residues 116 to 122 of the EHV-1 gB protein. This epitope was found to be conserved with EHV-4 gB but not other herpesvirus gB-like proteins. This is an important protective epitope on EHV-1 gB since hamsters immunised with 3F6 were protected against a subsequent lethal EHV-1 challenge (Stokes *et al.*, 1989). This particular epitope seems especially important in EHV-4 gB since 15% of total equine antibody was specifically directed against this epitope in EHV-4-infected foals, a higher proportion than was seen in EHV-1-infected and

EHV-1-vaccinated foals (G. Allen, EVRF Grantholders Meeting, Newmarket, October 1989).

An obvious problem when expressing any protein as a fusion product with, for example, β-galactosidase in E.coli is that the conformation of the β-galactosidase moiety influences the folding of the foreign protein. Since the foreign protein is consequently not presented in its native conformation, disruption of discontinuous epitopes will inevitably occur. Furthermore, the inability of E.coli to glycosylate proteins and the limitation on the size of the foreign protein that can be expressed as a fusion product in E.coli results in altered tertiary protein structure and, concomitant with this, the loss of discontinuous epitopes. These limitations should be borne in mind when expressing proteins in E.coli. Such problems can be partially overcome by cleavage of the desired protein from the carrier protein. The pGEX vector system has been designed for this purpose. The pGEX-2T and pGEX-3X vectors possess protease cleavage sites specific for thrombin and factor X, respectively (Figure 5.2b). The GST fusion protein could be cleaved to remove the GST carrier from the desired protein and both the carrier and any uncleaved fusion protein removed by absorption on glutathione-agarose beads to leave the pure, desired protein product. Alternatively, several other expression systems are available which would permit the synthesis of proteins in their naturally glycosylated and conformational state with retention of their biological properties. These include mammalian cell expression systems (Seidel-Dugan et al., 1988; Su and Courtney, 1988; Manservigi et al., 1988), murine cell expression systems (Blacklaws et al., 1987; Fitzpatrick et al., 1988) and the use

of live virus vectors such as vaccinia virus and human adenovirus. The use of live virus vectors will be described in Chapter 7 of this thesis. The purification of EHV-4 gB from viral extracts by immunoaffinity chromatography using monoclonal antibodies is a particularly appealing approach. Despite the problems associated with protein expression in E.coli, an immunoreactive HSV-1 gD protein has been successfully synthesised using this method (Watson et al., 1982). Fusion of HSV-1 gD with β-galactosidase gave a fusion product that stimulated the production of virus neutralising antibodies to HSV-1 and HSV-2 in rabbits (Weis et al., 1983). Although prokaryotic expression systems do have limitations, they cannot be dismissed as a simple, cheap and safe method for the production of large amounts of recombinant proteins.

Synthetic peptides have recently been employed in vaccine development strategies. The rationale for this is that antigenic peptides representing surface regions of viral proteins can induce the production of specific antibodies that confer humoral immunity to that virus. Immunisation of hamsters with several EHV-4 gB peptides did not stimulate the production of virus neutralising antibodies or protect animals from a lethal EHV-1 challenge, although it induced the proliferation of lymphocytes in response to whole virus antigen in vitro. The inability of EHV-4 gB peptides to stimulate the production of virus neutralising antibodies may be attributed to both the lesser role of EHV-4 gB as a major target for virus neutralising antibodies, as predicted earlier, and the fact that the bulk of the antibodies to EHV-4 gB are probably directed against conformational epitopes. Similarly, synthetic peptides from the

MDV gB sequence raised anti-peptide antibodies in rabbits which could not neutralise virus infectivity *in vitro* (Ross *et al.*, 1989). However, synthetic peptides from the HSV-1 gD sequence have been successfully used to elicit the production of virus neutralising antibodies in rabbits and mice (Cohen *et al.*, 1984; Weijer *et al.*, 1988) and to protect mice from a lethal challenge with HSV-2 (Eisenberg *et al.*, 1985). A 23 amino acid B cell determinant from HSV-1 gD has been shown to be antigenic for proliferating T cells (Heber-Katz *et al.*, 1988) and a T cell epitope has been identified in HSV-1 gD by the use of synthetic peptides (DeFreitas *et al.*, 1985). Peptides with predicted amphipathic properties (Margalit *et al.*, 1987) from the VZV gII and gIV sequences were demonstrated to elicit T cell responses as measured by proliferation and cytotoxicity assays (Hayward, 1990). The algorithm of Margalit *et al.* (1987) therefore successfully predicted T cell epitopes on the basis of a potential amphipathic helical structure in this case. Other structures can also elicit T cell responses since the peptide containing the T cell epitope identified for HSV-1 gD by DeFreitas *et al.* (1985) was not particularly amphipathic but possessed hydrophobic residues which contribute to an alternative structural model proposed for a T cell antigenic site (Rothbard *et al.*, 1988). Such structural models should prove useful in identifying T cell epitopes on EHV-4 gB.

The construction of an antigenic map of EHV-4 gB is a prerequisite for fully understanding the contribution it makes in immunity. Our knowledge of the HSV-1 gB protein has permitted the construction of such a map to be initiated for this protein. The most widely employed approach to map functional epitopes has

been by analysis of several mutants resistant to potent neutralising antibodies (mar mutants) (Kousoulas et al., 1984, 1988; Pellett et al., 1985b). These studies demonstrated that epitopes clustered in two distinct regions of the surface domain of the HSV-1 gB molecule. Another approach involved the construction of truncated derivatives, internal deletion mutants and oligopeptides of HSV-1 gB to map epitopes for a panel of monoclonal antibodies to HSV-1 gB (Pereira et al., 1989). Using this strategy, seven neutralising epitopes were localised to a segment of continuous residues between positions 1 and 47 of the molecule, five of which were specific for HSV-1 but not HSV-2. A further eleven neutralising epitopes mapped to a discontinuous domain composed of residues from the amino-terminal half of the molecule, of which four (between residues 273 and 298) corresponded to sites recognised by neutralising antibodies used in mapping studies with mar mutants (Kousoulas et al., 1984, 1988; Pellett et al., 1985b). Comparison of the reactivity of parental HSV-1 gB and a recombinant HSV containing a HSV-1/HSV-2 gB hybrid with type-specific monoclonal antibodies identified a subset of type-specific epitopes mapping in the amino-terminal half of the HSV-1 gB molecule (Kousoulas et al., 1989).

The mapping of epitopes on HCMV gB has also progressed. By testing the reactivity of truncated derivatives of the HCMV gB molecule expressed in COS cells and in a CHO cell line with a panel of neutralising antibodies, neutralising epitopes were demonstrated to map in at least two domains located in a discontinuous region of amino acids from residues 461 to 680 of the molecule (Banks et al., 1989). Two linear epitopes have been mapped on HCMV gB, one for a neutralising and the other for a

non-neutralising monoclonal antibody, by expressing overlapping fragments of the carboxyl-terminal part of the molecule in E.coli as β-galactosidase fusion products and testing their reactivity with these monoclonal antibodies; both antibodies recognised sequences between residues 608 and 625 of the molecule (Utz et al., 1989). It was also found that the non-neutralising monoclonal antibody could inhibit the neutralising activity of the other monoclonal antibody, which led the authors to suggest that future vaccine preparations would be less detrimental to the host if they were to include only the immunogenic regions eliciting protective responses and not entire virion glycoprotein components. In this way, the possibility of antibody inhibition of neutralising antibodies to that virion component would be minimised.

Although a panel of monoclonal antibodies to EHV-1 gB have been generated, there is a great need for the generation of monoclonal antibodies to EHV-4 gB if accurate epitope mapping of the molecule is to be accomplished using approaches such as those described for HSV-1 gB and HCMV gB. In the meantime, prediction of the best antigenic regions of the molecule can be accomplished by using quantities such as protein hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982), mobility (Westhof et al., 1984), antibody accessibility (Novotny et al., 1986) and protrusion (Thornton et al., 1986).

The accuracy of predictive algorithms is well proven. The prediction of amino acid residues likely to be exposed on the molecule surface using the Surfaceplot algorithm (Parker et al., 1986) permitted the synthesis of twelve peptides, containing thirteen predicted surface sites, from the HSV-1 gD sequence

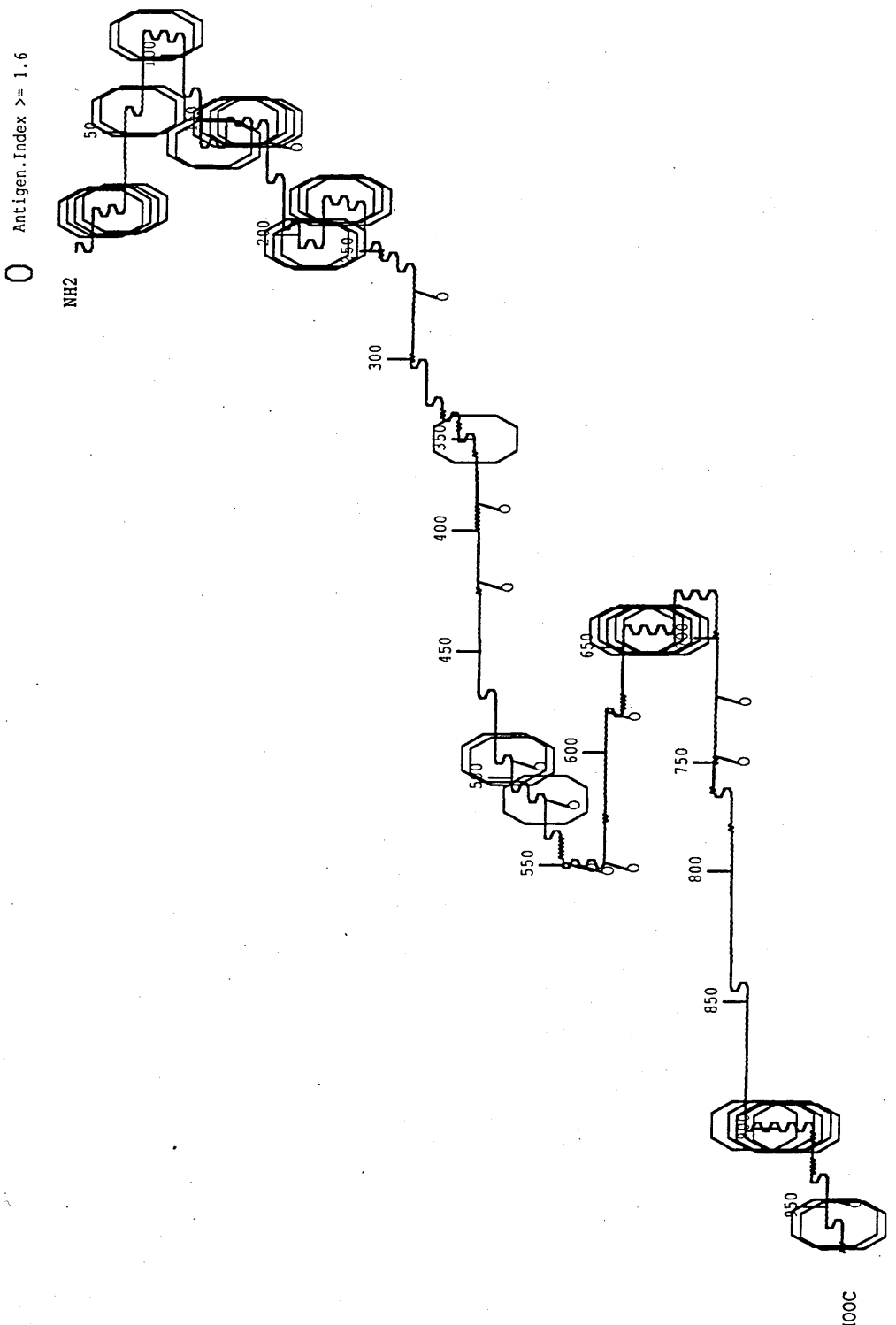
which all produced positive immune responses in rabbits; eight of the peptides produced anti-peptide antibodies that reacted with intact virions, which suggested the presence of the peptide sequences on the surface of the gD molecule in the virion, and five antisera contained virus neutralising activity (Strynadka et al., 1988). It is noteworthy that seven of the predicted surface sites were not predicted by the parameters of Hopp and Woods (1981), of which three generated virus neutralising antibodies and all produced anti-peptide antibodies that bound to HSV-1 virions.

A predicted antigenic map of the EHV-4 gB protein is shown in Figure 5.9. Peptides corresponding to some of these predicted antigenic regions could be synthesised and further evaluation of their immunogenicity carried out in hamsters. Ultimately, important epitopes of the EHV-4 gB molecule could be characterised by the Pepscan method (Geysen et al., 1984, 1985) using horse neutralising antiserum or, when available, neutralising monoclonal antibodies to EHV-4 gB.

Figure 5.9 Prediction of the most antigenic sites of the EHV-4 gB protein. Antigenic sites were predicted by the method of Jameson and Wolf (1988) using PEPTIDESTRUCTURE and PLOTSTRUCTURE from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984). Octagons represent sites with an antigenic index of at least 1.6. Predicted antigenic sites are superimposed on a two-dimensional plot representing secondary structure predicted by the method of Chou and Fasman (1978). Potential N-linked glycosylation sites are denoted by ball-and-stem structures.

F I G U R E 5.9

Predicted Antigenic Map of EHV-4 gB



CHAPTER 6

DNA Sequence of the EHV-4 Genome Between 0.067 and 0.122 Map Units and Analysis of the Predicted Gene Products

INTRODUCTION

The genetic relatedness of several alphaherpesviruses has been established by the utilisation of molecular hybridisation techniques. Using this approach the genomes of HSV-1 and HSV-2 were shown to share a directly colinear arrangement and those of EHV-1 and VZV demonstrated colinearity with the I_L/I_{SL} arrangement of HSV (Davison and Wilkie, 1983). The genome of PRV has also been shown to be grossly colinear with the I_L/I_{SL} arrangement of HSV with the exception of a region between 0.07 and 0.39 m.u. which appears to be inverted relative to HSV (Ben-Porat et al., 1983; Davison and Wilkie, 1983). The most highly conserved genes were those encoding the major DNA binding protein, major capsid protein, DNA polymerase, immediate-early protein V_{mw}IE175 and one or both of the two subunits of ribonucleotide reductase. However, the close linear relationship shared by these herpesvirus genomes applied only to the L segment; the only detectable homology in the S segment was in IR_S/TR_S and was due to the presence of the highly conserved V_{mw}IE175 gene. The S segment was thus demonstrated to be the most heterologous region of the alphaherpesvirus genomes.

The first alphaherpesvirus genome for which the complete DNA sequence was elucidated was that of VZV (Davison and Scott, 1986a). Comparison of amino acid sequences of the predicted gene products with those available for HSV-1 permitted the establishment of a clearly defined genetic relationship between these viruses. These analyses confirmed the highly conserved nature of the genes reported by Davison and Wilkie (1983) and demonstrated that many other genes were located in similar

genomic positions and that their genomes were generally colinear, at least within U_L.

However, gene conservation is not limited to the alphaherpesvirus genomes. The available DNA sequence of the genome of the gammaherpesvirus EBV (Baer et al., 1984) permitted the identification of several genes whose products had counterparts in HSV-1. These included the genes encoding the major DNA binding protein and DNA polymerase (Quinn and McGeoch, 1985), major capsid protein (Davison and Scott, 1986b), ribonucleotide reductase (Gibson et al., 1984), exonuclease (McGeoch et al., 1986b), glycoproteins gB (Pellett et al., 1985b) and gH (McGeoch and Davison, 1986), thymidine kinase (Honess et al., 1989), homologues of HSV-1 genes UL5 and UL52 which are involved in DNA replication (McGeoch et al., 1988b), and another gene of unknown function comprising a spliced mRNA (Costa et al., 1985). In a similar vein, investigations into the genetic relatedness of VZV and EBV identified EBV counterparts to 29 of the 67 genes specified by VZV (Davison and Taylor, 1987). Conserved genes were grossly colinear and detected only within the U_L region of each genome. These included the genes of known function conserved between HSV-1 and EBV as described above and also homologues of the virus-specified enzyme deoxyuridine-5'-triphosphate nucleotidohydrolase (dUTPase) (Preston and Fisher, 1984) and the immediate-early protein IE63 (Sacks et al., 1985; Perry and McGeoch, 1988).

In contrast, very little is known regarding gene organisation in the genomes of EHV-1 and EHV-4. Their genomes are directly colinear with each other and with the I_L/I_{SL} arrangement of the HSV genomes (Davison and Wilkie, 1983;

Cullinane et al., 1988). Insufficient sequence data is available for EHV-1 and EHV-4 to enable precise comparisons to be made regarding genetic structure with the genomes of HSV-1, VZV and EBV. The only data available for EHV-4, apart from the DNA sequence of the gB gene as presented in Chapter 4 of this thesis, is that of the BamHI-L genomic fragment (Cullinane et al., 1988). This fragment spans the junction between U_S and TR_S and contains four ORFs, all of which have counterparts in HSV-1 and VZV. The gene products of ORFs 1, 2, 3 and 4 were demonstrated to be homologous to those of HSV-1 US8 (gE)/VZV gene 68 (gpI), HSV-1 US9/VZV gene 65, HSV-1 US10/VZV gene 64 and HSV-1 US1/VZV gene 63, respectively. The arrangement of the EHV-4 genes was intermediate between that observed for HSV-1 and VZV; ORFs 1 and 2 were similar in genomic location and orientation to their HSV-1 counterparts whereas ORFs 3 and 4 more closely resembled their VZV counterparts in these respects.

Derivation of a precise genetic relationship between EHV-4 and other herpesviruses awaits determination of the DNA sequence of its genome. The studies presented in this chapter represent the first step of such an objective. Gene arrangement in a selected region of the U_L region of the EHV-4 genome was investigated and compared with the gene arrangement in the corresponding region of the HSV-1 and VZV genomes and their gene products analysed. The genomic region to be studied was selected in light of the studies of Allen and Yeargan (1987) who mapped the genes for the six major glycoproteins of EHV-1 (Figure 3.18). The genomic location within the U_L region of three of these genes indicated that EHV-1 specifies three glycoprotein species (gp2, gp10 and gp21/22a) for which no genetic homologues

had been detected in other herpesviruses. These regions of the EHV-1 genome are prime candidates for containing a gene arrangement divergent from that seen in HSV-1 and VZV. The genomes of EHV-1 and EHV-4 are colinear and therefore likely to share a similar genetic organisation. Furthermore, the structural protein profiles of EHV-1 and EHV-4 virions clearly show that both viruses specify the gp2, gp10 and gp21/22a species (Table 1.2). Taken together, these findings clearly suggest that EHV-4 will also specify these glycoprotein species and that their genes would be expected to map in a similar position to their EHV-1 counterparts.

It was decided to investigate the gene arrangement around the predicted genetic locus for gp10, an epitope of which mapped to between 0.093 and 0.114 m.u. on the EHV-1 genome (Allen and Yeargan, 1987). The corresponding region of the EHV-4 genome was identified by virtue of colinearity and analysed by DNA sequencing. Additional DNA sequence data for EHV-4 BamHI-Q is also presented in this chapter.

MATERIALS AND METHODS

Subcloning of EHV-4 DNA

The plasmid containing the 14.1kb BamHI-B genomic fragment cloned in pUC9 was digested with SmaI and the two fragments generated (9.7kb and 7.1kb) were separated by electrophoresis. The 7.1kb fragment was purified as previously described and self-ligated to generate the recombinant plasmid pUC4S, which contained the rightmost 4.4kb of BamHI-B in the form of a BamHI/SmaI fragment attached to pUC9 sequences. The plasmid containing the 9.5kb BamHI-G genomic fragment cloned in pUC9 was digested with SalI and the resulting 1.9kb SalI fragment (which contained the 1.9kb BamHI/SalI left-terminal subfragment of BamHI-G), and the adjacent 0.9kb SalI subfragment of BamHI-G, were separately cloned into the SalI site of plasmid vectors Bluescript M13+ and pUC8, respectively. The recombinant plasmids generated were named pBS1.9G and pUC0.9G, respectively.

DNA Sequencing

The EHV-4 DNA inserts in plasmids pUC4S, pBS1.9G and pUC0.9G were sequenced by the dideoxy chain termination method using the double-stranded DNA method for template preparation as described in Chapter 4 and both commercially available and custom made primers. The DNA sequences of EHV-4 BamHI-Q and the left-terminal region of EHV-4 BamHI-M were determined by sequencing of the plasmids containing these genomic fragments as described above. All computer analyses of DNA sequences were performed as described in Chapter 4.

Purification of Recombinant λgt11 Bacteriophage

A single colony of E.coli Y1090 was inoculated into 100ml of NZCYM medium (Table 2.1) containing ampicillin at 100ug/ml and 0.2% maltose, and grown overnight at 37°C with vigorous shaking in an orbital incubator. Cells were collected by centrifugation (2000rpm/10min) and suspended in an equal volume of 10mM $MgSO_4$ to give an OD (600nm) reading of about 0.5. This suspension was kept on ice until required and used the same day. 10^8 plaque forming units (10ul) of recombinant λgt11 bacteriophage carrying a putative EHV-1 gp10 DNA insert (kindly provided by Professor G. Allen, University of Kentucky, USA) was mixed with 10^{10} pelleted E.coli Y1090 cells, prepared as described above (OD of 0.5 at 600nm = $4x10^8$ cells/ml; 25ml of E.coli Y1090 represents 10^{10} cells), in 3ml of SM (Table 2.1). The mixture was incubated at 37°C for 20min to allow bacteria to absorb bacteriophage particles and then used to infect 500ml of NZCYM medium containing 100ug/ml ampicillin and 0.2% maltose. This was incubated overnight at 37°C with vigorous shaking until complete lysis of bacteria had occurred, which was evident from the considerable amount of bacterial cell debris in the culture. Following chilling of the lysed culture to room temperature, pancreatic DNase and RNase (Table 2.1) were each added to a final concentration of 1ug/ml and the culture incubated at room temperature for 30min. Solid sodium chloride was added to a final concentration of 1M (29.2g/500ml), dissolved by gentle mixing and incubated on ice for 1hr. Debris was removed by centrifugation (7000rpm/10min/4°C) in a Beckman JS7.5 rotor. Solid PEG-6000 was added to a final concentration of 10% (50g) and dissolved by stirring. The solution was left to stand on ice

for 1hr and the precipitated bacteriophage particles were collected by centrifugation (7000rpm/10min/4^{o}C) in a Beckman JS7.5 rotor. The bacteriophage pellet was resuspended in 8ml of SM. An equal volume of chloroform was added to the bacteriophage suspension, mixed thoroughly and the phases separated by centrifugation (2000rpm/5min) in a benchtop centrifuge. Caesium chloride (0.75g/ml) was added to the upper aqueous phase containing the bacteriophage and dissolved by mixing. The contents were transferred to a 12ml Beckman 'Quick-Seal' centrifuge tube, sealed and bacteriophage particles banded by centrifugation (40000rpm/24hr/4^{o}C) in a Beckman 50Ti rotor. The bacteriophage particles, visible as a bluish-grey band, were collected by puncturing the side of the tube with a 19-gauge needle and withdrawal into a 2ml syringe. Bacteriophage particles were dialysed for 2 x 1hr at room temperature against 2 litres of 10mM NaCl/50mM TrisHCl pH8.0/10mM $MgCl_2$ to remove caesium chloride.

Extraction of Recombinant Bacteriophage DNA

To the dialysed bacteriophage suspension were added EDTA, proteinase K and SDS (Table 2.1) to final concentrations of 20mM, 50ug/ml and 0.5%, respectively. The contents were mixed by gentle inversion, incubated at 65^{o}C for 1hr and extracted once with phenol, once with phenol/chloroform and once with chloroform as previously described. The aqueous phase was dialysed for 3 x 2hr against 2 litres of TE and bacteriophage DNA precipitated with two volumes of ethanol in the presence of 0.3M sodium acetate (pH 6.0) at -70^{o}C for 30min. DNA was pelleted by centrifugation (13000rpm/10min) in a benchtop

microcentrifuge, washed with 70% ethanol, dried in a vacuum dessicator and resuspended in TE. Purity of the DNA was determined by gel electrophoresis as previously described. Over 1mg of bacteriophage DNA was obtained.

Subcloning of Recombinant Bacteriophage DNA Containing EHV-1 Sequences

Purified recombinant λgt11 bacteriophage DNA was double-digested with KpnI and XbaI and a resulting 6.5kb fragment (containing the EHV-1 DNA insert in the EcoRI site) purified and cloned between the KpnI and XbaI sites of Bluescript M13+ using standard techniques. The EHV-1 DNA insert was sequenced using a λgt11-specific primer close to the EcoRI site as previously described.

RESULTS

Structure of Recombinant Plasmids

The structure of the recombinant plasmids pUC4S, pBS1.9G and pUC0.9G, which contain the rightmost 4.4kb BamHI/SmaI subfragment of BamHI-B, the leftmost 1.9kb BamHI/SalI and adjacent 0.9kb SalI subfragments of BamHI-G, respectively, is shown in Figure 6.1. Their structure was confirmed by restriction endonuclease analysis (Figure 6.2).

Determination of the EHV-4 DNA Sequence

Sequencing of the DNA inserts in the above plasmids was initiated using commercially available primers (M13, Reverse, T7, KS) and then completed using custom made primers to rapidly generate sequence data for both strands. Each nucleotide was sequenced at least three times until all ambiguities were removed. Sequencing was extended for a further 0.6kb downstream of the subcloned region of BamHI-G by priming on intact BamHI-G using custom made primers. The data were compiled and analysed as previously described. An autoradiograph of one of the sequencing gels for this region is shown in Figure 6.3. The region sequenced consisted of 7868 nucleotides, extending rightwards from a few nucleotides downstream of the SmaI site in BamHI-B to a region approximately 3.4kb downstream of the left terminus of BamHI-G (Figure 6.4).

Analysis of the EHV-4 DNA Sequence

Sequence data was analysed for ORFs by identifying potential ATG initiation codons (Kozak, 1984, 1986) and

polyadenylation signals (Berget, 1984). The ORFs identified were designated B1 to B7 and their arrangement and genomic location are shown in Figure 6.4. The complete DNA sequence of this region, the gene layout and the amino acid sequence of the predicted gene products are shown in Figure 6.5, and a summary of the salient features of these genes is presented in Table 6.1. In the analyses which follow, the DNA sequences of the VZV genome (Davison and Scott, 1986a) and the U_L region of the HSV-1 genome (McGeoch et al., 1988a) are the reference sources against which the EHV-4 sequence is compared.

The sequences surrounding each predicted initiation codon suggest that they could all function as efficient initiation sites, conforming well with the optimal sequence ACCATGG identified for translation initiation (Kozak, 1984, 1986). The initiation codon for ORF B1 was identified at nucleotide 592 and this ORF extends back to nucleotide 1 in a leftward direction. This partial ORF is analogous to HSV-1 UL52/VZV gene 6. ORF B2 extends for 732 nucleotides from nucleotide 591 to the termination codon at nucleotide 1323 in a rightward direction, with a polyadenylation signal at nucleotide 1338. The initiation codon for this gene overlaps with the initiation codon of ORF B1 on the opposite strand. A second in-frame ATG in ORF B2 occurs at nucleotide 606, but the sequences surrounding it suggest that it could not function as an efficient initiation site. ORF B2 is analogous and similar in size to HSV-1 UL51 (732 nucleotides) and VZV gene 7 (777 nucleotides). ORF B3 is 978 nucleotides in length and extends leftwards from nucleotide 2394 to the termination codon at nucleotide 1416, with a polyadenylation signal at nucleotide 1375. This ORF is analogous to HSV-1

UL50/VZV gene 8, although it is significantly smaller than HSV-1 UL50 (1113 nucleotides) and VZV gene 8 (1188 nucleotides). The remaining ORFs in the EHV-4 sequence extend in a rightward direction. ORF B4 comprises 870 nucleotides, initiating at nucleotide 2860 and terminating at nucleotide 3730, 36 nucleotides downstream of which lies a polyadenylation signal. This ORF is analogous and similar in size to HSV-1 UL49 (903 nucleotides) and VZV gene 9 (906 nucleotides). ORF B5 extends from nucleotide 3845, continues into BamHI-G and would appear to terminate at nucleotide 4514. However, this would yield an ORF much smaller than the analogous gene in HSV-1 (UL48, 1470 nucleotides) and VZV (gene 10, 1230 nucleotides). A feasible explanation for this is that a small BamHI fragment (0.6 to 0.8kb) has been omitted from the original restriction map of the EHV-4 genome between BamHI-B and BamHI-G (Figure 1.3). The most likely termination codon for this ORF is at nucleotide 4620, six nucleotides prior to a polyadenylation signal. Furthermore, an ORF extends back to the start of BamHI-G from this termination codon, and the predicted amino acid sequence of this region is indicated in brackets in Figure 6.5. B6 is the largest ORF in the EHV-4 sequence presented here, initiating at nucleotide 4993 and terminating at nucleotide 7609. This 2616 nucleotide ORF is considerably larger than the corresponding ORF in HSV-1 (UL47, 2079 nucleotides) and VZV (gene 11, 2457 nucleotides). The likely initiation codon of ORF B7, the EHV-4 homologue of HSV-1 UL46 and VZV gene 12, is at nucleotide 7734, although the sequence has only been extended for 135 nucleotides. Since no polyadenylation signal is present for ORF B6 in the region sequenced, it seems probable that the 3' end of its mRNA is

coterminal with that of ORF B7, an arrangement that has also been reported for the corresponding genes of HSV-1 (McKnight et al., 1987).

It is difficult to assign transcriptional regulatory elements to EHV-4 genes B1 to B7 shown in Figure 6.5 with any certainty on the basis of DNA sequence data alone. The sequence TATAAAA at nucleotide 2712, 148 nucleotides prior to the initiation codon, could tentatively be assigned as the TATA box for ORF B4, with a putative mRNA initiation site at nucleotide 2745 within the sequence TCATTC. The analogous VZV gene 9 also has the potential TATA box TATAAAA in a similar position, 92 nucleotides prior to the initiation codon. The sequence TATAAAA at nucleotide 4882, 111 nucleotides prior to the initiation codon, could be the functional TATA box for ORF B6, with a putative mRNA initiation site at nucleotide 4922 within the sequence CTATCTA. The TATAAAA sequence occurs in the analogous VZV gene 11, 89 nucleotides prior to the initiation codon, and also in the HSV-1 homologue UL47, 223 nucleotides prior to the initiation codon. Additionally, the variant sequence TATAACA at nucleotide 473 may represent the TATA box of ORF B2.

The ORFs are compactly arranged in the EHV-4 sequence, the largest non-coding region being 465 nucleotides long and located between ORFs B3 and B4. The corresponding genes in HSV-1 and VZV show a similar compact arrangement. An overall G+C content of 49% is reported for the EHV-4 sequence, a value similar to the 47% G+C content of the genomic region encoding the glycoprotein gB gene of EHV-4.

These EHV-4 sequence data demonstrate that the EHV-4 genome has an identical gene arrangement between 0.067 and 0.122 m.u.

as the HSV-1 (I_L) and VZV genomes.

Analysis of Amino Acid Sequences

The amino acid sequences of the predicted gene products of EHV-4 ORFs B1 to B7 were analysed and compared with the corresponding gene products of HSV-1 and VZV. Analyses employed were protein alignment, hydropathic analysis and dot matrix similarity comparisons, all of which were carried out as described in Chapter 4. Percentage amino acid homology values for pairwise comparisons of analogous gene products of EHV-4, HSV-1 and VZV are shown in Table 6.2.

i) EHV-4 B1/HSV-1 UL52/VZV Gene 6

Of the 197 amino acids available for analysis for EHV-4 B1, 42 residues are shared by all three proteins over a total length of 220 residues (Figure 6.6a). This is indicative of a moderate level of conservation of this protein in all three viruses. The HSV-1 and VZV proteins show a 37% amino acid identity over their entire length and the HSV-1 protein is one of several proteins identified as being required for the replication of viral DNA (McGeoch et al., 1988b; Wu et al., 1988). A homologous protein is also specified by EBV BSLF1, which accentuates the important conserved role of these species in the DNA replication process.

ii) EHV-4 B2/HSV-1 UL51/VZV Gene 7

EHV-4 B2 is predicted to be 244 amino acids long with a molecular mass of 26.2kDa, which compares favourably with the predicted sizes of the homologous proteins of HSV-1 (244 amino acids; 25.5kDa) and VZV (259 amino acids; 28.2kDa). The codon usage and amino acid composition of EHV-4 B2 are shown in Table 6.3. Alignment of EHV-4 B2 with the analogous proteins of HSV-1

and VZV reveals that they are quite well conserved with 67 of the amino acids being shared by all three proteins over a total length of 262 residues, corresponding to an overall identity of 26% (Figure 6.7). The amino-terminal portion is the best conserved region, with 32 residues being identically matched in all three proteins in a region of 55 amino acids corresponding to residues 36 to 90 of EHV-4 B2. The centre of the molecules show moderate conservation of amino acid sequence, with 36% of the residues being shared in a region stretching from residues 91 to 164 in EHV-4 B2. Of the remaining amino acids towards the carboxyl-terminus of the molecules, only one is shared by all three proteins. Thus, an important and conserved function can be predicted for the amino-terminal region of the molecules whilst the carboxyl-terminal region may be divergent in function. Most highly conserved regions include the sequences TRRLV (residues 64 to 68) and LARTY (residues 73 to 77) in the EHV-4 protein which are totally conserved with the HSV-1 and VZV proteins. The quite high amino acid homology between EHV-4 B2 and the analogous proteins of HSV-1 and VZV is summarised in dot matrix similarity comparisons (Figure 6.8). The hydropathic profiles of these proteins are very similar and show that the proteins do not possess extreme properties, although their carboxyl-terminal regions are generally hydrophilic (Figure 6.9). However, the function of these proteins is unknown.

iii) EHV-4 B3/HSV-1 UL50/VZV Gene 8

The codon usage and amino acid composition of EHV-4 B3 are shown in Table 6.4, and an alignment of this protein with its HSV-1 and VZV homologues is shown in Figure 6.10. EHV-4 B3 is 326 amino acids long with a predicted molecular mass of 35.2kDa,

which is somewhat smaller than the sizes predicted for the analogous proteins of HSV-1 (371 amino acids; 39.1kDa) and VZV (396 amino acids; 44.8kDa). The proteins possess highly divergent amino acid sequences, with only 49 amino acids being shared by all three proteins over a total length of 453 residues (11% identity). Generally, the amino acid homology is patchy and distributed over the whole length of the molecules. The best conserved region is towards the carboxyl-terminus where 39 amino acids are shared by all three proteins in a region corresponding to the carboxyl-terminal 155 amino acids of the EHV-4 protein. In particular, the sequences GFGST (residues 320 to 324) and GRSS (residues 217 to 220) in the EHV-4 protein are shared by all three proteins. The central region of the molecules are most divergent, with the VZV protein containing multiple insertions relative to the EHV-4 and HSV-1 proteins between residues 169 and 229. In a similar vein, the HSV-1 protein contains a 15 amino acid insertion between residues 96 and 110. The dot matrix similarity analysis in Figure 6.11 demonstrates the generally low level of amino acid conservation between these proteins. The hydropathic profiles of these proteins demonstrate that they possess no extreme features (Figure 6.12). However, a very hydrophilic peak is observed in their hydropathic profiles, which is indicated by a shaded box. These regions correspond to amino acid residues 171 to 181, 212 to 222 and 231 to 241 of the EHV-4, HSV-1 and VZV proteins, respectively, which are particularly well conserved between the proteins (Figure 6.10). Additionally, the HSV-1 protein contains a very hydrophilic short stretch of amino acids (residues 181 to 187) of which four residues are arginine. The gene product of HSV-1 UL50, first

identified by Preston and Fisher (1984), is known to be the enzyme dUTPase which has an important role in viral DNA metabolism. dUTPase catalyses the hydrolysis of dUTP to dUMP and pyrophosphate and serves to provide the cell with a supply of dUMP which can be converted to TMP by thymidylate synthetase, and also serves to reduce the intracellular concentration of dUTP (Preston and Fisher, 1984).

iv) EHV-4 B4/HSV-1 UL49/VZV Gene 9

The EHV-4 B4 protein is predicted to be 290 amino acids long (31.5kDa), which compares favourably with the predicted size of the analogous proteins of HSV-1 (301 amino acids; 32.2kDa) and VZV (302 amino acids; 32.2kDa). The codon usage and amino acid composition of the EHV-4 B4 protein are shown in Table 6.5. The alignment of these proteins (Figure 6.13) reveals that 40 amino acids are shared by all three proteins over a total length of 344 residues. The amino-terminal half of the molecules display the greatest level of divergence, with limited and patchy amino acid conservation. Conversely, the carboxyl-terminal half of the molecules is the best conserved region and a stretch of 79 amino acids between residues 165 and 243 of EHV-4 B4 shares 36% of its residues with both the HSV-1 and VZV proteins. In particular, the sequence FNKNVFCAAV at residues 183 to 192 of the EHV-4 protein shares nine out of ten residues with the HSV-1 protein and eight out of ten residues with the VZV protein. The extent of amino acid homology between EHV-4 B4 and its HSV-1 and VZV homologues is summarised by means of dot matrix similarity comparisons in Figure 6.14. The hydropathic profiles of the proteins demonstrate their hydrophilic nature (Figure 6.15). Analysis of the amino acid composition of EHV-4

B4 (Table 6.5) shows that the protein is highly charged, with the acidic residues Asp and Glu accounting for 10% of the total amino acids. Strongly basic amino acids, Arg and Lys, further account for 13% of the total amino acids, giving the protein a net basic character. The HSV-1 and VZV proteins show a very similar composition of acidic and basic residues, with acidic/basic compositions of 10%/14% and 12%/14%, respectively. The function of these proteins is still unknown.

v) EHV-4 B5/HSV-1 UL48/VZV Gene 10

An alignment of the predicted gene products of EHV-4 B5, HSV-1 UL48 and VZV gene 10 is shown in Figure 6.16. Only the first 211 amino acids of the EHV-4 protein were available for comparison and the complete HSV-1 and VZV protein sequences are shown. Alignment of the sequences shows that 43 amino acids are totally conserved between the three proteins over a total length of 219 residues in the amino-terminal half of the molecules. Conserved amino acids are distributed over this region in small blocks. The hydrophilic sequence ELRAREE at residues 175 to 181 of the EHV-4 protein is perfectly conserved with residues 160 to 166 of the HSV-1 protein and residues 164 to 170 of the VZV protein. Similarly, the sequence TWNEDLFS at residues 83 to 90 of the HSV-1 protein aligns with identical residues at position 88 to 95 in the VZV protein and shares six of these residues with the sequence KWNEDMFS at residues 94 to 101 in the EHV-4 protein. Since homology is evenly distributed in small patches no larger than five amino acids over the remaining length of the HSV-1 and VZV proteins, it seems likely that these two well conserved regions may have an important role in protein function. Dot matrix similarity comparisons of the amino-

terminal 211 amino acids of the EHV-4 B5 protein with the corresponding region of the HSV-1 and VZV proteins are shown in Figure 6.17 and serve to illustrate the well conserved nature of the amino acid sequences in this region. The gene product of HSV-1 UL48 has a role in the transactivation of IE genes (Campbell et al., 1984; Pellett et al., 1985c). Analysis of the gene product of HSV-1 UL48, also known as the α-trans-inducing factor (α-TIF), has revealed that it is highly charged, consisting of large proportions of acidic (14%) and basic (9%) residues (Dalrymple et al., 1985). The amino acid composition of the amino-terminal half of the EHV-4 B5 protein (Table 6.6) identifies it as also being of highly charged and acidic character, with acidic and basic amino acid contents of 15% and 7%, respectively. This compares well with acidic/basic amino acid compositions of 12%/8% and 15%/8% for the corresponding region of the HSV-1 and VZV proteins, respectively. In common with the relatively high leucine content reported for the HSV-1 α-TIF protein (Dalrymple et al., 1985), this amino acid is the most abundant residue in the region of the EHV-4 B5 protein analysed (Table 6.6). This suggests that these proteins may have a well defined and conserved secondary structure. The hydropathic profiles of the proteins (Figure 6.18) demonstrate that they do not possess extreme features. The peak corresponding to the strongly hydrophilic sequence ELRAREE conserved in all three proteins is indicated by a shaded box in each hydropathic profile.

vi) EHV-4 B6/HSV-1 UL47/VZV Gene 11

The EHV-4 B6 gene product is predicted to be 872 amino acids long with a molecular mass of 97.4kDa, which is

significantly larger than the analogous proteins of HSV-1 (693 amino acids; 73.8kDa) and VZV (819 amino acids; 91.8kDa). It is also the least well conserved of the proteins analysed in this chapter (Table 6.2). The codon usage and amino acid composition of the EHV-4 B6 protein are shown in Table 6.7 and an alignment of the proteins encoded by EHV-4 B6, HSV-1 UL47 and VZV gene 11 is presented in Figure 6.19. Only 66 amino acids are shared by all three proteins over a total length of 920 residues, emphasising the highly divergent nature of these species. A significant point, however, is that the EHV-4 B6 protein is more closely related to its VZV homologue (27% homology) than to its HSV-1 homologue (17% homology), whilst the HSV-1 and VZV proteins share only 18% of their sequences (Table 6.2). Comparison of the EHV-4 protein with its HSV-1 and VZV counterparts by the dot matrix method (Figure 6.20) clearly shows that the carboxyl-terminal half of these proteins is better conserved than the amino-terminal half, which exhibits particular divergence of amino acid sequence. Despite their extensive divergence of amino acid sequence, the proteins still share some similar features. Their hydropathic profiles display a conserved, strongly hydrophilic region corresponding to about the first 200 amino acids of each protein (Figure 6.21). This region is highly charged and acidic in character in each protein. In the case of the EHV-4 protein, 30% of the amino acids in this region are charged and compares with a value of 21% for the remainder of the molecule. A similar distribution of charged residues is predicted for the HSV-1 protein (36% and 19%) and the VZV protein (40% and 19%). Of particular note is the clustering of glutamic acid residues in the amino-terminal

region of the EHV-4 and VZV proteins. A region between residues 111 and 201 of the EHV-4 protein contains 26 glutamic acid residues and the corresponding region of the VZV protein contains 40 glutamic acid residues. In contrast, the HSV-1 protein contains only 10 glutamic acid residues in this region. The HSV-1 protein is unique in possessing an arginine-rich domain (residues 63 to 75) in which nine out of thirteen residues are arginine. The VZV protein contains multiple reiterations of the sequences DAIDDE and GEAEE between residues 117 and 217. No such reiterated sequences occur in the EHV-4 and HSV-1 proteins. Overall, acidic residues account for 14.2%, and basic residues for 9.4%, of the total amino acids in the EHV-4 protein. The VZV protein is even more acidic with an acidic/basic amino acid composition of 17.5%/7.3%. In contrast, the HSV-1 protein shows no overall acidic character (11.1% acidic, 12.4% basic residues). The HSV-1 protein has a high alanine (16.2%) and arginine (11.5%) content compared to the EHV-4 (alanine 8.8%, arginine 7.3%) and VZV (alanine 9.3%, arginine 5.7%) proteins. The gene product of HSV-1 UL47 has been shown to reduce the α-TIF-dependent induction of IE gene expression (McKnight et al., 1987).

vii) EHV-4 B7/HSV-1 UL46/VZV Gene 12

Although little data is presented for the EHV-4 B7 protein, alignment of the amino-terminal residues of the three proteins clearly shows that EHV-4 ORF B7 does indeed encode a protein with detectable homology to the gene products of HSV-1 UL46 and VZV gene 12, with 14 residues being shared by all three proteins over a total length of 76 residues (Figure 6.6b). Of particular note is a block of amino acids GCLLPTP at residues 24 to 30 in

the EHV-4 protein which is perfectly conserved with residues 38 to 44 of the HSV-1 protein and with six of seven residues in the VZV protein. The gene product of HSV-1 UL46 has been shown to increase the α-TIF-dependent activation of IE gene expression (McKnight et al., 1987).

Characterisation of the EHV-1 gp10 Epitope Sequence Contained Within a Recombinant λgt11 Bacteriophage

Since DNA sequence analysis of the EHV-4 genome between 0.067 and 0.122 m.u., a region to which the EHV-4 gp10 gene was expected to map, demonstrated that none of the genes in this region encode a species with features typical of envelope glycoproteins ie. a hydrophobic signal sequence at the amino terminus and a hydrophobic transmembrane domain towards the carboxyl terminus, it was necessary to profer an explanation for these somewhat unexpected data. This was achieved by determining the DNA sequence of the EHV-1 insert in the recombinant bacteriophage expressing a gp10 epitope. The DNA sequence of the EHV-1 insert and the amino acids encoded by the partial ORF it specifies are shown in Figure 6.22a. The EHV-1 gp10 sequence is actually part of an ORF encoding the EHV-1 protein analogous to the gene products of HSV-1 UL47/VZV gene 11/EHV-4 B6, and an alignment of the 71 amino acids available for the EHV-1 protein with the corresponding amino acids of the protein specified by EHV-4 ORF B6 is presented in Figure 6.22b. The two sequences share amino acid homology of 75%. The map position of EHV-4 ORF B6 corresponds to the region to which Allen and Yeargan (1987) mapped the EHV-1 gp10 gene. It is concluded that ORF B6 is the EHV-4 gp10 gene, but its predicted gene product does not possess

features typical of envelope glycoproteins.

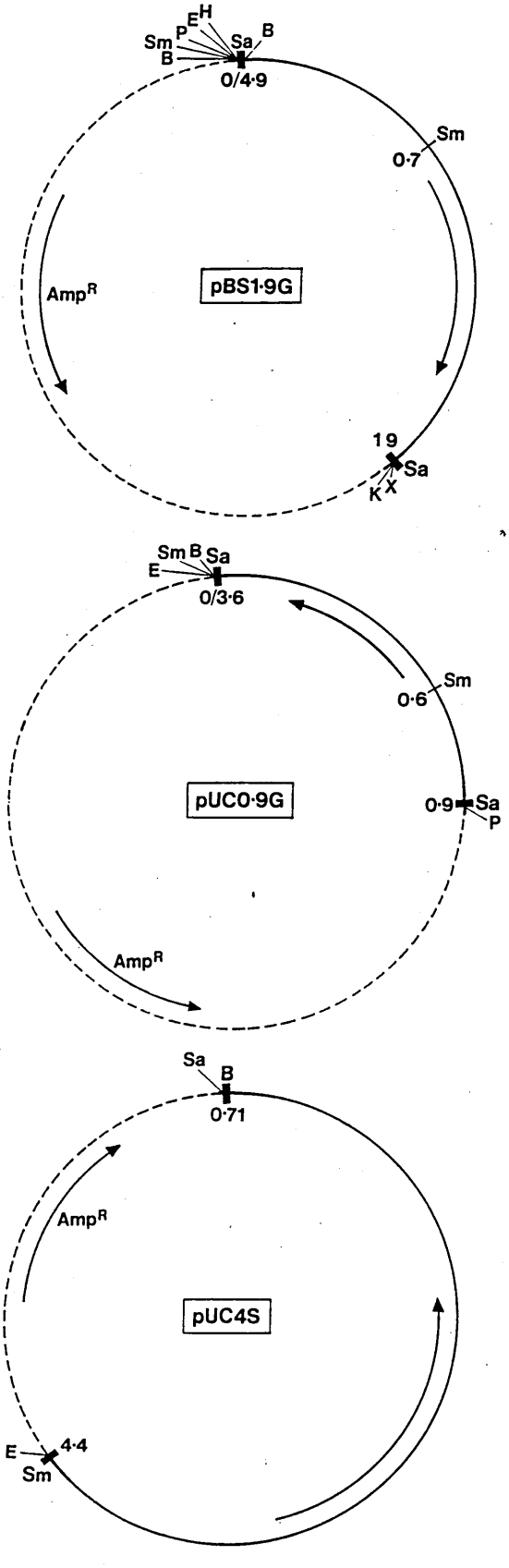
DNA Sequence of a Region of the EHV-4 Genome Containing Part of the Gene Encoding the Major DNA Binding Protein

The DNA sequence of EHV-4 BamHI-Q and the left terminus of BamHI-M, and the amino acid sequence of the partial ORF it specifies, are presented in Figure 6.23a. This partial ORF encodes part of the major DNA binding protein. An alignment of the amino acid sequence available for the EHV-4 protein with its HSV-1 (UL29) and VZV (gene 29) homologues is shown in Figure 6.23b. This demonstrates that the proteins are highly conserved between these three alphaherpesviruses, with 117 amino acids being shared by all three proteins over a total length of 271 residues, and is consistent with the finding that this is one of the most highly conserved proteins between HSV-1 and VZV (McGeoch et al., 1988a).

Figure 6.1 Physical maps of plasmids pBS1.9G, pUC0.9G and pUC4S. The broken lines represent vector DNA and the solid lines represent EHV-4 DNA. Sizes are shown in kilobases. Sites shown are those for BamHI (B), EcoRI (E), HindIII (H), KpnI (K), PstI (P), SalI (Sa), SmaI (Sm) and XhoI (X). The orientation of EHV-4 DNA is denoted by an arrow.

F I G U R E 6.1

Physical Maps of Plasmids pBS1.9G, pUC0.9G and pUC4S



F I G U R E 6.2

Restriction Endonuclease Analysis of Plasmids
pBS1.9G, pUC0.9G and pUC4S

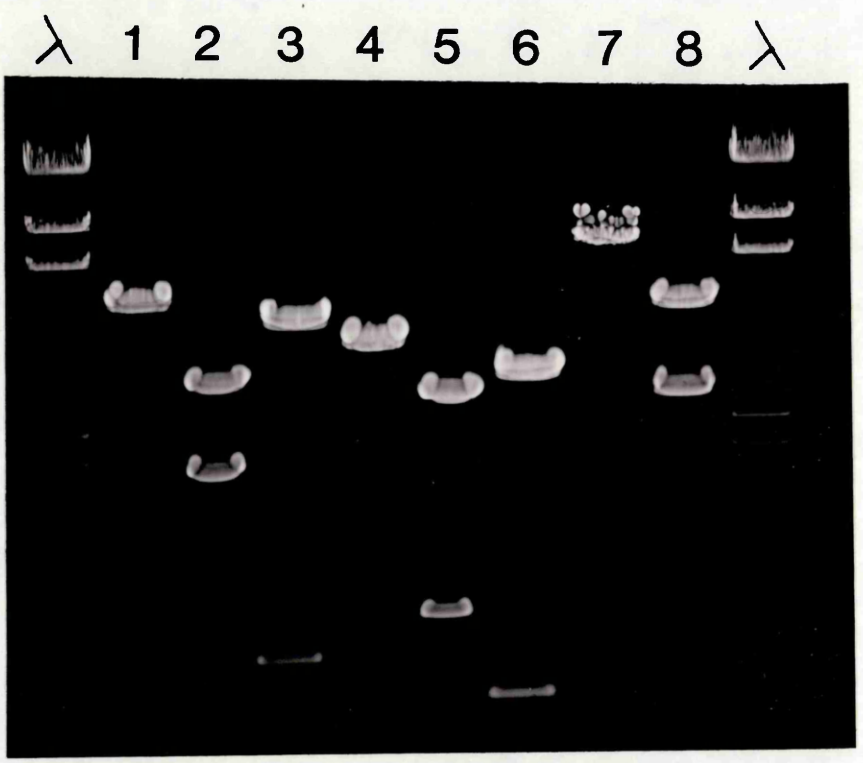


Figure 6.2 Restriction endonuclease analysis of plasmids pBS1.9G, pUC0.9G and pUC4S. Plasmid pBS1.9G was digested with BamHI (lane 1), SalI (lane 2) and SmaI (lane 3), plasmid pUC0.9G with BamHI (lane 4), SalI (lane 5) and SmaI (lane 6), and plasmid pUC4S with BamHI (lane 7) and BamHI/EcoRI (lane 8). HindIII digested λ DNA fragments were used as size markers.

Figure 6.3 Autoradiograph of a typical sequencing gel obtained in determining the DNA sequence of the EHV-4 genome between 0.067 and 0.122 map units. The primers used were M13 on the upper strand and Reverse on the lower strand. These primers were annealed to single-stranded DNA template generated from plasmid pUC4S and sequencing reactions then carried out. The samples shown on the gel are as follows:-
M13 primer (long run)- A track (lane 1), C track (lane 2), G track (lane 3) and T track (lane 4); Reverse primer (long run)- A track (lane 5), C track (lane 6), G track (lane 7) and T track (lane 8); M13 primer (short run)- A track (lane 9), C track (lane 10), G track (lane 11) and T track (lane 12); Reverse primer (short run)- A track (lane 13), C track (lane 14), G track (lane 15) and T track (lane 16).
Sequence data generated from the M13 primer reaction corresponds to that in a rightward direction from nucleotide 1 in Figure 6.5, and that from the Reverse primer reaction corresponds to that in a leftward direction on the opposite strand from the BamHI site in Figure 6.5.

FIGURE 6.3

Autoradiograph of a Typical Sequencing Gel Obtained in Determining the DNA Sequence of the EHV-4 Genome Between 0.067 and 0.122 Map Units

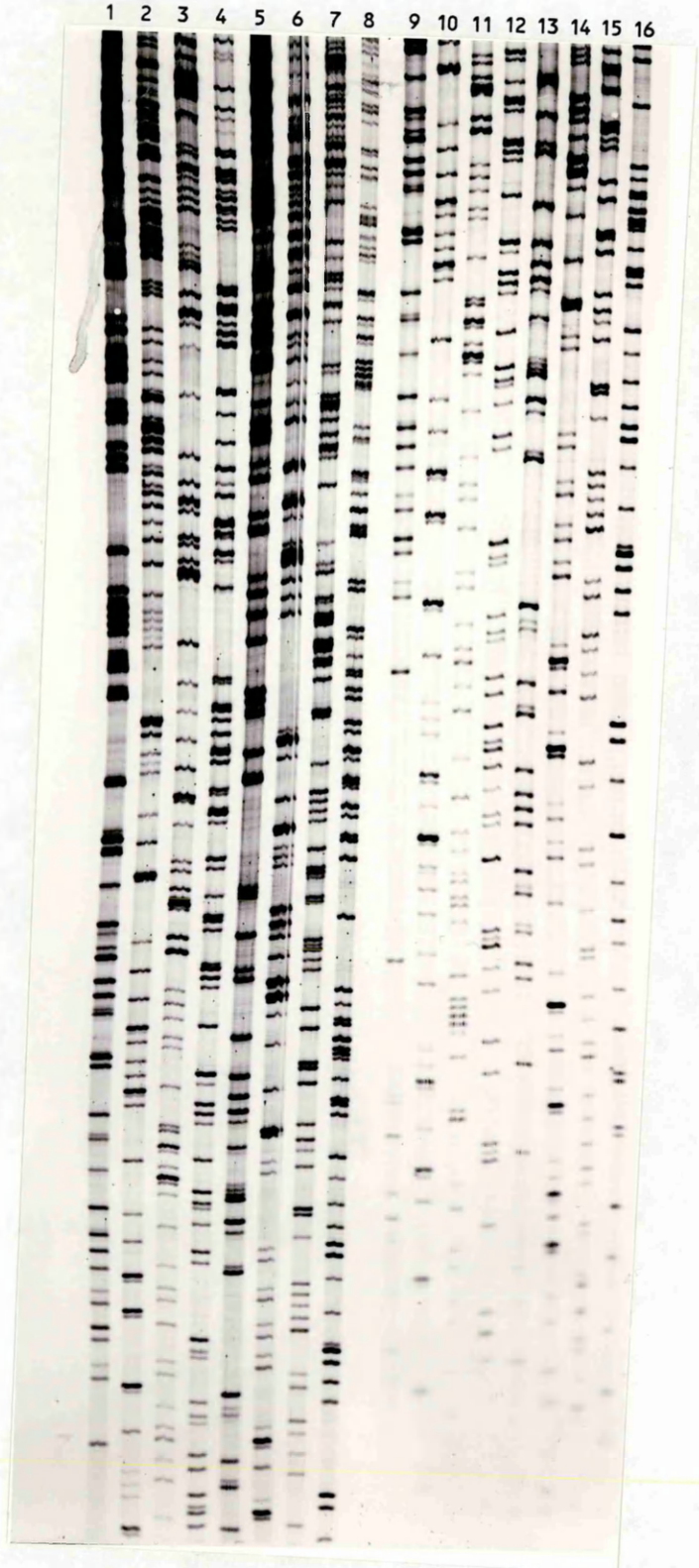


Figure 6.4 Map of the EHV-4 genome showing the restriction endonuclease sites and DNA fragments used in studying gene arrangement between 0.067 and 0.122 map units. (a) Structure of the EHV-4 genome. (b) Location of BamHI sites in the viral genome. (c) Genomic location of DNA fragments sequenced during this study. The 7868bp region sequenced is indicated by a thick bar. (d) Gene arrangement in the region of the genome sequenced. Complete ORFs B2, B3, B4 and B6 are indicated by arrows. Broken arrows indicate the location of partial ORFs B1, B5 and B7. Dots represent the 5′ end and arrowheads the 3′ end of each ORF.

F I G U R E 6.4

Gene Arrangement in the EHV-4 Genome Between 0.067 and 0.122 Map Units

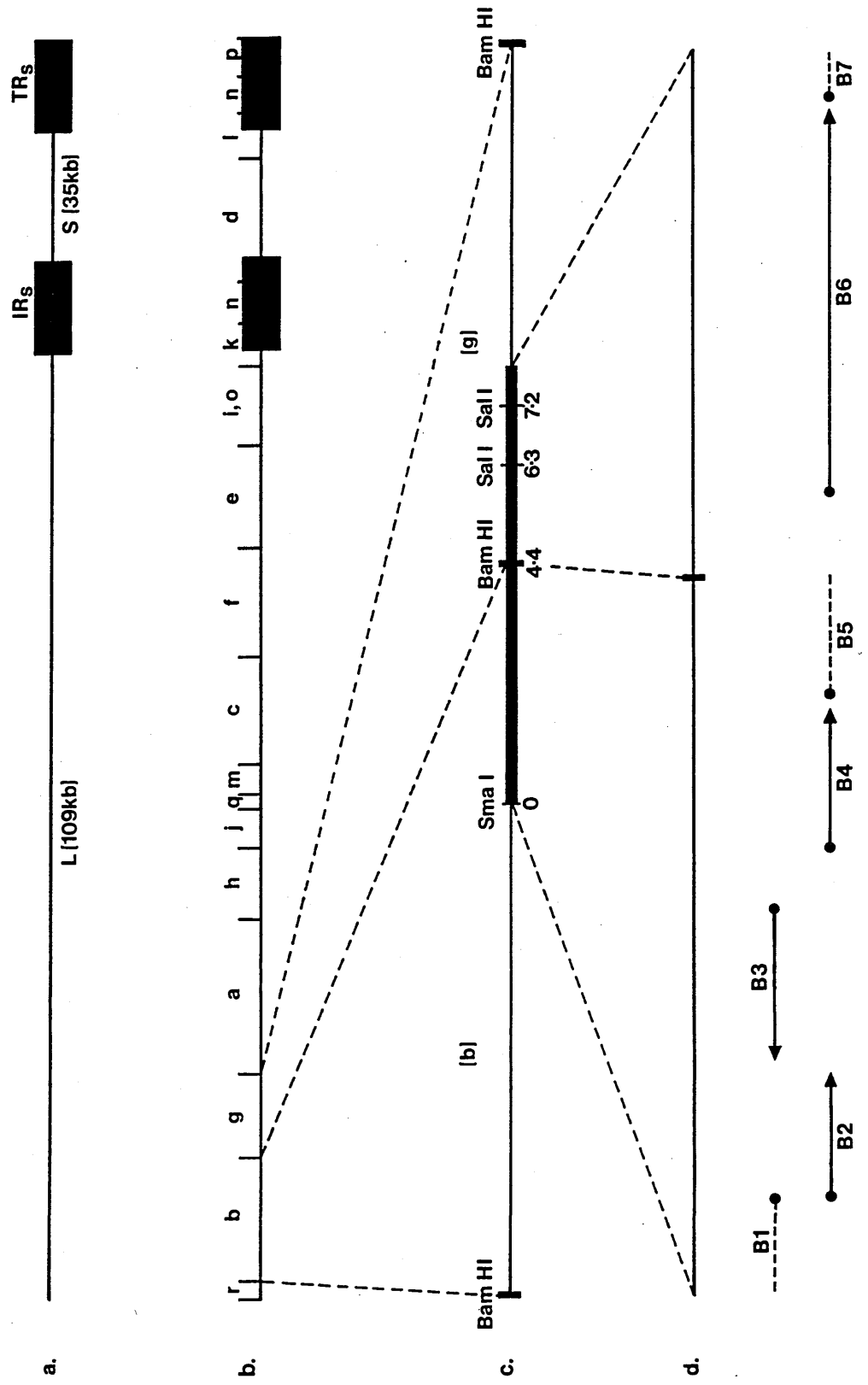


Figure 6.5 DNA sequence of the rightmost 4477bp of EHV-4 BamHI-B and leftmost 3397bp of BamHI-G. The amino acid sequences of the gene products of ORFs B1 to B7 are shown below (leftward encoded) or above (rightward encoded) the sequence. Potential polyadenylation signals are underlined and tentative TATA elements are shown in bold type. Since a small BamHI fragment appears to have been omitted from the EHV-4 library between BamHI-B and BamHI-G, only the first 211 amino acids of the gene product of ORF B5 are shown and the most likely carboxyl-terminal amino acids of this protein are shown in brackets.

FIGURE 6.5

DNA Sequence of the EHV-4 Genome Between 0.067 and 0.122 Map Units

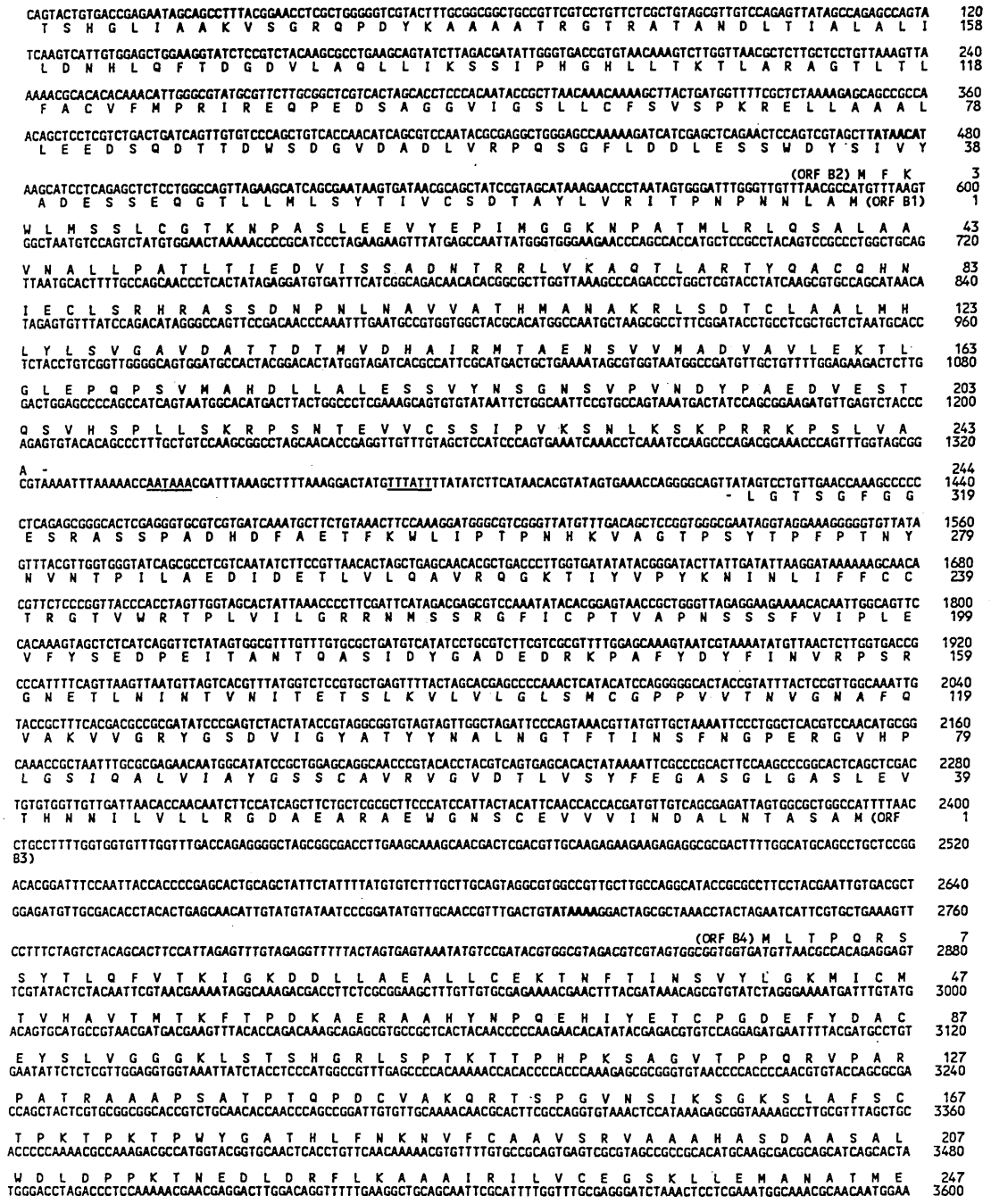
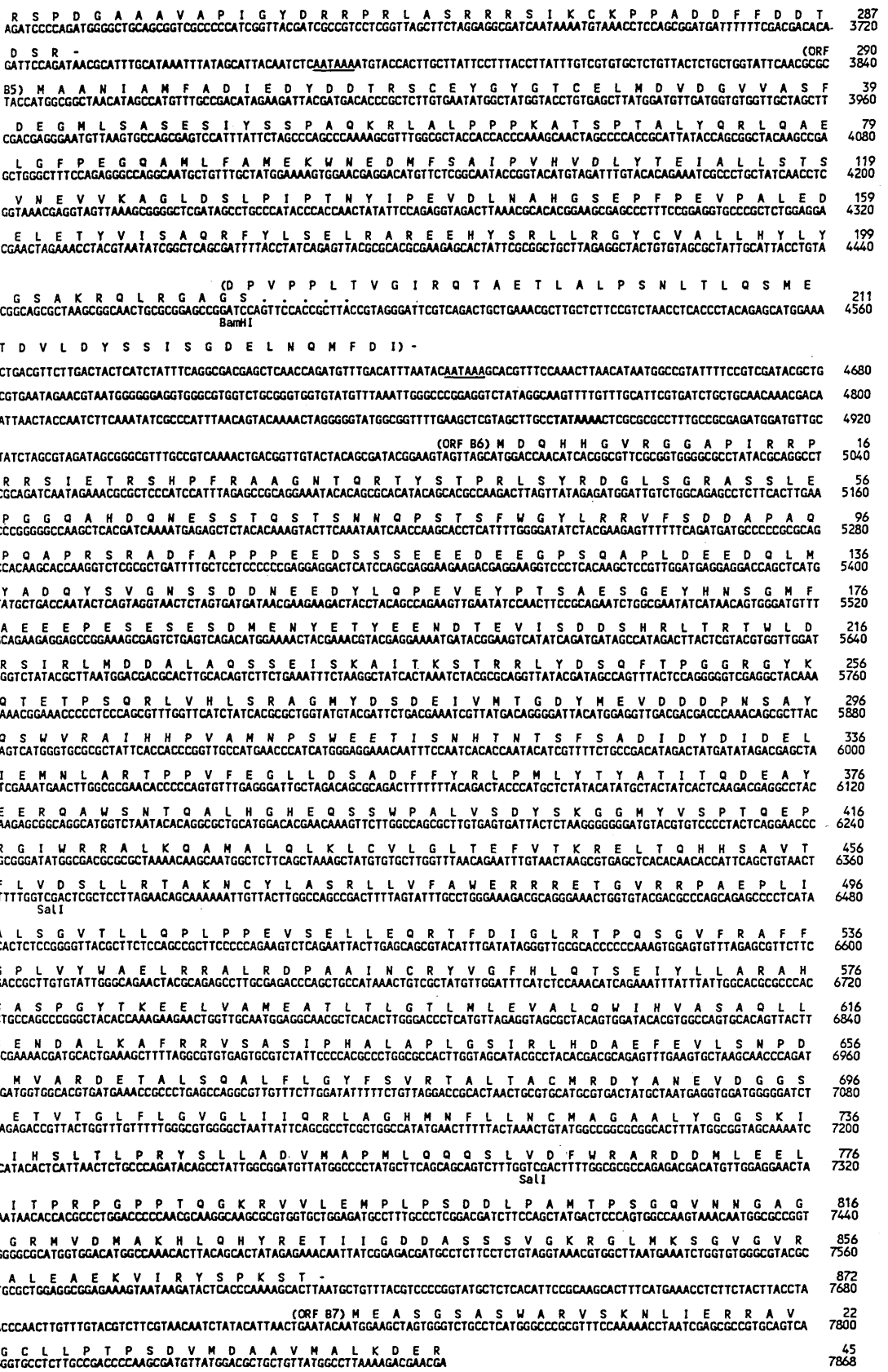


Figure 6.5- continued



T A B L E 6.1

Summary of EHV-4 Sequence Data

ORF	ORF start	ORF end	ATG context sequence	TATA sequence	Position of TATA sequence	Length (amino acids)	Molecular mass (kDa)
B1	592	(1)[a]	AACATGG	?	?	(197)[a]	-
B2	591	1323	GCCATGT	TATAACA	473	244	26.2
B3	2394	1416	AAAATGG	?	?	326	35.2
B4	2860	3730	GTGATGT	TATAAAA	2712	290	31.5
B5	3845	(4620)[b]	ACCATGG	?	?	(211)[b]	-
B6	4993	7609	AGCATGG	TATAAAA	4882	872	97.4
B7	7734	(7868)[a]	ACAATGG	?	?	(45)[a]	-

[a] Incomplete ORF.
[b] Incomplete ORF, probable termination site identified.
? No obvious TATA sequence 5′ to initiation codon.

Table 6.1 Summary of EHV-4 sequence data. ORFs, putative TATA sequences, and lengths and molecular masses of the predicted gene products are shown.

TABLE 6.2

Homology Between the Gene Products of EHV-4, HSV-1 and VZV

Gene Product	EHV-4/HSV-1	EHV-4/VZV	HSV-1/VZV
B2/UL51/Gene 7	38	45	36
B3/UL50/Gene 8	31	29	26
B4/UL49/Gene 9	29	28	26
B5/UL48/Gene 10	38*	34*	28
B6/UL47/Gene 11	17	27	18

* Denotes value derived from alignment of the first 211 amino acids of the EHV-4 protein.

Table 6.2 Percentage homologies for pairwise comparisons of the gene products of the alphaherpesviruses EHV-4, HSV-1 and VZV.

F I G U R E 6.6

Alignment of the Predicted Partial Amino Acid Sequences of EHV-4 B1 and B7 With the Analogous Gene Products of HSV-1 and VZV

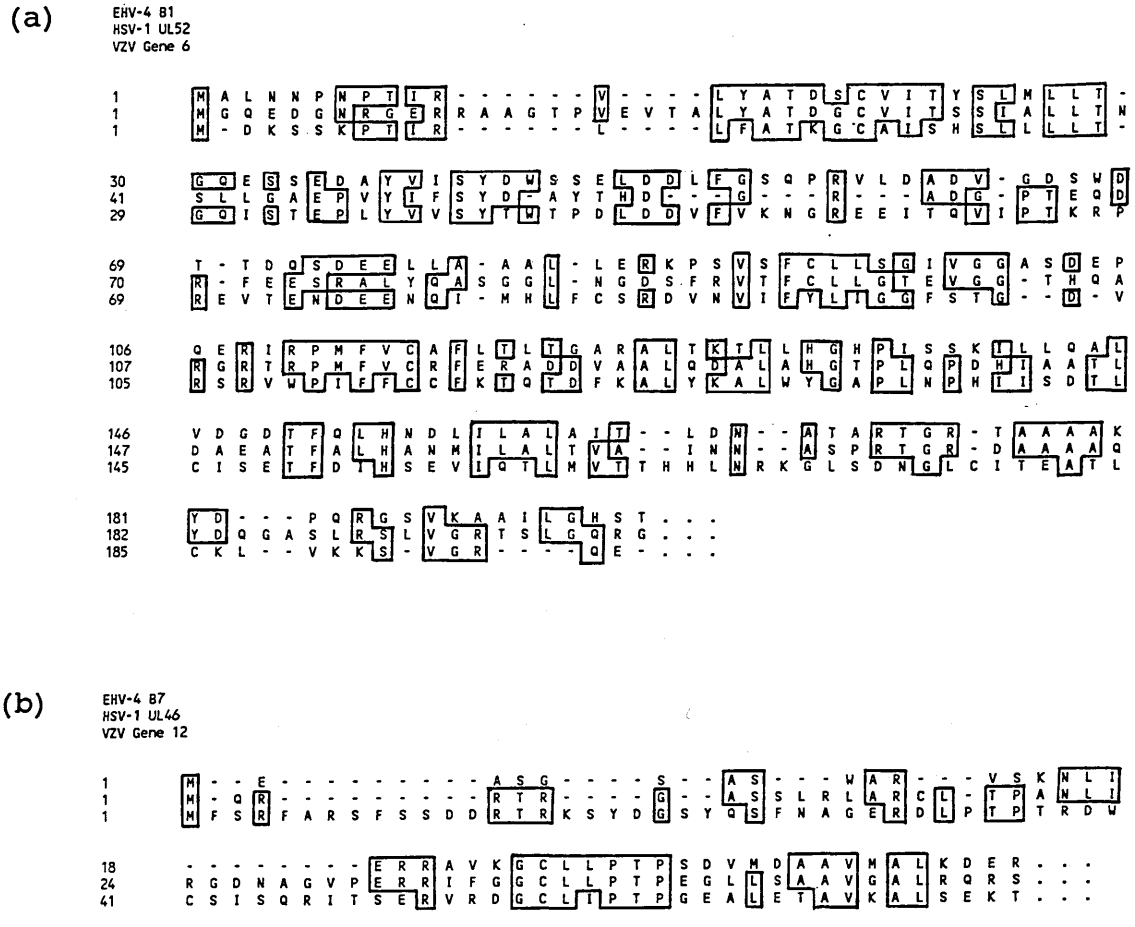


Figure 6.6 Direct alignments of the predicted partial amino acid sequences of the gene products of EHV-4 B1 and B7 with the analogous gene products of HSV-1 and VZV. (a) Alignment of EHV-4 B1 with the corresponding region of the gene products of HSV-1 UL52 and VZV gene 6. (b) Alignment of EHV-4 B7 with the corresponding region of the gene products of HSV-1 UL46 and VZV gene 12. Identical residues are boxed and dashes denote spaces introduced to maximise alignment of the sequences.

FIGURE 6.7

Alignment of the Predicted Amino Acid Sequence of EHV-4 B2 With the Analogous Gene Products of HSV-1 and VZV

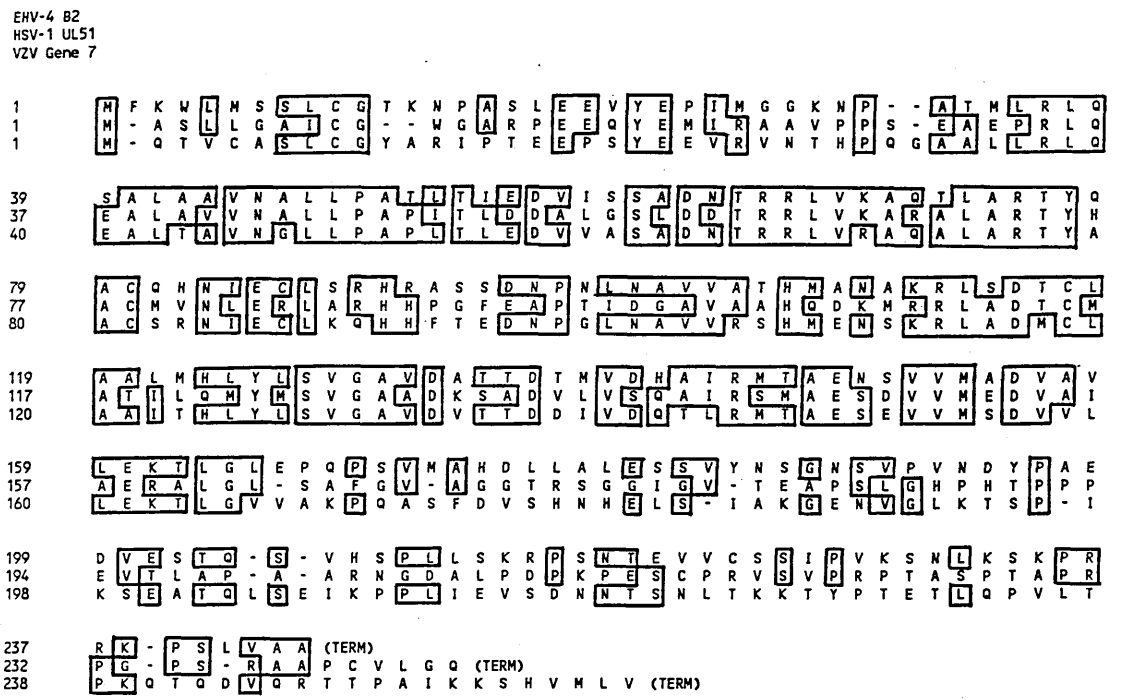


Figure 6.7 Direct alignment of the predicted amino acid sequence of the gene product of EHV-4 B2 with the analogous gene products of HSV-1 UL51 and VZV gene 7. Identical residues are boxed and dashes denote spaces introduced to maximise alignment of the sequences.

Figure 6.8 Dot matrix similarity analysis of the EHV-4 B2 amino acid sequence against those of the gene products of HSV-1 UL51 and VZV gene 7. Analysis was carried out using COMPARE and DOTPLOT from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984). In each plot, the horizontal axis is the EHV-4 B2 amino acid sequence. Vertical axes represent the amino acid sequences of the gene products of HSV-1 UL51 (a) and VZV gene 7 (b). Points are plotted when at least 15 amino acids in a moving window of 30 are identical. Large regions of similarity are indicated by uninterrupted diagonal lines from lower left to upper right.

F I G U R E 6.8

Dot Matrix Similarity Analysis of the EHV-4 B2 Amino Acid Sequence Against Those of the Gene Products of HSV-1 UL51 and VZV Gene 7

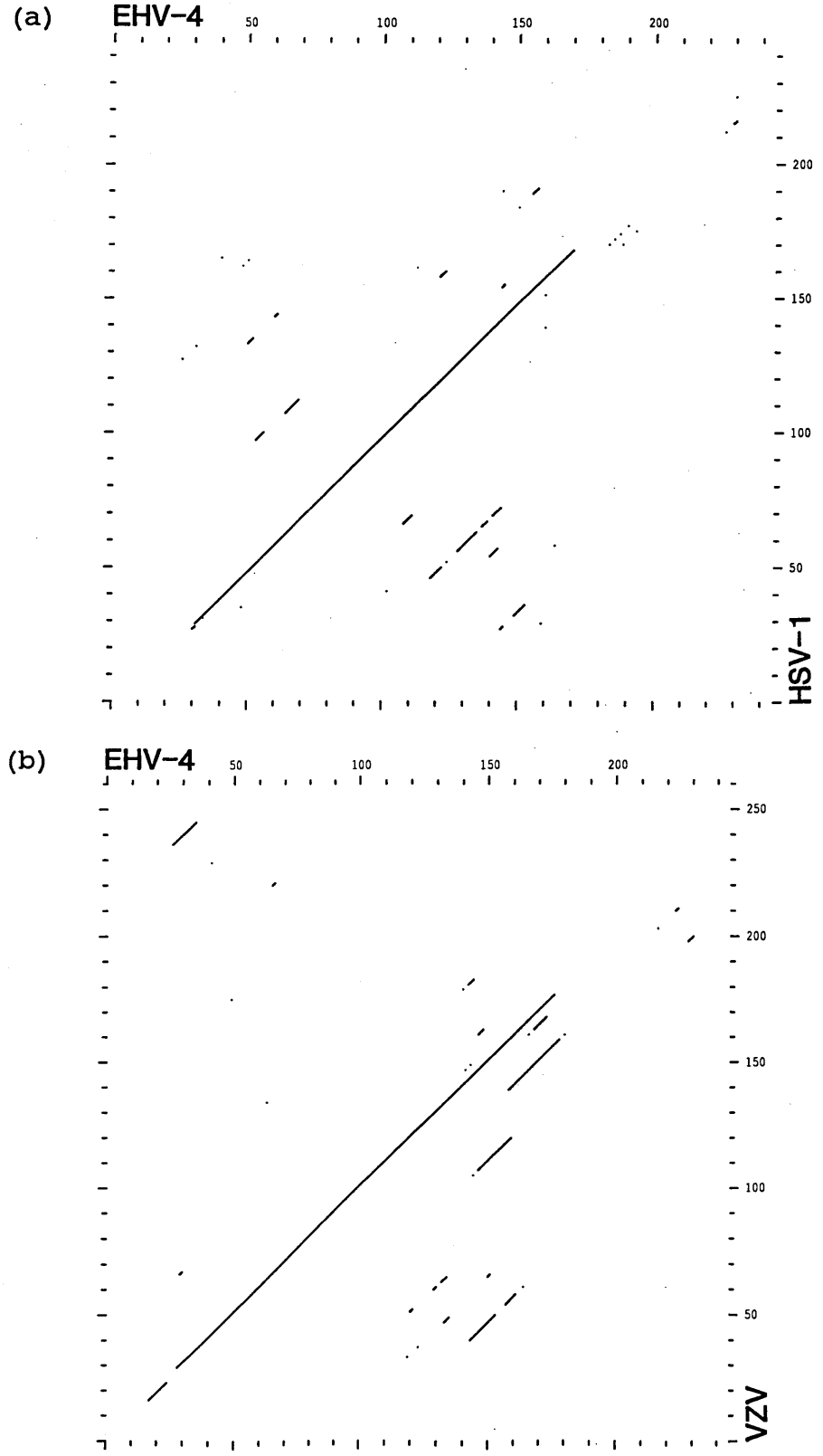


FIGURE 6.9

Hydropathic Analysis of the Gene Products of EHV-4 B2, HSV-1 UL51 and VZV Gene 7

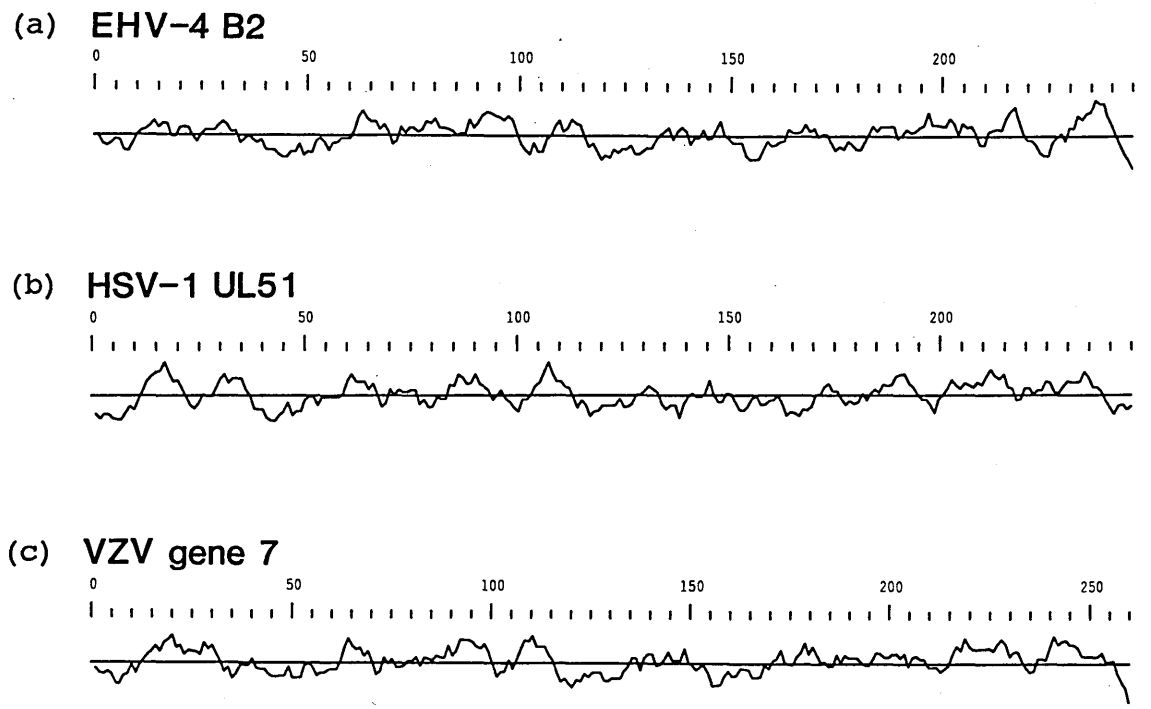


Figure 6.9 Hydropathic plots of the gene products of EHV-4 B2 (a), HSV-1 UL51 (b) and VZV gene 7 (c) as determined by the method of Kyte and Doolittle (1982). Points above the horizontal line represent regions of above average hydrophilicity.

T A B L E 6.3

Composition of EHV-4 Protein B2

Codon (amino acid)	Frequency	% of total	Codon (amino acid)	Frequency	% of total
TTT (Phe)	1	0.4	TAT (Tyr)	4	1.6
TTC (Phe)	0	0.0	TAC (Tyr)	1	0.4
TTA (Leu)	2	0.8	TAA (End)	0	0.0
TTG (Leu)	6	2.5	TAG (End)	0	0.0
CTT (Leu)	3	1.2	CAT (His)	3	1.2
CTC (Leu)	6	2.5	CAC (His)	4	1.6
CTA (Leu)	5	2.0	CAA (Gln)	1	0.4
CTG (Leu)	6	2.5	CAG (Gln)	5	2.0
ATT (Ile)	3	1.2	AAT (Asn)	8	3.3
ATC (Ile)	1	0.4	AAC (Asn)	7	2.9
ATA (Ile)	2	0.8	AAA (Lys)	5	2.0
ATG (Met)	10	4.1	AAG (Lys)	6	2.5
GTT (Val)	9	3.7	GAT (Asp)	6	2.5
GTC (Val)	0	0.0	GAC (Asp)	5	2.0
GTA (Val)	6	2.5	GAA (Glu)	5	2.0
GTG (Val)	8	3.3	GAG (Glu)	7	2.9
TCT (Ser)	2	0.8	TGT (Cys)	3	1.2
TCC (Ser)	9	3.7	TGC (Cys)	2	0.8
TCA (Ser)	3	1.2	TGA (End)	0	0.0
TCG (Ser)	3	1.2	TGG (Trp)	1	0.4
CCT (Pro)	2	0.8	CGT (Arg)	1	0.4
CCC (Pro)	4	1.6	CGC (Arg)	5	2.0
CCA (Pro)	8	3.3	CGA (Arg)	0	0.0
CCG (Pro)	0	0.0	CGG (Arg)	2	0.8
ACT (Thr)	6	2.5	AGT (Ser)	5	2.0
ACC (Thr)	7	2.9	AGC (Ser)	5	2.0
ACA (Thr)	1	0.4	AGA (Arg)	2	0.8
ACG (Thr)	2	0.8	AGG (Arg)	1	0.4
GCT (Ala)	8	3.3	GGT (Gly)	1	0.4
GCC (Ala)	10	4.1	GGC (Gly)	1	0.4
GCA (Ala)	7	2.9	GGA (Gly)	2	0.8
GCG (Ala)	4	1.6	GGG (Gly)	2	0.8

Table 6.3 Codon usage and predicted amino acid composition of EHV-4 B2 (244 amino acids).

FIGURE 6.10

Alignment of the Predicted Amino Acid Sequence of EHV-4 B3 With the Analogous Gene Products of HSV-1 and VZV

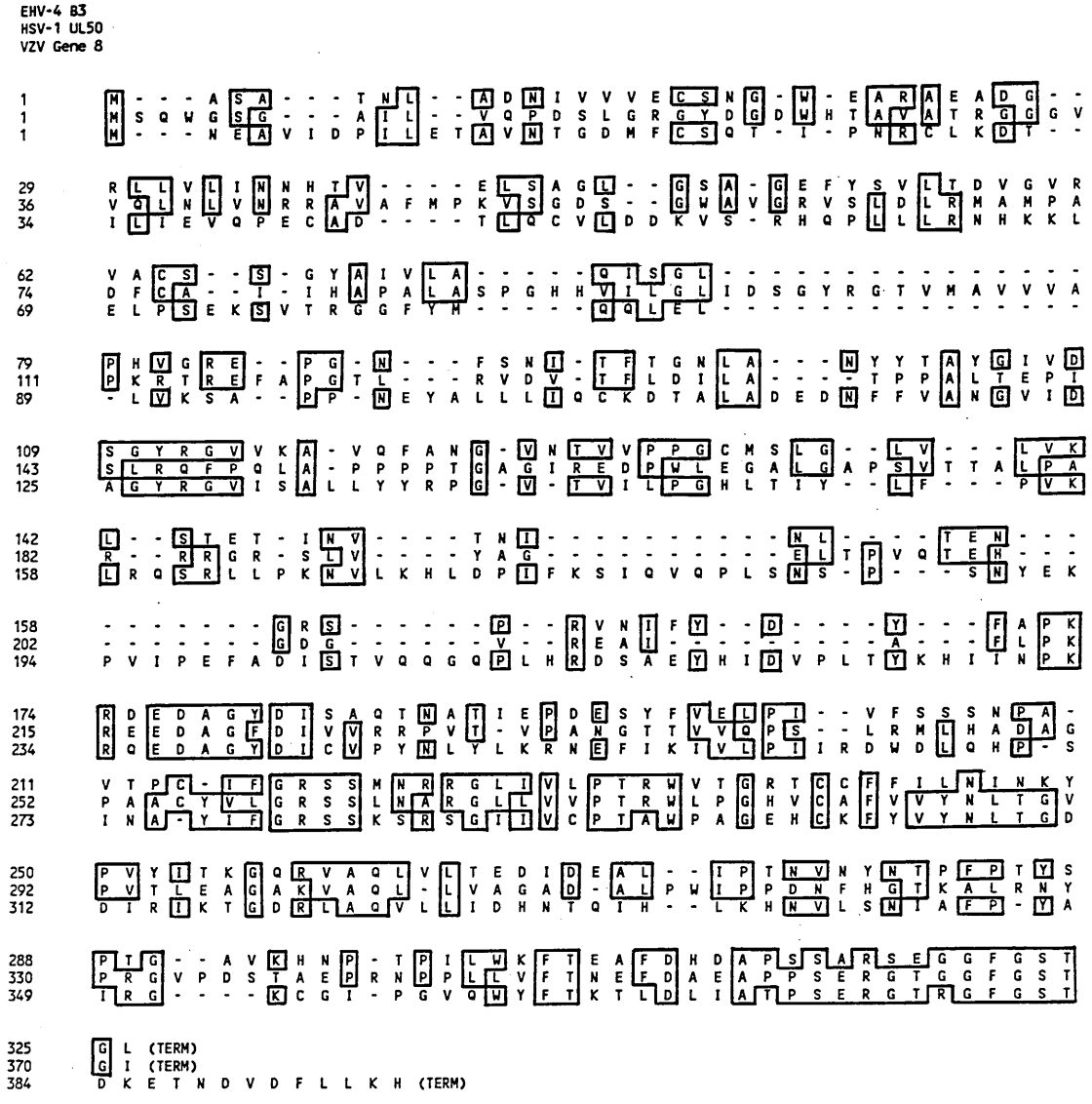


Figure 6.10 Direct alignment of the predicted amino acid sequence of the gene product of EHV-4 B3 with the analogous gene products of HSV-1 UL50 and VZV gene 8. Identical residues are boxed and dashes denote spaces introduced to maximise alignment of the sequences.

Figure 6.11 Dot matrix similarity analysis of the EHV-4 B3 amino acid sequence against those of the gene products of HSV-1 UL50 and VZV gene 8. Analysis was carried out using COMPARE and DOTPLOT from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984). In each plot, the horizontal axis is the EHV-4 B3 amino acid sequence. Vertical axes represent the amino acid sequences of the gene products of HSV-1 UL50 (a) and VZV gene 8 (b). Points are plotted when at least 15 amino acids in a moving window of 30 are identical. Large regions of similarity are indicated by uninterrupted diagonal lines from lower left to upper right.

F I G U R E 6.11

Dot Matrix Similarity Analysis of the EHV-4 B3 Amino Acid Sequence Against Those of the Gene Products of HSV-1 UL50 and VZV Gene 8

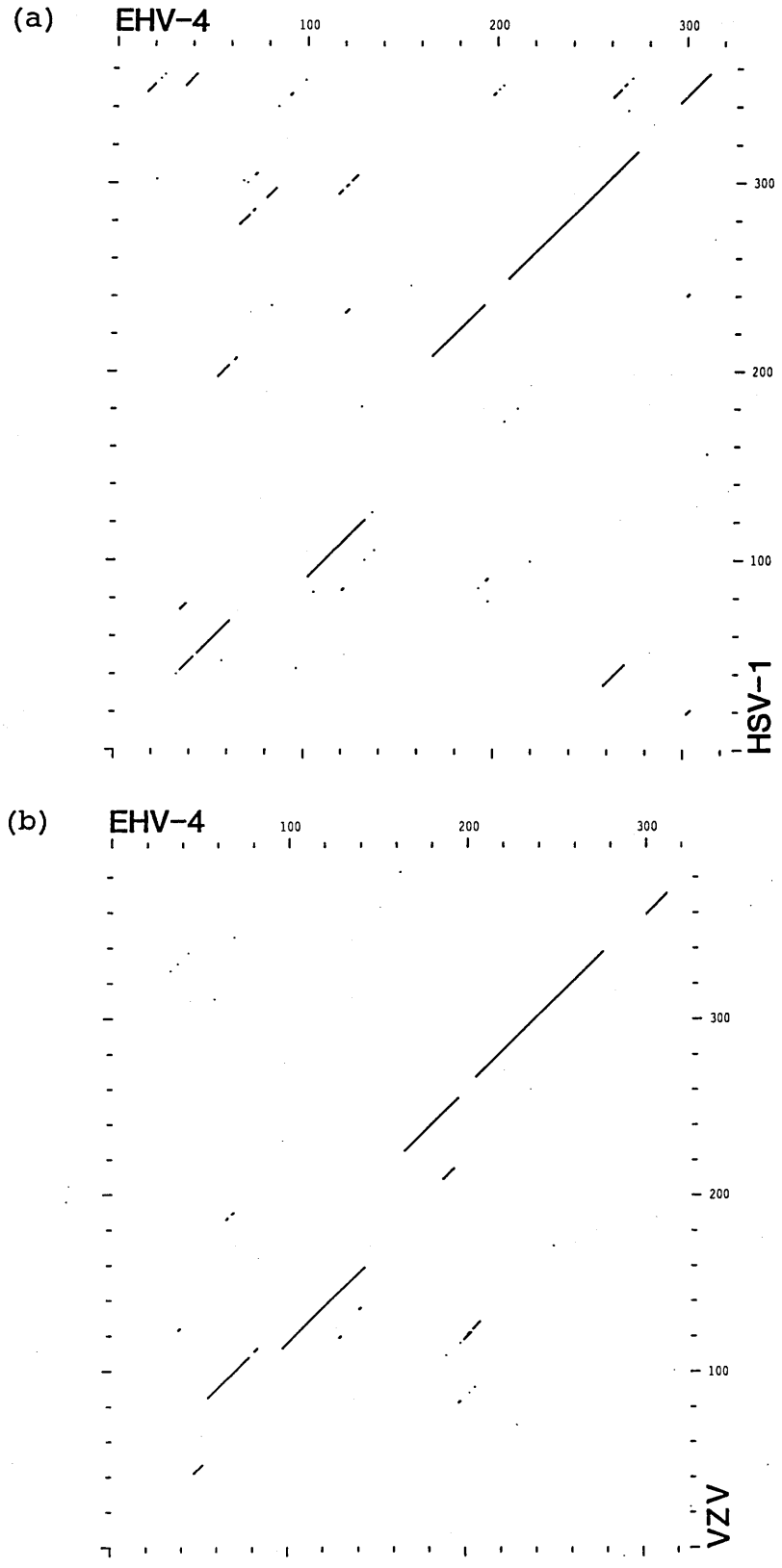


FIGURE 6.12

Hydropathic Analysis of the Gene Products of EHV-4 B3, HSV-1 UL50 and VZV Gene 8

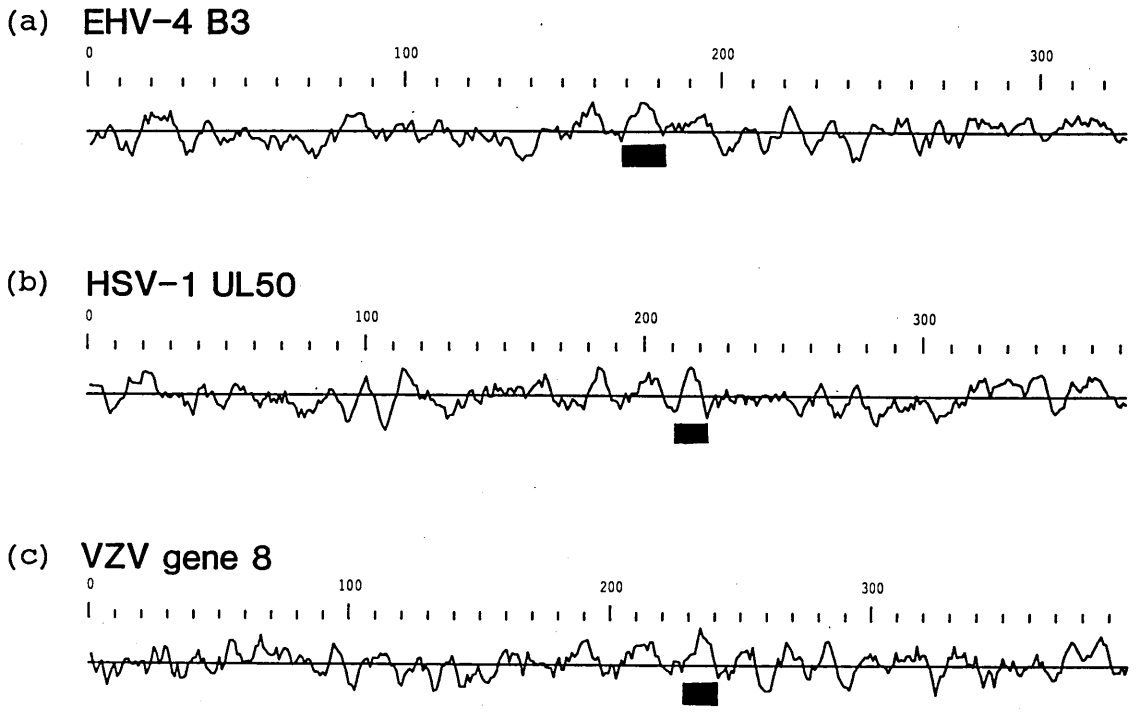


Figure 6.12 Hydropathic plots of the gene products of EHV-4 B3 (a), HSV-1 UL50 (b) and VZV gene 8 (c) as determined by the method of Kyte and Doolittle (1982). Points above the horizontal line represent regions of above average hydrophilicity. In each plot, the shaded box indicates a region of very hydrophilic amino acids which is highly conserved between the proteins (see text for details).

T A B L E 6.4

Composition of EHV-4 Protein B3

Codon (amino acid)	Frequency	% of total	Codon (amino acid)	Frequency	% of total
TTT (Phe)	15	4.6	TAT (Tyr)	8	2.5
TTC (Phe)	0	0.0	TAC (Tyr)	6	1.8
TTA (Leu)	4	1.2	TAA (End)	0	0.0
TTG (Leu)	4	1.2	TAG (End)	0	0.0
CTT (Leu)	3	0.9	CAT (His)	2	0.6
CTC (Leu)	5	1.5	CAC (His)	2	0.6
CTA (Leu)	5	1.5	CAA (Gln)	3	0.9
CTG (Leu)	3	0.9	CAG (Gln)	2	0.6
ATT (Ile)	5	1.5	AAT (Asn)	9	2.8
ATC (Ile)	7	2.1	AAC (Asn)	16	4.9
ATA (Ile)	8	2.5	AAA (Lys)	4	1.2
ATG (Met)	3	0.9	AAG (Lys)	3	0.9
GTT (Val)	7	2.1	GAT (Asp)	5	1.5
GTC (Val)	3	0.9	GAC (Asp)	8	2.5
GTA (Val)	10	3.1	GAA (Glu)	10	3.1
GTG (Val)	12	3.7	GAG (Glu)	6	1.8
TCT (Ser)	4	1.2	TGT (Cys)	4	1.2
TCC (Ser)	2	0.6	TGC (Cys)	2	0.6
TCA (Ser)	2	0.6	TGA (End)	0	0.0
TCG (Ser)	4	1.2	TGG (Trp)	3	0.9
CCT (Pro)	3	0.9	CGT (Arg)	2	0.6
CCC (Pro)	7	2.1	CGC (Arg)	4	1.2
CCA (Pro)	6	1.8	CGA (Arg)	2	0.6
CCG (Pro)	3	0.9	CGG (Arg)	2	0.6
ACT (Thr)	8	2.5	AGT (Ser)	6	1.8
ACC (Thr)	8	2.5	AGC (Ser)	7	2.1
ACA (Thr)	4	1.2	AGA (Arg)	3	0.9
ACG (Thr)	6	1.8	AGG (Arg)	2	0.6
GCT (Ala)	5	1.5	GGT (Gly)	6	1.8
GCC (Ala)	10	3.1	GGC (Gly)	3	0.9
GCA (Ala)	5	1.5	GGA (Gly)	12	3.7
GCG (Ala)	6	1.8	GGG (Gly)	7	2.1

Table 6.4 Codon usage and predicted amino acid composition of EHV-4 B3 (326 amino acids).

F I G U R E 6.13

Alignment of the Predicted Amino Acid Sequence of EHV-4 B4 With the Analogous Gene Products of HSV-1 and VZV

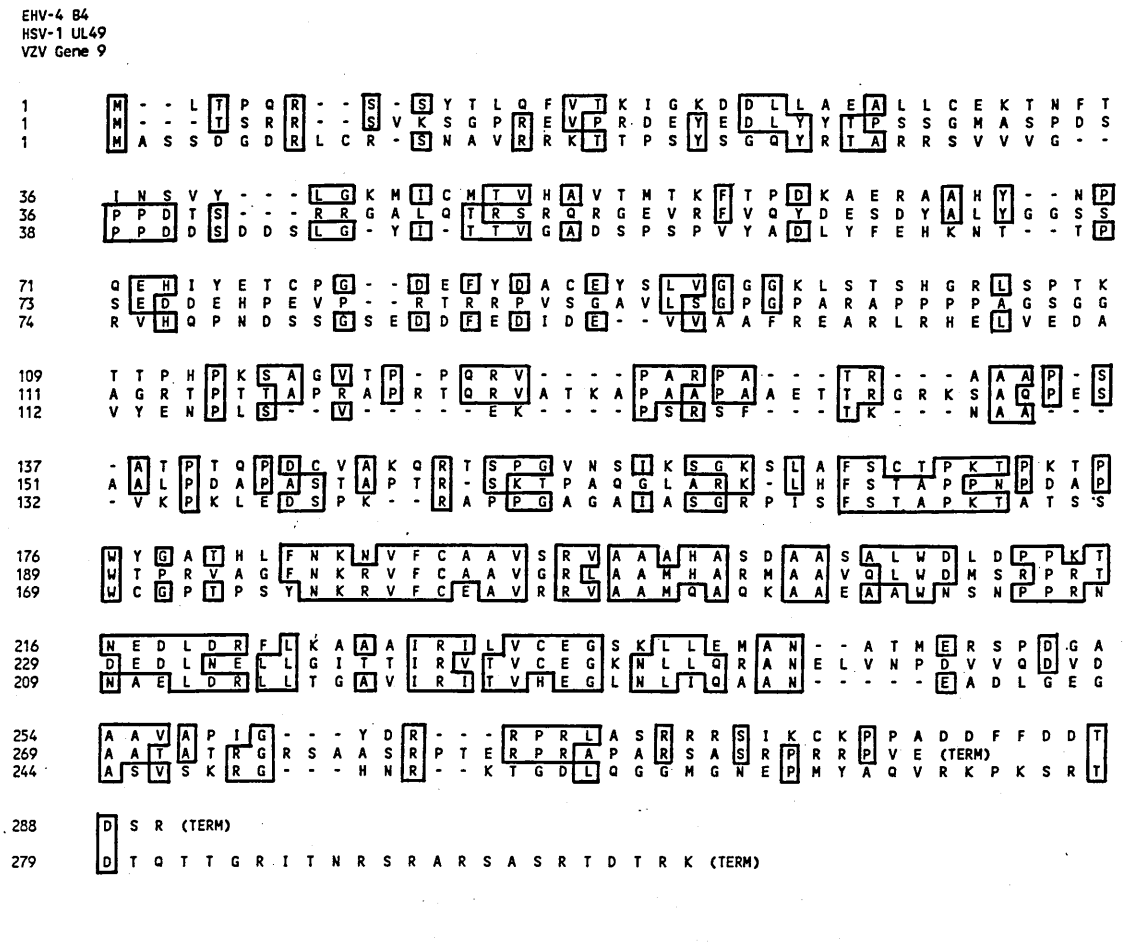
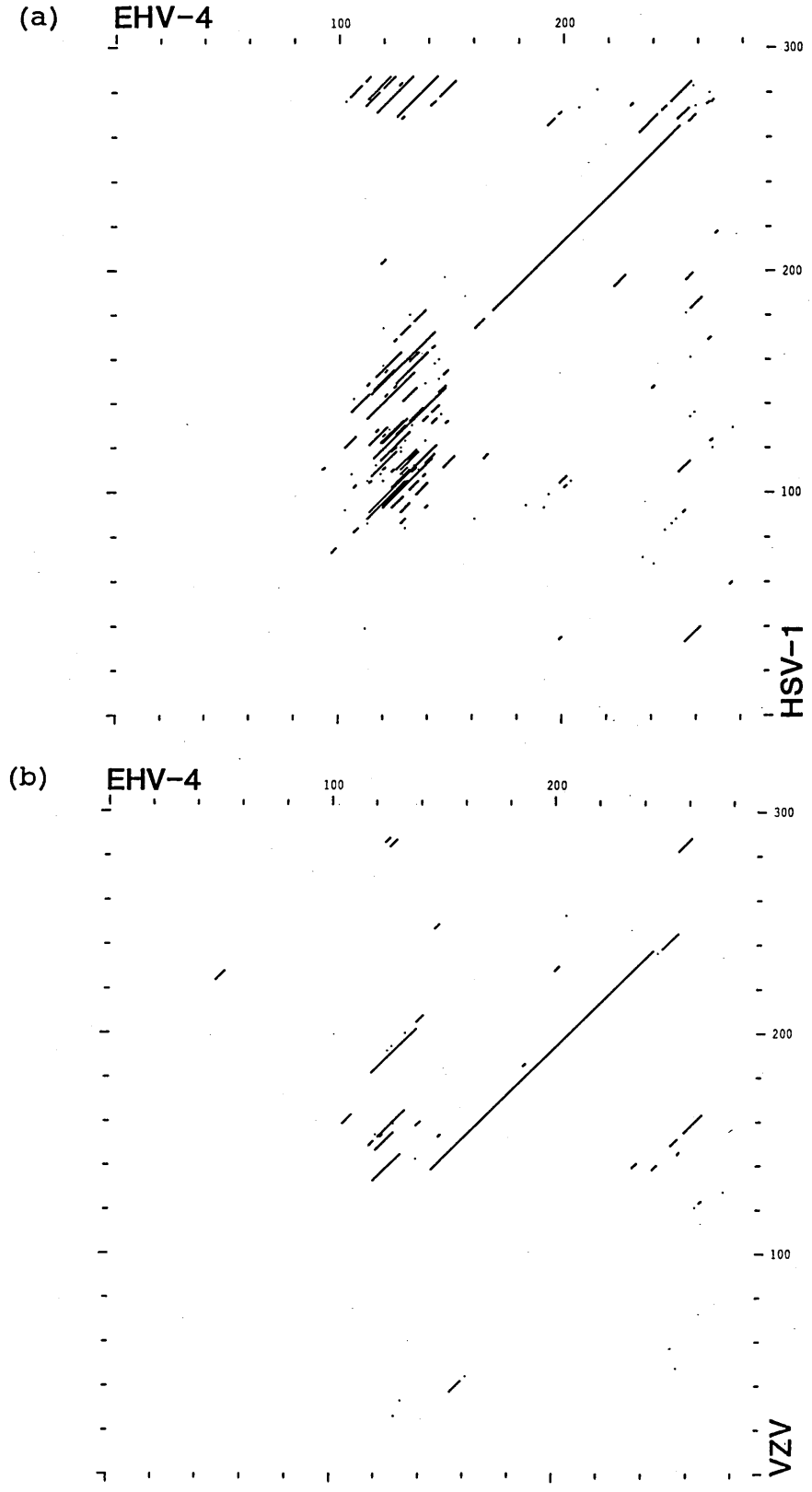


Figure 6.13 Direct alignment of the predicted amino acid sequence of the gene product of EHV-4 B4 with the analogous gene products of HSV-1 UL49 and VZV gene 9. Identical residues are boxed and dashes denote spaces introduced to maximise alignment of the sequences.

Figure 6.14 Dot matrix similarity analysis of the EHV-4 B4 amino acid sequence against those of the gene products of HSV-1 UL49 and VZV gene 9. Analysis was carried out using COMPARE and DOTPLOT from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984). In each plot, the horizontal axis is the EHV-4 B4 amino acid sequence. Vertical axes represent the amino acid sequences of the gene products of HSV-1 UL49 (a) and VZV gene 9 (b). Points are plotted when at least 15 amino acids in a moving window of 30 are identical. Large regions of similarity are indicated by uninterrupted diagonal lines from lower left to upper right.

F I G U R E 6.14

Dot Matrix Similarity Analysis of the EHV-4 B4 Amino Acid Sequence Against Those of the Gene Products of HSV-1 UL49 and VZV Gene 9



F I G U R E 6.15

Hydropathic Analysis of the Gene Products of EHV-4 B4, HSV-1 UL49 and VZV Gene 9

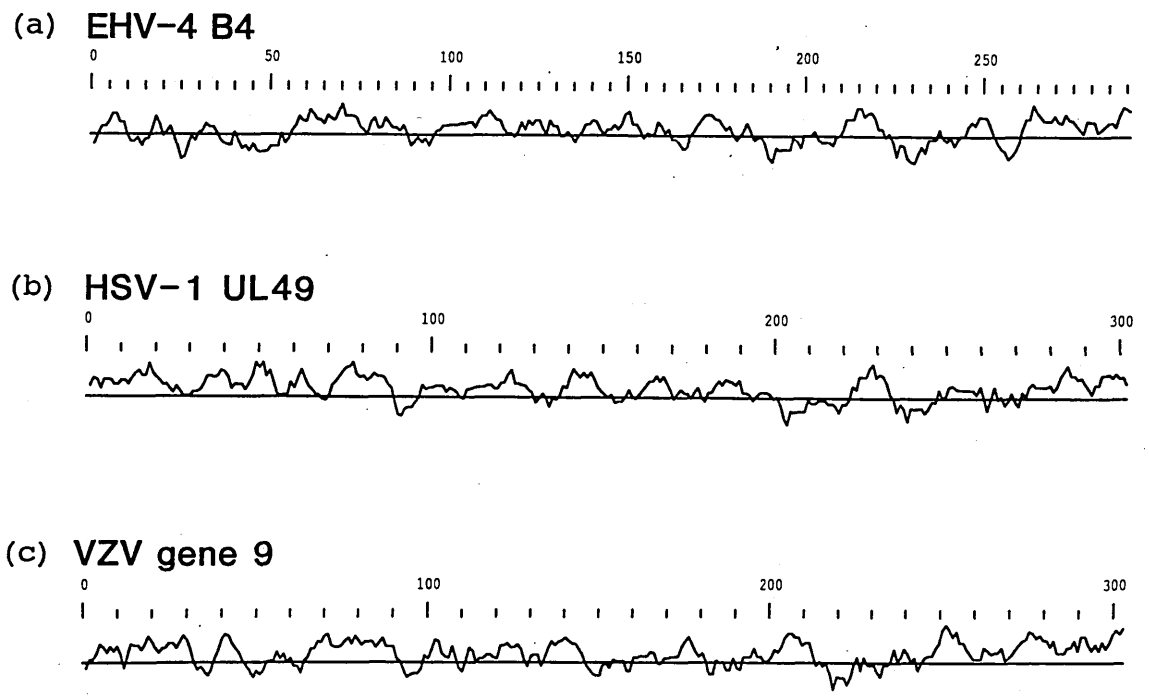


Figure 6.15 Hydropathic plots of the gene products of EHV-4 B4 (a), HSV-1 UL49 (b) and VZV gene 9 (c) as determined by the method of Kyte and Doolittle (1982). Points above the horizontal line represent regions of above average hydrophilicity.

TABLE 6.5

Composition of EHV-4 Protein B4

Codon (amino acid)	Frequency	% of total	Codon (amino acid)	Frequency	% of total
TTT (Phe)	7	2.4	TAT (Tyr)	3	1.0
TTC (Phe)	3	1.0	TAC (Tyr)	5	1.7
TTA (Leu)	3	1.0	TAA (End)	0	0.0
TTG (Leu)	6	2.1	TAG (End)	0	0.0
CTT (Leu)	2	0.7	CAT (His)	3	1.0
CTC (Leu)	4	1.4	CAC (His)	4	1.4
CTA (Leu)	4	1.4	CAA (Gln)	4	1.4
CTG (Leu)	1	0.3	CAG (Gln)	2	0.7
ATT (Ile)	3	1.0	AAT (Asn)	0	0.0
ATC (Ile)	1	0.3	AAC (Asn)	8	2.8
ATA (Ile)	5	1.7	AAA (Lys)	15	5.2
ATG (Met)	6	2.1	AAG (Lys)	5	1.7
GTT (Val)	3	1.0	GAT (Asp)	8	2.8
GTC (Val)	1	0.3	GAC (Asp)	10	3.4
GTA (Val)	6	2.1	GAA (Glu)	6	2.1
GTG (Val)	4	1.4	GAG (Glu)	5	1.7
TCT (Ser)	5	1.7	TGT (Cys)	6	2.1
TCC (Ser)	4	1.4	TGC (Cys)	3	1.0
TCA (Ser)	2	0.7	TGA (End)	0	0.0
TCG (Ser)	2	0.7	TGG (Trp)	2	0.7
CCT (Pro)	3	1.0	CGT (Arg)	5	1.7
CCC (Pro)	6	2.1	CGC (Arg)	4	1.4
CCA (Pro)	14	4.8	CGA (Arg)	2	0.7
CCG (Pro)	2	0.7	CGG (Arg)	1	0.3
ACT (Thr)	4	1.4	AGT (Ser)	2	0.7
ACC (Thr)	5	1.7	AGC (Ser)	7	2.4
ACA (Thr)	7	2.4	AGA (Arg)	2	0.7
ACG (Thr)	10	3.4	AGG (Arg)	4	1.4
GCT (Ala)	6	2.1	GGT (Gly)	7	2.4
GCC (Ala)	7	2.4	GGC (Gly)	2	0.7
GCA (Ala)	16	5.5	GGA (Gly)	3	1.0
GCG (Ala)	8	2.8	GGG (Gly)	2	0.7

Table 6.5 Codon usage and predicted amino acid composition of EHV-4 B4 (290 amino acids).

FIGURE 6.16

Alignment of the Predicted Partial Amino Acid Sequence of EHV-4 B5 With the Analogous Gene Products of HSV-1 and VZV

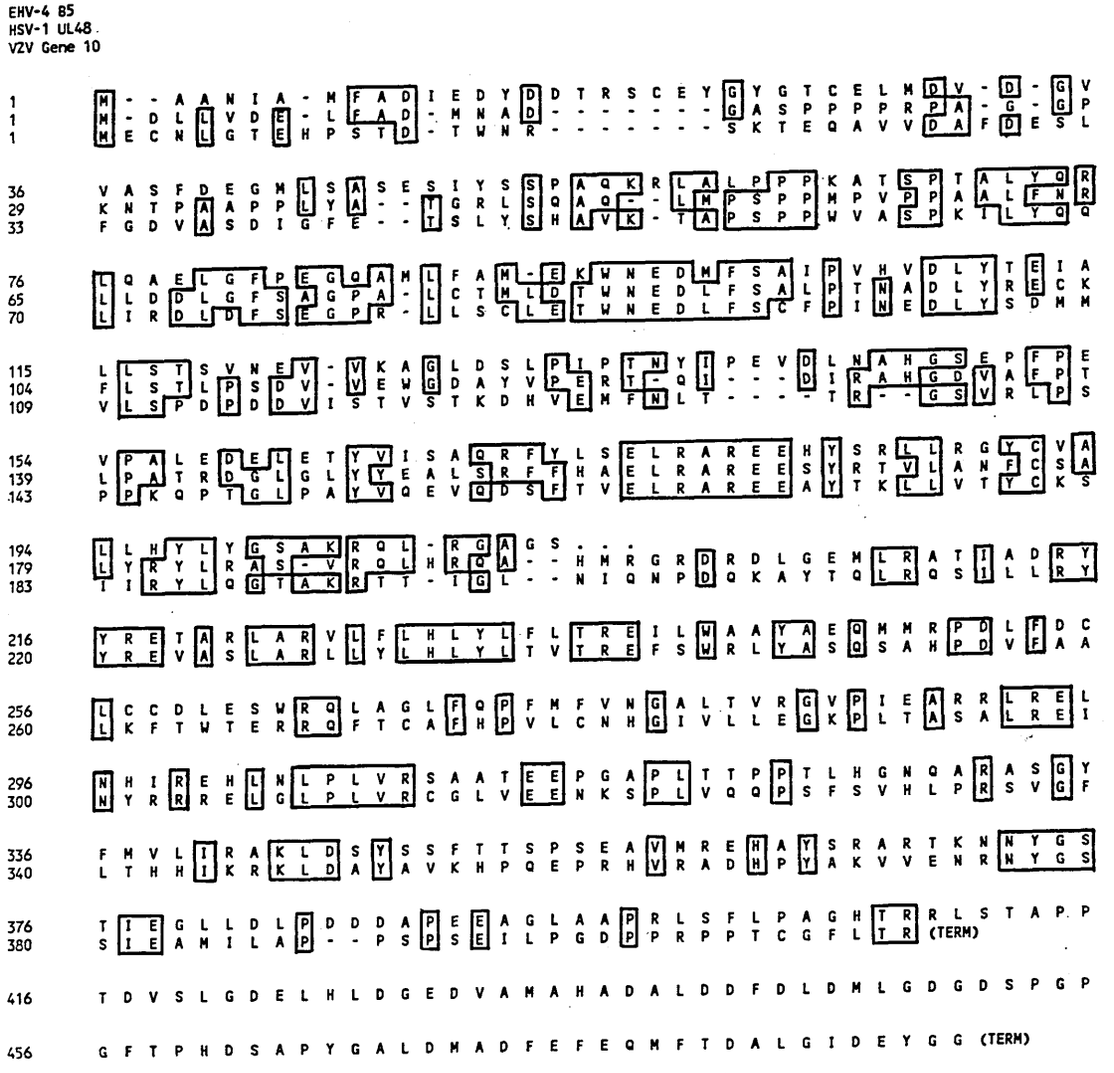
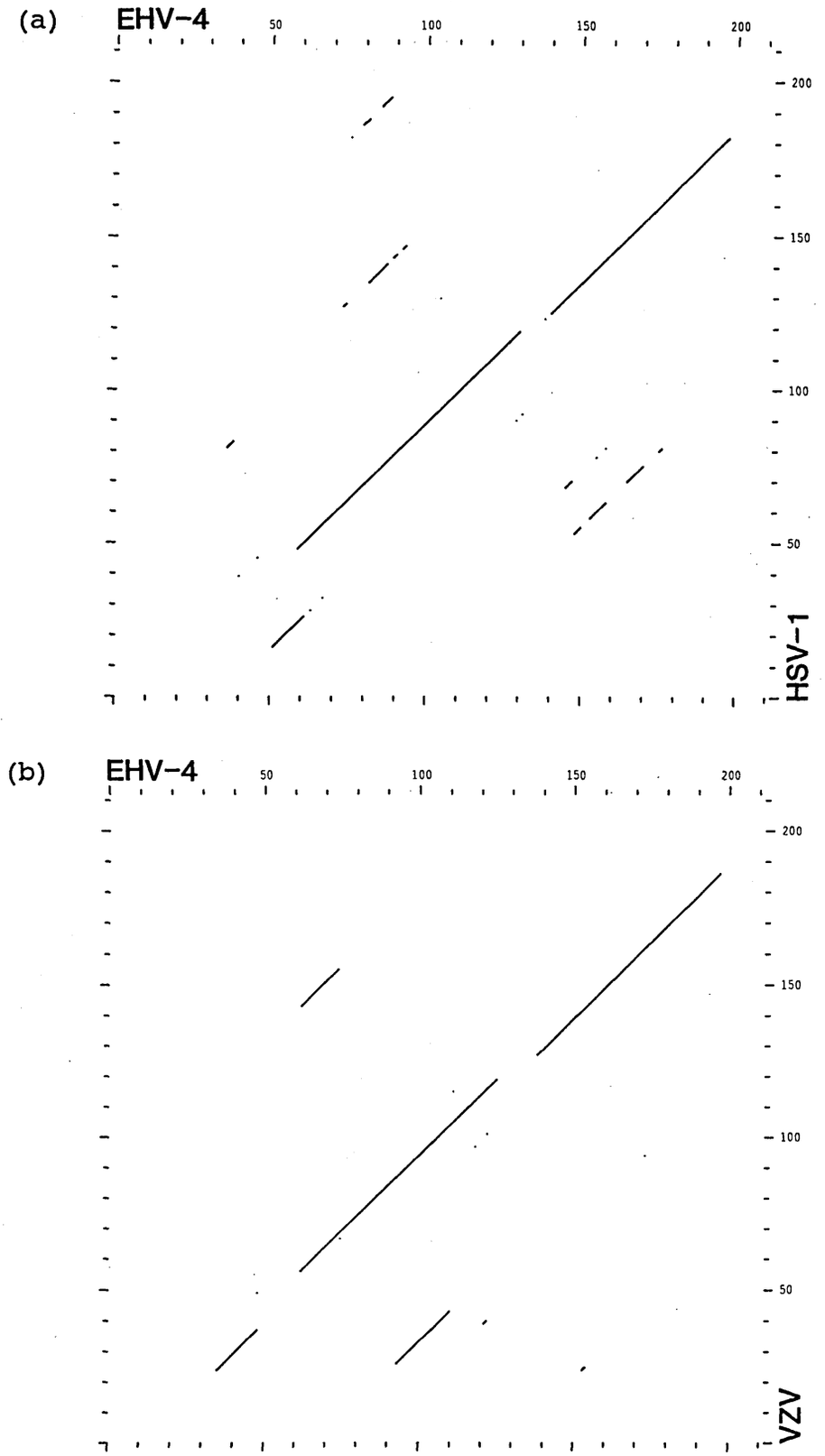


Figure 6.16 Direct alignment of the predicted amino acid sequence of the partial gene product of EHV-4 B5 with the analogous gene products of HSV-1 UL48 and VZV gene 10. The available 211 amino acids of the EHV-4 gene product, and the entire HSV-1 and VZV gene product sequences, are shown. Identical residues are boxed and dashes denote spaces introduced to maximise alignment of the sequences.

Figure 6.17 Dot matrix similarity analysis of the EHV-4 B5 partial amino acid sequence against those of the corresponding region of the gene products of HSV-1 UL48 and VZV gene 10. Analysis was carried out using COMPARE and DOTPLOT from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984). In each plot, the horizontal axis is the EHV-4 B5 partial amino acid sequence. Vertical axes represent the amino acid sequences of the corresponding region of the gene products of HSV-1 UL48 (a) and VZV gene 10 (b). Points are plotted when at least 15 amino acids in a moving window of 30 are identical. Large regions of similarity are indicated by uninterrupted diagonal lines from lower left to upper right.

F I G U R E 6.17

Dot Matrix Similarity Analysis of the EHV-4 B5 Partial Amino Acid Sequence Against Those of the Corresponding Region of the Gene Products of HSV-1 UL48 and VZV Gene 10



F I G U R E 6.18

Hydropathic Analysis of the Partial Gene Product of EHV-4 B5 and the Corresponding Region of the Gene Products of HSV-1 UL48 and VZV Gene 10

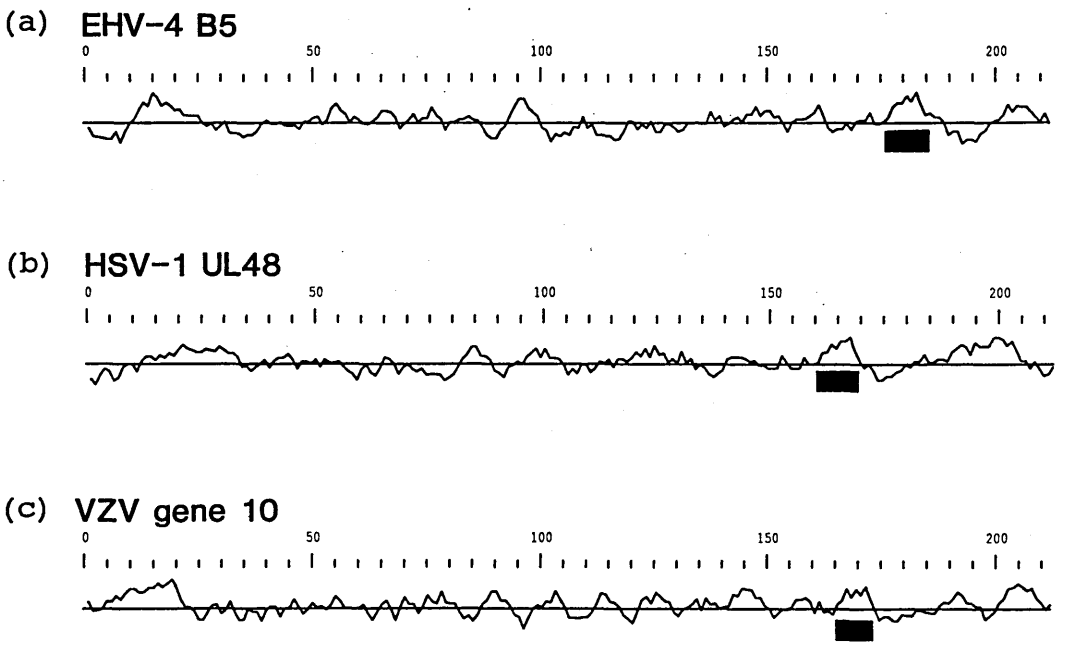


Figure 6.18 Hydropathic plots of the partial gene product of EHV-4 B5 (a) and the corresponding region of the gene products of HSV-1 UL48 (b) and VZV gene 10 (c) as determined by the method of Kyte and Doolittle (1982). Points above the horizontal line represent regions of above average hydrophilicity. In each plot, the shaded box indicates a region of very hydrophilic amino acids which is highly conserved between the proteins (see text for details).

TABLE 6.6

Partial Composition of EHV-4 Protein B5

Codon (amino acid)	Frequency	% of total	Codon (amino acid)	Frequency	% of total
TTT (Phe)	5	2.4	TAT (Tyr)	5	2.4
TTC (Phe)	2	0.9	TAC (Tyr)	8	3.8
TTA (Leu)	4	1.9	TAA (End)	0	0.0
TTG (Leu)	3	1.4	TAG (End)	0	0.0
CTT (Leu)	2	0.9	CAT (His)	2	0.9
CTC (Leu)	1	0.5	CAC (His)	2	0.9
CTA (Leu)	6	2.8	CAA (Gln)	3	1.4
CTG (Leu)	8	3.8	CAG (Gln)	3	1.4
ATT (Ile)	2	0.9	AAT (Asn)	0	0.0
ATC (Ile)	1	0.5	AAC (Asn)	5	2.4
ATA (Ile)	5	2.4	AAA (Lys)	2	0.9
ATG (Met)	7	3.3	AAG (Lys)	3	1.4
GTT (Val)	3	1.4	GAT (Asp)	6	2.8
GTC (Val)	0	0.0	GAC (Asp)	6	2.8
GTA (Val)	7	3.3	GAA (Glu)	7	3.3
GTG (Val)	2	0.9	GAG (Glu)	13	6.2
TCT (Ser)	2	0.9	TGT (Cys)	3	1.4
TCC (Ser)	2	0.9	TGC (Cys)	0	0.0
TCA (Ser)	2	0.9	TGA (End)	0	0.0
TCG (Ser)	4	1.9	TGG (Trp)	1	0.5
CCT (Pro)	0	0.0	CGT (Arg)	1	0.5
CCC (Pro)	6	2.8	CGC (Arg)	4	1.9
CCA (Pro)	5	2.4	CGA (Arg)	1	0.5
CCG (Pro)	2	0.9	CGG (Arg)	3	1.4
ACT (Thr)	1	0.5	AGT (Ser)	1	0.5
ACC (Thr)	6	2.8	AGC (Ser)	7	3.3
ACA (Thr)	1	0.5	AGA (Arg)	1	0.5
ACG (Thr)	0	0.0	AGG (Arg)	0	0.0
GCT (Ala)	6	2.8	GGT (Gly)	2	0.9
GCC (Ala)	7	3.3	GGC (Gly)	5	2.4
GCA (Ala)	6	2.8	GGA (Gly)	4	1.9
GCG (Ala)	4	1.9	GGG (Gly)	1	0.5

Table 6.6 Partial codon usage and predicted amino acid composition of EHV-4 B5 (amino-terminal 211 amino acids).

FIGURE 6.19

Alignment of the Predicted Amino Acid Sequence of EHV-4 B6 With the Analogous Gene Products of HSV-1 and VZV

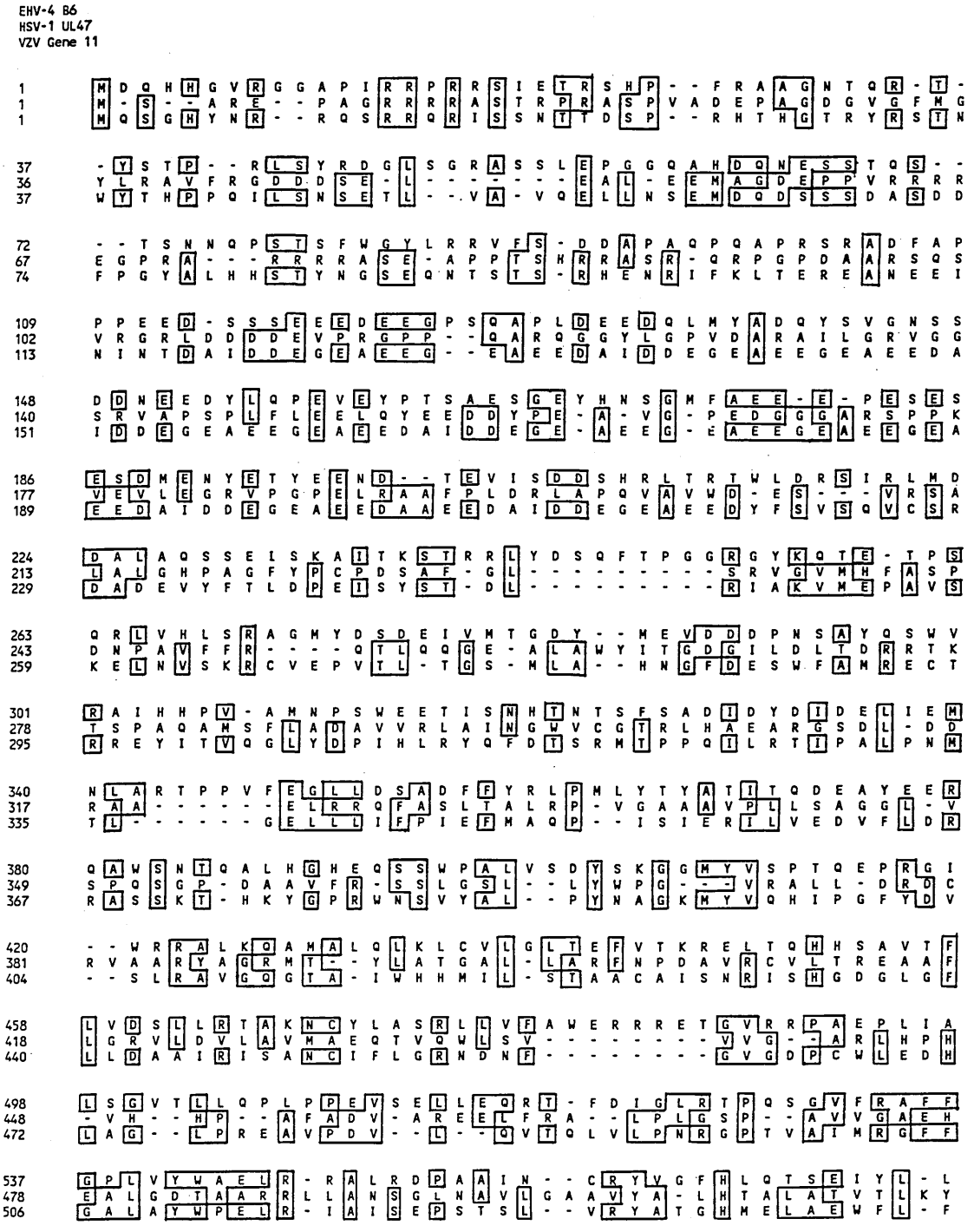


Figure 6.19- continued

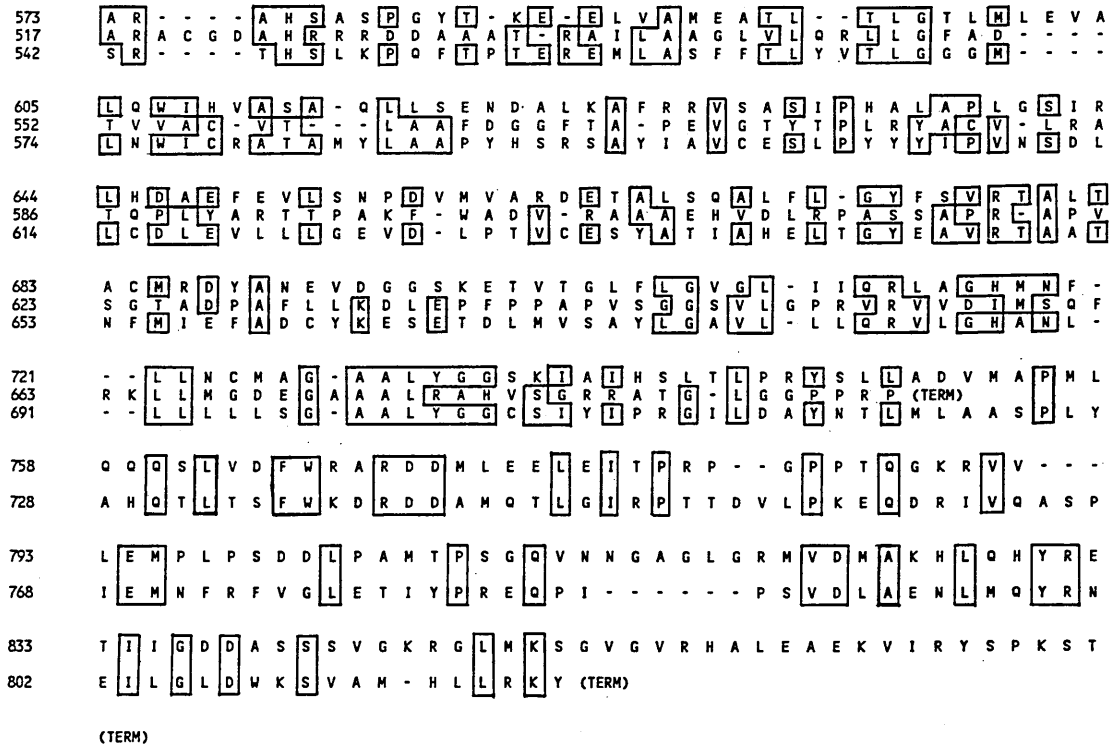
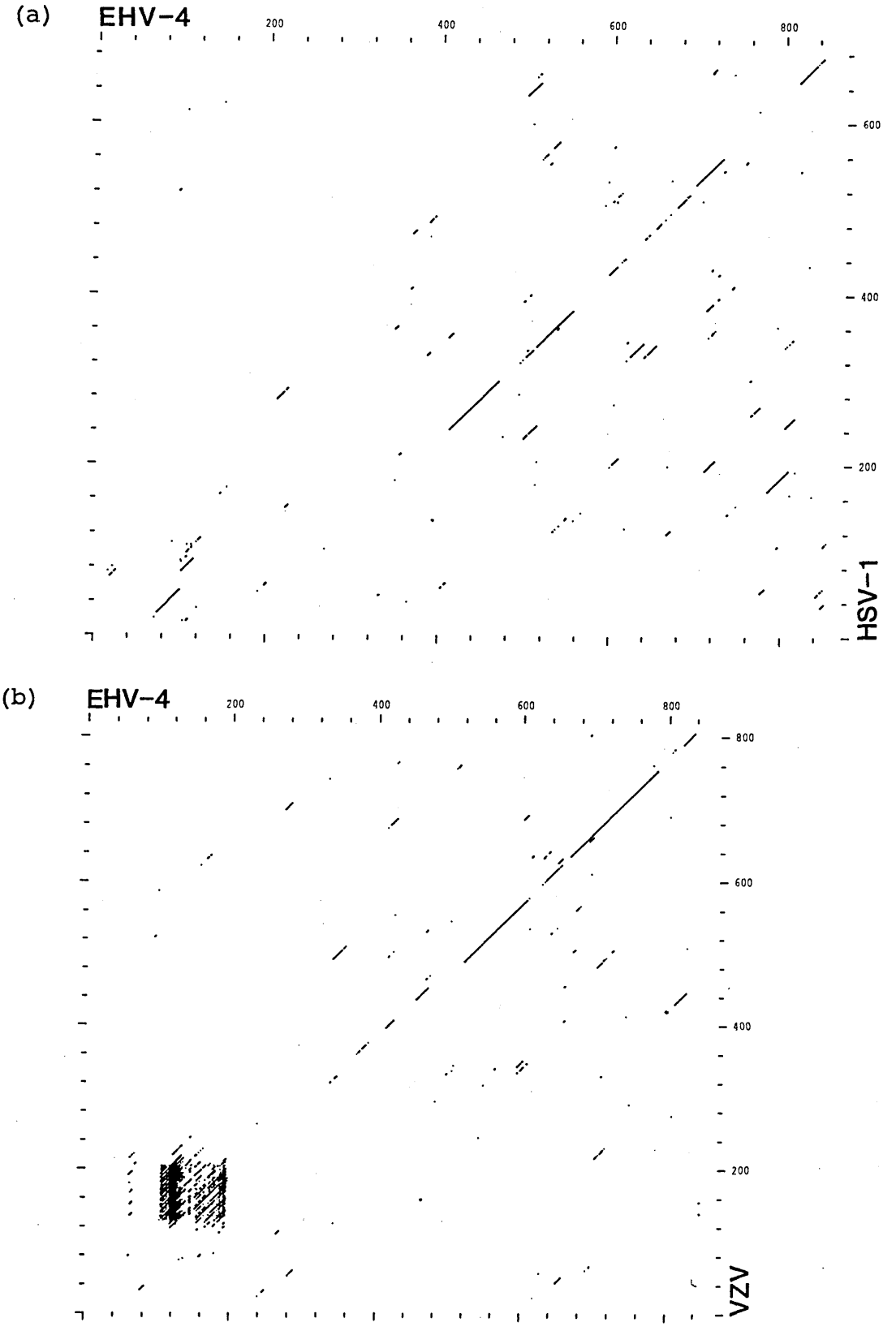


Figure 6.19 Direct alignment of the predicted amino acid sequence of the gene product of EHV-4 B6 with the analogous gene products of HSV-1 UL47 and VZV gene 11. Identical residues are boxed and dashes denote spaces introduced to maximise alignment of the sequences.

Figure 6.20 Dot matrix similarity analysis of the EHV-4 B6 amino acid sequence against those of the gene products of HSV-1 UL47 and VZV gene 11. Analysis was carried out using COMPARE and DOTPLOT from the University of Wisconsin Genetics Computer Group programmes (Devereux et al., 1984). In each plot, the horizontal axis is the EHV-4 B6 amino acid sequence. Vertical axes represent the amino acid sequences of the gene products of HSV-1 UL47 (a) and VZV gene 11 (b). Points are plotted when at least 15 amino acids in a moving window of 30 are identical. Large regions of similarity are indicated by uninterrupted diagonal lines from lower left to upper right.

F I G U R E 6.20

Dot Matrix Similarity Analysis of the EHV-4 B6 Amino Acid Sequence Against Those of the Gene Products of HSV-1 UL47 and VZV Gene 11



F I G U R E 6.21

Hydropathic Analysis of the Gene Products of EHV-4 B6, HSV-1 UL47 and VZV Gene 11

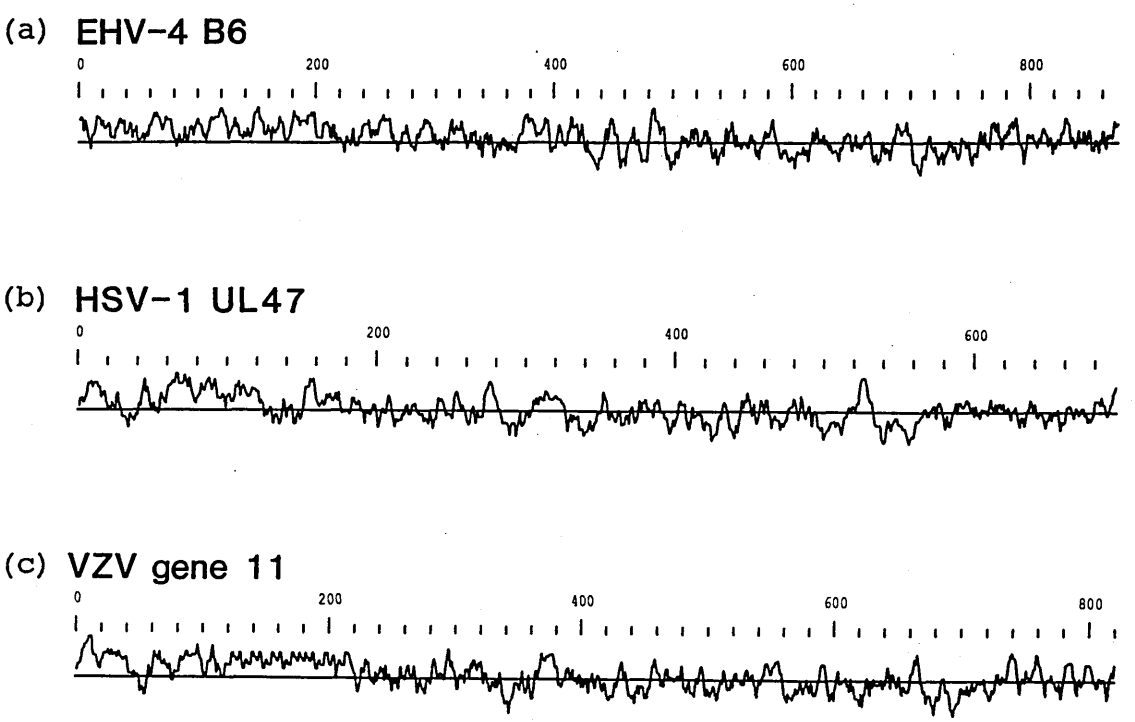


Figure 6.21 Hydropathic plots of the gene products of EHV-4 B6 (a), HSV-1 UL47 (b) and VZV gene 11 (c) as determined by the method of Kyte and Doolittle (1982). Points above the horizontal line represent regions of above average hydrophilicity.

TABLE 6.7

Composition of EHV-4 Protein B6

Codon (amino acid)	Frequency	% of total	Codon (amino acid)	Frequency	% of total
TTT (Phe)	23	2.6	TAT (Tyr)	15	1.7
TTC (Phe)	2	0.2	TAC (Tyr)	18	2.1
TTA (Leu)	12	1.4	TAA (End)	0	0.0
TTG (Leu)	19	2.2	TAG (End)	0	0.0
CTT (Leu)	20	2.3	CAT (His)	10	1.1
CTC (Leu)	11	1.3	CAC (His)	14	1.6
CTA (Leu)	18	2.1	CAA (Gln)	17	1.9
CTG (Leu)	8	0.9	CAG (Gln)	22	2.5
ATT (Ile)	8	0.9	AAT (Asn)	11	1.3
ATC (Ile)	6	0.7	AAC (Asn)	13	1.5
ATA (Ile)	15	1.7	AAA (Lys)	13	1.5
ATG (Met)	27	3.1	AAG (Lys)	5	0.6
GTT (Val)	13	1.5	GAT (Asp)	25	2.9
GTC (Val)	4	0.5	GAC (Asp)	29	3.3
GTA (Val)	10	1.1	GAA (Glu)	40	4.6
GTG (Val)	18	2.1	GAG (Glu)	30	3.4
TCT (Ser)	25	2.9	TGT (Cys)	4	0.5
TCC (Ser)	8	0.9	TGC (Cys)	1	0.1
TCA (Ser)	18	2.1	TGA (End)	0	0.0
TCG (Ser)	3	0.3	TGG (Trp)	11	1.3
CCT (Pro)	7	0.8	CGT (Arg)	8	0.9
CCC (Pro)	18	2.1	CGC (Arg)	25	2.9
CCA (Pro)	20	2.3	CGA (Arg)	7	0.8
CCG (Pro)	6	0.7	CGG (Arg)	1	0.1
ACT (Thr)	16	1.8	AGT (Ser)	10	1.1
ACC (Thr)	9	1.0	AGC (Ser)	18	2.1
ACA (Thr)	17	1.9	AGA (Arg)	16	1.8
ACG (Thr)	10	1.1	AGG (Arg)	7	0.8
GCT (Ala)	17	1.9	GGT (Gly)	13	1.5
GCC (Ala)	22	2.5	GGC (Gly)	15	1.7
GCA (Ala)	22	2.5	GGA (Gly)	12	1.4
GCG (Ala)	16	1.8	GGG (Gly)	14	1.6

Table 6.7 Codon usage and predicted amino acid composition of EHV-4 B6 (872 amino acids).

FIGURE 6.22

Characterisation of the EHV-1 DNA Sequence Encoding a gp10 Epitope

(a) λgt11/EHV-1 insert

```
      Q  A  P  R  P  R  A  D  F  A  P  P  S  G  E  E  S  S     18
GAATTCCAAGCACCCAGACCTCGCGCGGACTTTGCACCGCCCTCCGGCGAGGAATCATCT   60
EcoRI

 S  E  E  E  E  E  E  G  P  A  Q  A  P  L  D  E  E  D  Q  L    38
AGCGAGGAAGAGGAGGAAGAGGGTCCCGCCCAAGCTCCGCTGGACGAGGAAGACCAGCTA  120

 M  Y  A  D  Q  Y  S  V  G  D  S  S  D  E  N  D  E  E  E  D    58
ATGTATGCTGACCAGTACTCTGTAGGGGACTCTAGTGACGAAAACGACGAGGAAGAAGAC  180

 P  R  L  G  S  D  Y  P  T  S  A  E  S                        71
CCCCGTCTAGGATCTGACTATCCCACGTCCGCCGAATCCAGAATTC                226
                                      EcoRI
```

(b)

```
EHV-4 B6
EHV-1 insert

98      Q A P R S R A D F A P P P E E D S S S E
        | | | |   | | | | | | |   | |   | | | |
1       Q A P R P R A D F A P P S G E E S S S E

118     E E D E E G P S Q A P L D E E D Q L M Y
        | |   | | | |   | | | | | | | | | | | |
21      E E E E E G P A Q A P L D E E D Q L M Y

138     A D Q Y S V G N S S D D N E E D Y L Q P
        | | | | | | |   | | |   | |   |
41      A D Q Y S V G D S S D E N D E E E D P R

158     E V E - Y P T S A E S
                | | | | | | |
61      L G S D Y P T S A E S
```

Figure 6.22 Sequence of the DNA insert in a recombinant λ gt11 bacteriophage known to encode an epitope of the EHV-1 glycoprotein gp10. (a) DNA sequence of the 226bp DNA insert and predicted amino acid sequence of the partial gene product it encodes. (b) Alignment of the amino acid sequence of the EHV-1 partial gene product in (a) with the corresponding region of the EHV-4 B6 gene product. The upper sequence is that of the EHV-4 partial gene product, and the lower sequence that of the EHV-1 partial gene product. Identical residues are indicated with a vertical bar, and dashes denote spaces introduced to maximise alignment of the two sequences. Residue 1 of the EHV-1 sequence aligns with residue 98 of the EHV-4 sequence.

FIGURE 6.23

DNA Sequence of EHV-4 BamHI-Q and the Adjoining Left-Hand End of BamHI-M

(a) EHV-4 major DNA binding protein (partial)

```
   I  L  F  Y  L  E  R  C  D  A  G  T  F  G  G  R  N  E  T  D  A  L  R  Y  L  A  N  T  L  E  S  E  V  P  C  G  L  C  T
GGATCCTATTTTACCTCGAAAGATGTGACGCTGGAACATTTGGGGGTCGCAACGAGACAGATGCACTGCGTTACTTGGCAAACACGCTAGAGTCTGAGGTACCATGTGGGTTGTGTACCC  120
BamHI-Q

P  A  T  R  P  A  C  A  H  T  T  L  H  R  L  R  Q  R  L  P  R  F  G  T  P  V  R  A  P  I  G  I  F  G  T  M  N  S  T  Y
CAGCTACGCGGCCGGCATGCGCTCATACCACGCTCCATCGTCTCCGGCAGCGTCTGCCACGCTTTGGAACGCCAGTTCGTGCTCCAATAGGAATATTTGGCACAATGAACAGCACGTATA  240

S  D  C  D  V  L  G  N  Y  A  S  Y  G  A  L  K  R  P  N  D  N  E  A  P  K  S  I  M  Q  D  T  Y  R  A  T  M  E  R  L  V
GCGACTGTGATGTACTGGGTAACTATGCTTCCTACGGGGCGCTAAAGCGACCCAATGACAACGAAGCCCCCAAAAGCATCATGCAGGATACGTATCGTGCTACTATGGAGCGACTGGTAA  360

N  D  L  E  Q  A  K  L  I  D  K  E  A  L  A  H  A  G  T  C  S  A  S  T  G  V  V  K  D  Q  A  S  F  I  N  L  L  S  T  I
ATGACCTGGAACAGGCTAAGCTTATTGACAAGGAAGCGCTGGCTCATGCCGGCACCTGCTCGGCCTCCACAGGCGTAGTAAAGGACCAGGCCAGCTTTATAAATCTTTTGTCTACAATCA  480

K  D  I  T  E  G  A  A  E  Q  F  M  R  T  L  V  E  V  R  D  F  K  I  R  E  G  L  A  D  A  N  H  T  M  S  I  S  L  D  P
AAGACATAACTGAGGGGGCAGCAGAGCAGTTTATGCGCACTTTGGTTGAGGTTCGCGATTTTAAAATCCGCGAAGGCCTGGCAGATGCAAACCATACCATGTCAATTTCCCTGGATCCAT  600
                                                                                                                BamHI-M

Y  S  S  S  F  C  P  V  T  S  F  L  S  R  R  T  I  F  A  V  L  Q  D  L  V  L  S  Q  C  H  C  L  F  Y  G  Q  S  V  E  G
ATTCCAGCAGTTTTTGTCCAGTTACATCATTTCTCTCGCGCCGCACCATTTTTGCTGTTTTGCAGGACCTAGTATTGAGCCAGTGTCACTGTCTTTTCTACGGTCAGTCGGTGGAGGGGC  720

R  N  F  R  N  Q  F  Q  P  V  L  R  R  R  F  L  D  M  L  N  G  G  F  I  T  A  K  T  V  T
GCAACTTTCGCAACCAGTTTCAGCCAGTTTTAAGACGTAGATTTTTAGATATGCTCAACGGGGGCTTTATCACTGCTAAAACCGTAACA                                809
```

(b) EHV-4 major DNA binding protein (partial)
HSV-1 UL29
VZV Gene 29

```
1    I L F Y L E R C D A G T F G G R N E T D A L R Y L A N T L E S E V P C G L C T P
465  M L F Y L E R C D G A V I V G R Q E M D V F R Y V A D S N Q T D V P C N L C T F
464  L L F Y L E R C D A G A F T G G H G - D A L K Y V T G T F D S E I P C S L C E K

41   A T R P A C A H T T L H R L R Q R L P R F G T P V R A P I G I F G T M N S T Y S
505  D T R H A C V H T T L M R L R A R H P K F A S A A R G A I G V F G T M N S M Y S
503  H T R P V C A H T T V H R L R Q R M P R F G Q A T R Q P I G V F G T M N S Q Y S

81   D C D V L G N Y A S Y G A L K R P N D N - E A P K S I M Q D T Y R A T M E R L V
545  D C D V L G N Y A A F S A L K R A D G S - E T A R T I M Q E T Y R A A T E R V M
543  D C D P L G N Y A P Y L T L R K P G D Q T E A A K A T M Q D T Y R A T L E R L F

120  N D L E Q A K L I D K E A L A H A - G T C S A S T G V V K D Q A S F I N L L S T
584  A E L E T L Q Y V D Q - A V P T A M G R L E T I I T N R E A L H T V V N N V R Q
583  I D L E Q E R L L D R G A P C S S E G L S S - - - - V I V D H P T F R R I L D T

159  I K D I T E G A A E Q F M R T L V E V R D F K I R E G L A D A N H T M S I S L D
623  V V D R - E V - - E Q L M R N L V E G R N F K F R D G L G E A N H A M S L T L D
619  L R A R I E Q T T T Q F M K V L V E T R D Y K I R E G L S E A T H S M A L T F D

199  P Y S S S F C P V T S F L S R R T I F A V L Q D L V L S Q C H C L F Y G Q S V E
660  P Y A C G P C P L L Q L L G R R S N L A V Y Q D L A L S Q C H G V F A G Q S V E
659  P Y S G A F C P I T N F L V K R T H L A V V Q D L A L S Q C H C V F Y G Q Q V E

239  G R N F R N Q F Q P V L R R R F L D M L N G G F I T A K T V T . . .
700  G R N F R N Q F Q P V L R R R V M D M F N N G F L S A K T L T . . .
699  G R N F R N Q F Q P V L R R R F V D L F N G G F T S T R S I T . . .
```

Figure 6.23 DNA sequence of EHV-4 BamHI-Q and the left-hand end of BamHI-M which contains a partial ORF encoding the major DNA binding protein. (a) DNA sequence of EHV-4 BamHI-Q and the left-hand end of BamHI-M and predicted amino acid sequence of the partial gene product it encodes. (b) Alignment of the partial amino acid sequence of the EHV-4 major DNA binding protein in (a) with the corresponding region of the major DNA binding proteins of HSV-1 (encoded by UL29) and VZV (encoded by gene 29). Identical residues are boxed and dashes denote spaces introduced to maximise alignment of the sequences. Residue 1 of the EHV-4 sequence aligns with residues 465 and 464 of the HSV-1 and VZV sequences, respectively.

D I S C U S S I O N

Determination of the DNA sequence of the EHV-4 genome between 0.067 and 0.122 m.u. has revealed a gene arrangement in this region identical to that in the HSV-1 (I_L) and VZV genomes. The proteins specified by the EHV-4 genes demonstrated weak to moderate homologies with the analogous gene products of HSV-1 and VZV (Table 6.2). The analysis of these predicted EHV-4 gene products and comparison with analogous proteins of HSV-1 and VZV sheds new light on the extent of their divergence of structure and permits the prediction of a likely function for some of these proteins on the basis of our knowledge of the molecular genetics of HSV-1.

The protein encoded by EHV-4 ORF B2 is the best conserved species of those analysed in this section and shows a significantly higher homology to the gene product of VZV gene 7 (45%) than to that of HSV-1 UL51 (38%). An important conserved function is predicted for the amino-terminal region of the molecules whereas the highly divergent carboxyl-terminal region may have a less conserved role in protein function. Davison and Taylor (1987) have proposed that an analogous protein may be specified by EBV BSRF1 on the basis of possessing a hydropathic profile similar to that of the gene product of VZV gene 7, although the proteins share little detectable amino acid homology. This indicates that these proteins may have an important conserved, albeit an as yet unknown, function in EHV-4, HSV-1, VZV and EBV.

The protein specified by EHV-4 ORF B3 shows more limited homology (about 30%) to the dUTPase of HSV-1 and VZV. The even

distribution of homology across the molecules makes it difficult to identify specific regions which may be essential for protein function, although the carboxyl-terminal half of the proteins shows the greatest identity. A dUTPase is also thought to be specified by EBV BLLF2 on the basis of predicted amino acid sequence homology (Davison and Taylor, 1987). The conservation of this protein in both alpha- and gammaherpesviruses reflects the essential role it plays in cell metabolism.

The gene product of EHV-4 ORF B4 shows about 30% homology to the gene products of HSV-1 UL49 and VZV gene 9 and only 11% of residues are shared by all three proteins. However, a relatively well conserved region in the carboxyl-terminal half of these proteins corresponding to residues 165 to 243 of the EHV-4 protein may have an important role in protein function.

Although only the first 211 amino acids of the EHV-4 B5 protein were available for analysis, alignment of these residues with the amino acid sequences of the analogous proteins of HSV-1 and VZV identified two well conserved peptides in the EHV-4 protein, ELRAREE and KWNEDMFS, which may be important for protein function. The protein specified by HSV-1 UL48, the α-TIF, is a particularly well studied virion component which activates, in trans, the expression of IE genes. Three temporal classes of genes have been distinguished for HSV-1: IE (α), early (β) and late (γ) (Honess and Roizman, 1974, 1975). Their expression is coordinately regulated and sequentially ordered in a cascade fashion. HSV-1 specifies five IE genes, referred to as IE1/IE110/ICP0 (two copies), IE2/IE63/ICP27, IE3/IE175/ICP4 (two copies), IE4/IE68/ICP22 and IE5/IE12/ICP47 (Watson et al., 1979; Murchie and McGeoch, 1982; Everett, 1984,

1986; McGeoch et al., 1985, 1986a; Sacks et al., 1985; Perry et al., 1986; Perry and McGeoch, 1988), which are the first genes to be expressed after infection and in the absence of de novo protein synthesis and whose expression is normally repressed at the onset of early transcription (Honess and Roizman, 1974, 1975; Preston, 1979; Dixon and Schaffer, 1980). One IE protein, IE175, has long been recognised as being required for continuous expression of early and late genes (Knipe et al., 1978; Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1980), although other IE proteins are also known to be involved in regulating transcription of later classes of genes (Everett, 1984, 1986; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985; Sacks et al., 1985; Sears et al., 1985). Transcription from all IE genes was shown to be stimulated by a virion structural component (Post et al., 1981; Mackem and Roizman, 1982; Cordingley et al., 1983; Preston et al., 1984). Analysis of the 5' regulatory regions of the HSV-1 IE genes identified a cis-acting element essential for this stimulatory response, TAATGARATTC, which was present in one or more copies upstream of all HSV-1 IE genes (Mackem and Roizman, 1982; Murchie and McGeoch, 1982; Whitton et al., 1983; Whitton and Clements, 1984), and it was concluded that activation of IE gene expression occurs by direct or indirect interaction of the virion structural component with this sequence. Campbell et al. (1984) were the first workers to identify the gene specifying the stimulatory virion component, a virion tegument structural protein that is the α-TIF. Recent studies have shown that α-TIF actually interacts with several other cellular factors to form a complex which then binds to TAATGARATTC (Kristie et al.,

1989).

The VZV genome contains three IE genes, namely gene 4, gene 63/70 and gene 62/71, which are analogous to HSV-1 IE63, IE68 and IE175, respectively (Davison and Scott, 1986a). Since the VZV IE genes do not possess an upstream TAATGARATTC sequence, it seems possible that the putative VZV α-TIF may either recognise a different upstream element or even lack the ability to stimulate the transcription of IE genes. EHV-1 has been demonstrated to encode four IE proteins generated from a single IE transcript present in two copies in IR_S/TR_S (Caughman et al., 1985; Gray et al., 1987a). The EHV-1 IE gene has been sequenced and its product shown to be homologous to the HSV-1 IE175 and VZV gene 62/71 gene products, and it has been reported that the EHV-1 IE gene contains a potential TAATGARATTC element upstream of its promoter (Grundy et al., 1989). On the basis of this, it seems likely that transcription from the EHV-1 IE gene may follow a mechanism similar to that identified for HSV-1 IE genes. An interesting point is that, in contrast to findings for EHV-1, a homologue of the HSV-1 IE68 gene has been identified in the EHV-4 genome (Cullinane et al., 1988). This suggests that EHV-4 may have a different number of IE genes than does EHV-1, since the EHV-4 genome is also known to specify a gene analogous to HSV-1 IE175 (Cullinane et al., 1988). Alternatively, the EHV-4 gene analogous to HSV-1 IE68 may be regulated as an early gene. Detailed characterisation of the EHV-1 and EHV-4 genomes will provide an answer to this. The gene product of EBV BMLF1 is homologous to the gene products of HSV-1 IE63 and VZV gene 4 and represents the only conserved IE protein specified by EBV (Baer et al., 1984; Davison and Taylor, 1987; McGeoch et al., 1988a).

The gene product of EHV-4 ORF B6 shows limited conservation of amino acid sequence with the analogous HSV-1 UL47 and VZV gene 11 gene products. Of particular note is the very hydrophilic nature of the amino-terminal 200 amino acids of these proteins, which may play an important role in protein function. The HSV-1 UL47 and UL46 gene products have been demonstrated to decrease and increase the α-TIF-dependent activation of IE genes, respectively (McKnight et al., 1987). McKnight et al. (1987) proposed a number of possible functions for the HSV-1 UL47 and UL46 gene products. Their suggestions included that these proteins may form a complex with the α-TIF and determine its functions, that they alter the physiological state of the cell such that it is not specific for α-TIF or, alternatively, that they are transcriptional factors in their own right. Further studies will be necessary to determine the precise function of these proteins in EHV-4, HSV-1 and VZV.

The gene product of EHV-4 ORF B6 is a particularly interesting species. The data presented in this chapter demonstrated that EHV-4 ORF B6 maps to a region of the genome known to contain the gene for glycoprotein gp10 (Allen and Yeargan, 1987). Glycoprotein gp10 of EHV-1 and EHV-4 has been regarded as a major glycoprotein on the basis of incorporation of [^{3}H]-glucosamine (Allen and Bryans, 1986; Allen and Yeargan, 1987) and additionally for EHV-1 by in vitro labelling with UDP-[^{14}C]-galactose and [^{3}H]-borohydride (Turtinen and Allen, 1982). It was therefore assumed that gp10 would possess features typical of herpesvirus envelope glycoproteins, namely a hydrophobic signal sequence at the amino terminus and a hydrophobic transmembrane domain towards the carboxyl terminus.

Since the gene product of EHV-4 ORF B6 possesses none of these features, it seems likely that gp10 may reside in the virion tegument as does the analogous protein specified by HSV-1 UL47. Further studies have demonstrated that, in accordance with the amino acid homology observed between EHV-4 B6 (gp10) and the gene product of HSV-1 UL47 in this chapter, these species are antigenically cross-reactive (Whittaker et al., submitted). Polyclonal antisera raised against VP13/14, the gene product of HSV-1 UL47, was shown to be cross-reactive with gp10 of both EHV-1 (120kDa) and EHV-4 (123kDa). By a combination of immune precipitation and Western blotting it was confirmed that this polyclonal antisera and a monoclonal antibody specific for EHV-1 gp10 (13A9) both reacted with the same protein in EHV-1-infected cells. Since EHV-4 gp10 has no signal sequence for membrane insertion, it may be glycosylated by the addition of O-glycosyl-linked N-acetylglucosamine within the cytoplasm of the cell. The molecular mass of 123kDa for mature gp10 in the EHV-4 virion, compared with the predicted molecular mass of about 97kDa for the gene product of EHV-4 ORF B6, suggests that carbohydrate residues on gp10 represent 26kDa. Further characterisation of gp10 of EHV-1 and EHV-4 should prove particularly interesting and help elucidate the precise functions of this novel, glycosylated tegument protein.

The DNA sequence of EHV-4 BamHI-Q and the left-hand terminus of the adjoining BamHI-M identified part of the gene specifying the major DNA binding protein. Homology has been demonstrated between the major DNA binding proteins of HSV-1 and VZV and the gene product of EBV BALF2 (Quinn and McGeoch, 1985; Davison and Taylor, 1987; McGeoch et al., 1988a). The highly

conserved nature of the major DNA binding protein of the alphaherpesviruses and the observation that an analogous protein is also specified by the distantly related EBV is consistent with an essential, conserved role for this protein in viral DNA replication (Conley et al., 1981; Weller et al., 1983; Challberg, 1986). In contrast to the generally low levels of homology displayed by the EHV-4 genes mapping in the region 0.067 to 0.122 m.u. to their HSV-1 and VZV counterparts, the EHV-4 major DNA binding protein gene is contained within a cluster of genes specifying proteins that are highly conserved between HSV-1 and VZV, and which have detectable homologues in the EBV genome. These genes are those encoding glycoprotein gB, ICP18.5, major DNA binding protein and DNA polymerase. Although sequencing of the EHV-4 genome was not sufficiently extended in this region to detect the DNA polymerase gene, it is certain that this gene will map to the left of the major DNA binding protein gene, within BamHI-J, on the basis of colinearity with the HSV-1 and VZV genomes. This gene cluster is located near the centre of U_L in the alphaherpesvirus genomes but occurs close to the right terminus of the EBV genome (Quinn and McGeoch, 1985). Furthermore, the EBV DNA polymerase gene is relocated relative to the gB and ICP18.5 genes. Conservation of this gene cluster has also been reported for the gammaherpesvirus HVS, and the order of individual genes within this cluster is identical to that seen in EBV although the gene cluster is located near the left terminus of the HVS genome and the genes are transcribed in the opposite orientation to that found in the EBV genome (Albrecht and Fleckenstein, 1990).

By virtue of transfection studies and characterisation of

mutants defective in DNA replication, the major DNA binding protein and DNA polymerase have been shown to be two of seven HSV-1 gene products required for origin-dependent DNA replication (Chartrand et al., 1979; Conley et al., 1981; Weller et al., 1983; Gibbs et al., 1985; Challberg, 1986; McGeoch et al., 1988b; Wu et al., 1988). This region of the HSV-1 genome (0.38 to 0.43 m.u.) is particularly important in DNA replication since it also contains an origin of replication (ori_L) between the major DNA binding protein and DNA polymerase genes (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller et al., 1985). The VZV and EBV genomes do not contain an origin of replication in this region (Baer et al., 1984; Davison and Scott, 1986a) and it would be of interest to determine whether the EHV-4 and EHV-1 genomes also lack such an element in this region. An additional origin of replication (ori_S) is found in two copies in the short repeat sequences of the genomes of HSV-1 (Stow, 1982, 1985; Stow and McMonagle, 1983), VZV (Stow and Davison, 1986) and EHV-1 (Baumann et al., 1989). Although no ori_L has been detected in the VZV genome, such an element appears to localise just downstream of the glycoprotein gH gene in the genomes of EHV-1 and EHV-4 (M. Whalley, personal communication; Nicolson et al., 1990a). The highly conserved nature of the major DNA binding protein across the herpesviruses as predicted from DNA sequence data is consistent with early studies which reported extensive antigenic cross-reactivity between the major DNA binding proteins of HSV-1, HSV-2, BHV-2, PRV and EHV-1 (Yeo et al., 1981), and which demonstrated that these cross-reacting proteins of all five viruses were very similar in structure by virtue of tryptic peptide analysis (Littler et al., 1981). More recently,

immunofluorescence and Western blot analysis of EBV-transformed cell lines has demonstrated the antigenic relatedness between the major DNA binding proteins of HSV and EBV (Littler et al., 1988).

All the EHV-4 genes identified in this chapter mapped to genomic locations colinear with the analogous genes in the HSV-1 (I_L) and VZV genomes. However, much work remains to be done in the characterisation of other EHV-4 genes and the determination of the functions of their gene products.

CHAPTER 7

General Discussion

The involvement of EHV-1 and EHV-4 in abortigenic, respiratory and neurological diseases is particularly worrying for the horse breeding industry worldwide due to the great economic losses associated with outbreaks of disease. Immunity to infection, whether by natural infection or vaccination, is weak and short lived and horses become reinfected at intervals of 3 to 6 months throughout their lifetime (Bryans, 1969). A deeper understanding of the horses immune response to viral infection is of paramount importance if the virus-induced diseases are to be effectively controlled. One means of achieving this is by characterisation of the viral envelope glycoproteins, the major immunogens of herpesviruses known to induce both humoral and cell-mediated immune responses. Most of the new generation of herpesvirus vaccines currently being developed are based upon the ability of the envelope glycoproteins to stimulate a wide range of host immune responses.

A major part of the studies presented in this thesis were concerned with the identification and DNA sequence determination of the EHV-4 gene encoding the major glycoprotein gB and subsequent evaluation of the immunogenicity of the gene product. Analysis of the predicted amino acid sequence of the EHV-4 gB protein and comparison with those available for the gB homologues of other herpesviruses demonstrated that the proteins possess highly conserved primary and predicted secondary structures which suggests important, conserved functions for these proteins in each virus. HSV-1 gB is known to play an essential role in virus entry and cell fusion (Little et al., 1981; DeLuca et al., 1982). However, the precise roles of EHV-4

gB in viral infection remain to be determined by further experimental studies.

Studies presented on the immunogenicity of EHV-4 gB indicated that this glycoprotein may play only a minor role in eliciting the production of virus neutralising antibodies and is probably more important in stimulating other immune mechanisms. This was inferred from the observation that neither EHV-4 gB fusion proteins or peptides derived from the EHV-4 gB sequence could stimulate the production of virus neutralising antibodies in hamsters although they elicited anti-protein and anti-peptide antibody responses. The protection from a lethal EHV-1 challenge of one hamster immunised with the EHV-4 gB fusion protein pUR0.6gB in the absence of virus neutralising antibodies indicated that other antibody-mediated immune effector mechanisms may have been mediating protection, such as ADCC or CDL. Additionally, the ability of EHV-4 gB peptides to induce the proliferation of lymphocytes in response to whole virus antigen <u>in vitro</u> indicated the presence of an important T cell epitope on the EHV-4 gB molecule. Although the studies presented in this thesis are only the first of a wide range of experimental approaches which will be necessary to fully elucidate the precise role of EHV-4 gB in the immune response to viral infection, it can be regarded as a glycoprotein suitable for vaccine development.

By analogy with HSV-1, another EHV-1 and EHV-4 glycoprotein also suitable for development as a vaccine is gC. EHV-1 gC (gp13) is the most extensively characterised EHV glycoprotein. The genes encoding EHV-1 gC (Allen and Coogle, 1988) and EHV-4 gC (Nicolson and Onions, 1990) have been identified and

sequenced. Allen et al. (1988) identified at least 16 epitopes on EHV-1 gC, of which were 85% were type-specific and 20% elicited virus neutralising antibody production. A predicted immunodominant site was localised to residues 145 to 150 (Glu-Arg-Lys-Lys-Ser-Arg) of EHV-1 gC (Allen and Coogle, 1988). The EHV-1 gC and EHV-4 gC molecules show a sequence identity of 79% and, given this high degree of conservation of amino acid sequences, it is somewhat surprising that the epitopes on the gC molecules are predominantly type-specific. Detailed characterisation of the epitopes of EHV-1 gC and EHV-4 gC should identify the precise antigenic relationship between these glycoproteins.

Two separate studies have illustrated the antigenic nature and importance of EHV-1 gC in protective immunity. Immunisation of guinea pigs with a recombinant vaccinia virus expressing EHV-1 gC elicited the production of EHV-1 neutralising antibodies and hamsters vaccinated with this recombinant virus were protected from a lethal EHV-1 challenge (Guo et al., 1989). Stokes et al. (1990) demonstrated that hamsters immunised with purified EHV-1 gC produced antibodies with virus neutralising activity and which participated in complement-mediated lysis of EHV-1-infected cells, and that these hamsters were also protected from a lethal EHV-1 challenge. Peptides from the EHV-4 gC sequence have been shown to elicit the production of anti-peptide antibodies upon immunisation of hamsters which in some instances reacted with whole virus, but could not protect hamsters from a lethal EHV-1 challenge (Stokes et al., 1990), a situation analogous to that seen when using peptides from the EHV-4 gB sequence. However, one EHV-4 gC peptide was predicted

to contain an important linear B cell epitope on the basis of strong reactivity with an EHV-1 gC-specific monoclonal antibody. Thus, studies so far carried out on the immunogenicity of EHV-1 gC clearly indicate that it is a highly antigenic molecule that plays a major role in protective immunity and, as such, is an ideal candidate for EHV vaccine development. These findings are in accordance with the similar role of the gC-like proteins of other alphaherpesviruses in both humoral and cell-mediated immunity. HSV-1 gC is an important antigen that can elicit the production of virus neutralising antibodies (Glorioso et al., 1984; Roberts et al., 1985; Weir et al., 1989), CTL responses (Glorioso et al., 1985; Rosenthal et al., 1987), and can confer protective immunity (Glorioso et al., 1984; Roberts et al., 1985; Weir et al., 1989) in mice. BHV-1 gIII has been shown to elicit the production of antibodies in cattle with virus neutralising activity and which participate in ADCC of BHV-1-infected cells (Babiuk et al., 1987; van Drunen Littel-van den Hurk et al., 1989) and to protect cattle from virus challenge (Babiuk et al., 1987). In mice, BHV-1 gIII elicited virus neutralising antibody production and was recognised by murine CTLs and antibody in immune-mediated cytotoxicity assays (Fitzpatrick et al., 1988). PRV gIII is a major target for virus neutralising antibodies (Ben-Porat et al., 1986a) and for murine and swine CTLs (Zuckermann et al., 1990), and monoclonal antibodies to PRV gIII can protect mice and swine against PRV challenge (Marchioli et al., 1988).

Although the development of individual antigenic glycoproteins as subunit vaccines by their expression as fusion products in E.coli is a well documented approach, the method

does have several drawbacks. In particular, the expressed protein product is not glycosylated by E.coli and does not retain its native conformation and biological activity and any immunity afforded is generally short lived. Chapter 5 of this thesis dealt with the expression of segments of the EHV-4 gB gene in E.coli as fusion products with β-galactosidase and glutathione-S-transferase. An obvious problem with β-galactosidase fusion proteins is that, due to the large size of the β-galactosidase moiety (116kDa), a major portion of any immune response may be directed against this region unless the expressed exogenous product is particularly antigenic. This potential problem can be partly overcome by expression as fusion products with the relatively small glutathione-S-transferase (26kDa). Furthermore, this expression system allows cleavage of the carrier protein from the protein of interest and rapid purification of protein products by immunoaffinity chromatography. In general, subunit vaccines developed by expression of glycoproteins in E.coli may be regarded as suitable for the initial characterisation of potentially immunogenic glycoproteins for incorporation into genetically attenuated virus vaccine strains but not as final vaccine products.

Expression of foreign antigens in systems derived from the insect baculovirus Autographa californica nuclear polyhedrosis virus is a more favoured approach for the development of glycoproteins as subunit vaccines (reviewed in Luckow and Summers, 1988). In this system, the foreign gene to be expressed is linked to the strong viral polyhedrin gene promoter by homologous recombination in insect cells. Foreign antigens

produced by this system are expressed to particularly high levels, although insect cells do not always authentically glycosylate and process the expressed protein. Many eukaryotic genes have been successfully expressed in this way (Prehaud et al., 1989; Koener and Leong, 1990; Tratner et al., 1990). In particular, HCMV gB has been successfully expressed in insect cells using a recombinant baculovirus (Wells et al., 1990). The mature protein was processed by proteolytic cleavage in insect cells, although this step was less efficient than in mammalian cells. More importantly, authentic processing and glycosylation of HCMV gB did not appear to be required for immunogenicity since immunisation of mice with the recombinant virus stimulated the production of virus neutralising antibodies to a level comparable with that previously reported following immunisation of mice with a vaccinia virus expressing HCMV gB (Britt et al., 1988).

Vaccinia virus and human adenoviruses have been used as biological delivery systems for the presentation of foreign antigens and in studying the nature of the immune responses elicited by these antigens. The use of vaccinia virus as a vector is by far the most widely exploited approach (Mackett et al., 1982; Mackett and Smith, 1986; Moss and Flexner, 1987). Vaccinia virus is the prototypic orthopoxvirus with a large genome of about 187kb, possessing great capacity for foreign DNA without destruction of infectivity and a wide host range. The insertion of foreign genes into the vaccinia virus genome is achieved using a two-step strategy. Firstly, a plasmid insertion vector is constructed which contains the foreign gene linked to a vaccinia virus promoter flanked by vaccinia virus DNA taken

from a non-essential region of the genome. The next step involves insertion of the chimeric gene into the vaccinia virus genome by transfection of the recombinant plasmid into cells infected with wild-type vaccinia virus. Homologous recombination in cells between the vaccinia virus DNA sequences flanking the chimeric gene and the vaccinia virus genome results in the generation of a recombinant virus containing the foreign gene. Recombinant genomes are replicated and packaged into infectious vaccinia virus. The site in the vaccinia virus genome at which the foreign gene is inserted is determined by the vaccinia virus DNA flanking the chimeric gene. Although any site non-essential for virus growth can be used for insertion of foreign genes, the TK gene is most often used since insertional inactivation of the TK gene greatly decreases the virulence of vaccinia virus and, as such, produces a safer, attenuated virus for vaccination purposes (Buller et al., 1985). Furthermore, TK-negative recombinant viruses can be readily selected by their resistance to 5-bromodeoxyuridine (BUdR) by plaquing on a TK-negative cell line in the presence of BUdR. Vaccinia virus recombinants expressing HSV-1 glycoproteins have been demonstrated to invoke humoral, cell-mediated and protective immune responses. A recombinant vaccinia virus expressing HSV-1 gB has been shown to induce the production of anti-HSV-1 CTLs (McLaughlin-Taylor et al., 1988), and virus neutralising antibodies and protective immunity (Cantin et al., 1987), in mice. Immunisation of mice with an HSV-1 gC-vaccinia virus recombinant virus elicited the production of virus neutralising antibodies and protected mice from a lethal HSV-1 challenge (Weir et al., 1989). Similarly, vaccinia virus recombinants expressing HSV-1 gD could also

induce virus neutralising antibody production in mice, protect mice from a lethal HSV-1 challenge and prevent the establishment of a latent HSV-1 infection (Paoletti et al., 1984; Cremer et al., 1985; Rooney et al., 1988), and were also shown to activate HSV-specific human CTLs (Zarling et al., 1986b) and other T cells in mice (Martin et al., 1987, 1989). Immunisation of mice with recombinant vaccinia viruses expressing the minor HSV-1 glycoproteins gG (Sullivan and Smith, 1987) and gI (Sullivan and Smith, 1988) resulted in the production of virus neutralising antibodies. Other herpesvirus glycoproteins expressed in vaccinia virus and shown to induce virus neutralising antibody production upon immunisation of animals include HCMV gB (Cranage et al., 1986; Britt et al., 1988), BHV-1 gI and gIII (van Drunen Littel-van den Hurk et al., 1989) and PRV gp50 (Marchioli et al., 1987a).

The use of human adenoviruses, particularly type 5 (Ad5), as model vaccine vector systems is well documented. Regions of the virus genome available for insertion of foreign genes by homologous recombination are the early regions 1 (E1) and 3 (E3). Sequences in the E3 region can be deleted without affecting the ability of Ad5 to replicate in cells permissive for human adenoviruses and up to 4kb of foreign DNA sequences can be inserted in the E3 region (Ghosh-Choudhury et al., 1986; Haj-Ahmad and Graham, 1986). Although most of the sequences in the E1 region can be deleted, the resulting mutant viruses can only replicate in 293 cells (Graham et al., 1977). Adenovirus vectors with deletions in both the E1 and E3 regions can accommodate up to 7.5kb of foreign DNA sequences (Ghosh-Choudhury et al., 1986; Haj-Ahmad and Graham, 1986). Recombinant

adenovirus vectors have been successfully used to express a wide range of foreign antigens in cell culture (Ballay et al., 1985; Haj-Ahmad and Graham, 1986; Davidson and Hassell, 1987; Prevec et al., 1989). In particular, human adenovirus vectors have been successfully used to express HSV-1 gB (Johnson et al., 1988b) and immunisation of mice with this recombinant adenovirus stimulated the production of virus neutralising antibodies to HSV-1 and HSV-2 and protected animals from lethal HSV-1 and HSV-2 challenges (McDermott et al., 1989). Adenovirus vectors expressing HSV-1 gB and gC have been used in the study of CTL responses specific for HSV in mice (Witmer et al., 1990). This study demonstrated that HSV-specific CTLs could lyse cells transfected with the Ad-gB recombinant virus but not with the Ad-gC recombinant virus.

Preferred herpesvirus vaccines are those which comprise whole virus. Killed virus vaccines prepared from whole virus have been used to induce the production of protective antibodies against a wide range of virus-induced diseases (Melnick, 1985). However, several disadvantages are associated with such vaccines. These include the production of only brief immunity which must be boosted, the ability of some vaccines to induce hypersensitivity to infection by wild-type virus and extreme care required in ensuring that all virulent live virus is removed from the vaccine preparation. Genetic manipulation techniques have made possible the development of safer, live attenuated viruses. Using this approach virus mutants with deletions in virulence genes and temperature-sensitive mutants have been generated to serve as live virus vaccines. In particular, the TK gene, which is known to play an important

role in herpesvirus virulence (Field and Wildy, 1978; Tenser et al., 1981; Ben-Hur et al., 1983) and can enhance herpesvirus replication in non-dividing cells (Jamieson et al., 1974), has been the focus of much attention. Several TK-negative attenuated herpesviruses with deletions in the TK gene that cannot revert to virulence have been successfully used in the control of herpesvirus-induced diseases. TK-negative PRV mutants have been shown to be immunogenic, avirulent and capable of protecting swine from a virulent PRV infection (Kit et al., 1985a,b; McGregor et al., 1985; Marchioli et al., 1987b). In a similar vein, a TK-negative BHV-1 mutant virus was also shown to be highly attenuated for calves and capable of protecting animals from challenge with virulent BHV-1 (Kit and Qavi, 1983; Kit et al., 1985c). Pregnant cows vaccinated with this mutant virus were also protected from BHV-1 challenge (Kit et al., 1986). A PRV virus vaccine with deletions in the TK, gI and gIII genes has also been constructed (Kit et al., 1987). The generation of TK-negative EHV-1 and EHV-4 viruses for use as vaccines in the horse could readily be accomplished since the TK genes of these viruses have been mapped and sequenced (Robertson and Whalley, 1988; Nicolson et al., 1990b). An EHV-1 TK-negative mutant has been prepared by passaging of the Vero-adapted TK-positive EHV-1 (RQ) strain in media containing the nucleoside analogues BUdR and 1-B-D arabinofuranosylthymine, and its safety and efficacy as a vaccine in young horses evaluated (Cornick et al., 1990). The vaccine was safe when administered either intramuscularly or intravenously, with no EHV-1 being shed intranasally during the 12 days following its administration, and was shown to be antigenic since serum neutralising antibody

levels in vaccinated animals increased significantly following intranasal challenge with virulent EHV-1. However, the vaccine could not prevent clinical signs of respiratory disease following intranasal challenge with virulent EHV-1. It was suggested that the inability of the vaccine to protect against respiratory disease may have been due to the requirement for more than a single inoculation to stimulate adequate immunity, possible over-attenuation of the vaccine virus prior to deletion of the TK gene or simply a result of the EHV-1 challenge dose used in the experiments being greater than that of a natural infection.

The avirulent PRV vaccine strains NIA-4, Norden and Bartha contain deletions in the U_S region which contribute to their reduced virulence (Lomniczi et al., 1984a,b; Berns et al., 1985). These strains do not contain the gene coding for the PRV glycoprotein gI which is analogous to HSV-1 gE (Mettenleiter et al., 1985a,b; Ben-Porat et al., 1986b). Additional studies demonstrated that the Bartha strain also contains a deletion in the gene for glycoprotein gp63 (Petrovskis et al., 1986b), the analogue of HSV-1 gI. Quint et al. (1987) constructed PRV mutants with deletions in U_S which, when inoculated into pigs, protected vaccinated animals from challenge with virulent wild-type PRV. Thus, the U_S region of the PRV genome encodes two glycoproteins, gI and gp63, which contribute to virulence and are not essential for viral growth. It would not be prudent to assume that the analogous glycoproteins of HSV-1 (gE and gI) and other alphaherpesviruses will also contribute to virus virulence and can also be deleted to produce avirulent vaccine strains. This approach for the development of attenuated EHV-1 and EHV-4

vaccine strains awaits the determination of the DNA sequence of the U_S region of their genomes. The generation of mutant viruses with further deletions in other virulence genes from other regions of the genome, including the TK gene, as well as deletions in sequences involved in cell transformation, should prove to be particularly safe and effective vaccines.

Both EHV-1 and EHV-4 have the upper respiratory tract as the site of entry and primary site of virus replication. Live intranasal vaccines are an attractive proposition if stable non-revertant temperature-sensitive mutants could be produced. This could be achieved by *in vitro* mutagenesis of cloned restriction endonuclease fragments from selected regions of the EHV-1 and EHV-4 genomes with nitrous acid. Transfection of these fragments into cells together with parental virus would generate recombinant viruses containing the engineered mutations. The screening of progeny virions for temperature-sensitivity and stability on passage and confirmation of the lesions by marker rescue studies, restriction mapping and DNA sequence analysis would identify suitable mutants for testing as vaccine viruses. Using this method, multiple lesions can be induced which eliminate spontaneous reversion to virulence. Herpesvirus temperature-sensitive mutants have proved to be safe and effective vaccines against disease caused by BHV-1 and FHV-1.

To effectively protect against both EHV-1- and EHV-4-induced diseases, a bivalent vaccine containing immunogenic components from both virus types is desirable. This would be achieved by development of EHV-1 and EHV-4 as virus vectors. Genes coding for the immunogenic glycoproteins, for example gB and gC, of one virus could be inserted into the TK gene of the

other virus. The resulting TK-negative recombinant viruses generated by homologous recombination would then be selected by their resistance to BUdR as previously described. Coexpression of gB and gC is particularly desirable since it has been shown that the expression of both EHV-1 gB and gC in vaccinia virus greatly enhances the protective efficacy in the hamster challenge model over that obtained with the singular expression of either gB or gC (Guo et al., 1990). Deletions of a combination of other non-essential genes contributing to virulence could also be incorporated into such vaccine viruses to create even higher levels of safety and efficiency.

An immune stimulating complex (ISCOM) vaccine prepared from the abortigenic EHV-1 strain V592, and which contains all the major viral glycoproteins, has been shown to fully protect hamsters from a lethal EHV-1 challenge (Cook et al., 1990). Studies with this vaccine are now underway to determine whether it can protect horses from challenge with virulent EHV-1.

Although studies on antiviral immunity to herpesvirus infection have concentrated on the surface glycoproteins as the principal immunogens against which antiviral responses are directed, it is now apparent that non-glycoprotein and non-structural antigens may also act as targets for such responses. For example, the IE gene products of human and murine cytomegaloviruses and HSV-1 have been shown to act as major, even dominant, targets for antiviral CTLs (Reddehase et al., 1984; Reddehase and Koszinowski, 1984; Koszinowski et al., 1987; Martin et al., 1988). The CTL response to herpesvirus infection would therefore appear to be a multi-faceted entity. The manner in which these IE proteins, normally confined to the nucleus,

can appear at the cell surface to act as targets for CTLs remains to be determined. In a similar vein, it was shown in Chapter 6 of this thesis that a major, antigenic glycoprotein of EHV-1 and EHV-4, gp10, resides not in the virus envelope as previously thought but probably in the virus tegument.

An alternative approach to a vaccination programme for the control of herpesvirus-induced diseases is the development of antiviral compounds which block specific virus-coded functions and consequently interfere with the production of infectious virus. One particular compound which has undergone much investigation is the nucleoside analogue acyclovir (ACV; 9-[2-hydroxyethoxymethyl]guanine). The triphosphate derivative of ACV acts as a competitive inhibitor with dGTP for the HSV-1 DNA polymerase, interfering with viral DNA synthesis and the production of infectious virus (Elion et al., 1977). Although ACV demonstrates activity against some human herpesviruses (Schaeffer et al., 1978; Collins and Bauer, 1979; Bridgen et al., 1981) it is not effective against HCMV (Plotkin et al., 1982; Wade et al., 1982). The HSV-1 ribonucleotide reductase, which is essential for virus pathogenicity in mice (Cameron et al., 1988), has been targeted as another viral component against which antivirals may be effective. The synthetic nonapeptide YAGAVVNDL, which represents the carboxyl-terminal nine amino acids of the small subunit of HSV-1 ribonucleotide reductase, has been shown to specifically inhibit the HSV-1 enzyme (Cohen et al., 1986; Dutia et al., 1986) by causing dissociation of the two subunits of the enzyme (McClements et al., 1988). Since serological studies indicated that the carboxyl-terminal region of the small subunit of ribonucleotide reductase was highly

conserved with that of other herpesviruses, it was speculated that this nonapeptide may also inhibit other herpesvirus ribonucleotide reductase enzymes (Dutia et al., 1986). This was borne out by the observations that the activities of the ribonucleotide reductase of PRV (Cohen et al., 1987) and EHV-1 (Telford et al., 1990) were also blocked by this nonapeptide. An antiviral drug based on this nonapeptide may be of value in treating EHV-1 and EHV-4 infections in the horse. Treatment of EHV-1-infected mice with the antiviral agent (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) resulted in the successful clearance of viraemia and reversed the progression of clinical signs, thereby demonstrating the potential for this compound in effective chemotherapy of EHV-1 (Field and Awan, 1990). HPMPA probably acts by directly inhibiting the viral DNA polymerase (Votruba et al., 1987). The development of antiviral drugs against other targets which contribute to virus pathogenicity is an exciting prospect for the future.

The data presented in Chapter 6 comprise the largest piece of DNA sequence so far reported for the EHV-4 genome and represents an early step in the characterisation of the genetic content of this virus. Knowledge of the DNA sequence of herpesvirus genomes will yield important information with respect to the genetic and antigenic relatedness of these viruses and provide an insight into the manner in which they have undergone evolutionary divergence at the genetic level. Comparison of the EHV-4 gene products analysed with the analogous gene products of HSV-1 and VZV suggests that EHV-4 may be more closely related to VZV than HSV-1, although this can only be confirmed by comparison of the complete DNA sequence of

the EHV-4 genome when this becomes available with those of the HSV-1 and VZV genomes. Comparison of the proteins and antigens of HSV-1, HSV-2, BMV, PRV and EHV-1 by SDS-PAGE of purified virus, and by virus neutralisation and agar gel immunodiffusion tests, has demonstrated that PRV and EHV-1 are more closely related to each other at the antigenic level than to BMV, HSV-1 or HSV-2 (Killington et al., 1977). Such findings made at the antigenic level can now be confirmed at the DNA level as the DNA sequences of these herpesvirus genomes come to light. The complete DNA sequence of the genomes of the alphaherpesviruses HSV-1 (McGeoch et al., 1985, 1986a, 1988a; Perry and McGeoch, 1988) and VZV (Davison and Scott, 1986a), the gammaherpesvirus EBV (Baer et al., 1984) and the betaherpesvirus HCMV (Chee et al., 1990) are currently available. Comparison of the genes specified by the genomes of HSV-1 and VZV revealed that the two viruses possess a near identical gene arrangement in U_L; the only differences are that VZV gene 13, which encodes a thymidylate synthetase, is replaced by a gene of unknown function (UL45) in HSV-1, whilst the genomic position occupied by VZV genes 1 and 2 at the start of U_L is occupied by the unrelated gene UL56 in HSV-1. Interestingly, the EHV-4 genome contains a gene in a position colinear with HSV-1 UL45 and VZV gene 13 whose predicted product is unrelated to the gene products of both HSV-1 UL45 and VZV gene 13 (Nicolson and Onions, 1990). The S segment of the HSV-1 and VZV genomes has undergone extensive divergence. HSV-1 contains 13 genes in this region, VZV only 7. Each VZV gene has a homologue in HSV-1 and the remaining 6 extra HSV-1 genes with no VZV homologues include those for glycoproteins gD and gG and the IE protein

IE12. The layout of conserved genes in the S segment is different but the S segments are clearly related and a mechanism has been proposed for their descent from the S segment of an ancestral herpesvirus genome by expansion and contraction of the inverted repeats (Davison and McGeoch, 1986). Comparison of the DNA sequence of the VZV genome with that of the distantly related EBV identified 29 VZV genes with detectable counterparts in EBV, all of which are contained within the U_L region of their genomes and located in a generally colinear fashion in three major regions, although these regions are arranged differently in the two genomes (Davison and Taylor, 1987). Conserved genes include those for the two subunits of ribonucleotide reductase, DNA polymerase, major capsid protein, exonuclease, dUTPase, major DNA binding protein, TK and glycoproteins gB and gH. The use of immunological probes in immunofluorescence and Western blot analyses of EBV-transformed cells identified EBV-coded proteins antigenically related to the HSV alkaline deoxyribonuclease, glycoprotein gB, TK, major DNA binding protein and infected cell-specific protein 34/35 (Littler et al., 1988).

DNA sequence analysis has also been employed in the determination of gene arrangement in other herpesviruses. For example, the A+T-rich γ_2-herpesvirus HVS genome has been shown to share a generally colinear arrangement with the G+C-rich γ_1-herpesvirus EBV genome, encoding closely related proteins, and this gene arrangement is in turn different from that of the alphaherpesviruses HSV-1 and VZV (Gompels et al., 1988). DNA sequence analysis has also led to the reclassification of some herpesviruses originally classified on the basis of biological

properties. MDV and HVT, originally classified as gammaherpesviruses due to their tropism for lymphocytes, are now regarded as alphaherpesviruses (Buckmaster et al., 1988; Ross et al., 1989). Large scale DNA sequencing has established that HHV-6, originally classified as a gammaherpesvirus, more closely resembles HCMV than EBV, HSV or VZV at the genetic level and is now regarded as a betaherpesvirus (Efstathiou et al., 1988; Lawrence et al., 1990; Littler et al., 1990).

Determination of the complete DNA sequence of the genomes of EHV-1 and EHV-4 would be expected to reveal a similar gene complement and organisation to that observed for HSV-1 and VZV, at least within U_L. However, the S segment will prove to be the most interesting region. This has been shown to be the most divergent region of the alphaherpesvirus genomes and it seems highly likely that differences in genetic layout may account for the differing pathogenicity and disease-forming potential of each herpesvirus. In particular, differences in the S segments of the EHV-1 and EHV-4 genomes may contribute to the differing disease spectra of these two viruses.

All of the 12 ORFs in the S component of the HSV-1 genome, with the exception of US6 (gD), have been deleted and shown to be dispensable for virus growth in cell culture (Post and Roizman, 1981; Longnecker and Roizman, 1986, 1987; Longnecker et al., 1987). These non-essential genes include those encoding the glycoproteins gG (US4), gI (US7) and gE (US8), and a protein kinase (US3). This pronounced clustering of genes not essential for viral growth is in contrast with the small number and wide scattering of such genes in the L component of the HSV-1 genome, which include those for gC and TK. One hypothesis proposed to

explain this clustering of dispensable genes is that these genes have evolved in the S component to allow HSV to survive in the ecological niche of its human host rather than to replicate or package its genome, and that they have evolved from DNA sequences acquired by a progenitor virus (Roizman, 1979). Genes non-essential for viral growth which are known to map in the S component of the PRV genome include those for the glycoproteins gI, gp63 and gX. It therefore seems highly probable that many genes non-essential for viral growth will also cluster in the S component of other herpesvirus genomes. Identification of such genes in the genomes of EHV-1 and EHV-4 will aid vaccine development. Genes non-essential for viral growth have generally been identified by site-specific deletion or insertional mutagenesis techniques. A rapid mutagenesis procedure for exploring herpesvirus genomes and identifying such genes is that of Tn5 mutagenesis (Weber et al., 1987).

The detection of EHV-1 and EHV-4 DNA in clinical specimens from natural infections has frequently been accomplished by the use of cloned viral DNA fragments as hybridisation probes. Morris and Field (1988) directly analysed infected tissue from aborted foetuses using dot blot and Southern blot hybridisation techniques and successfully confirmed the presence of EHV-1 DNA sequences in these specimens. Moreover, using this approach it was possible to distinguish between EHV-1 and EHV-4, to detect intratypic strain variation and to reveal information on the conformational state of the viral genome. However, the Southern blot method is relatively labour intensive, although the dot blot method was reported to have potential as a rapid diagnostic procedure. The problem of non-specific hybridisation is always

possible, leading to false positive results. The extremely sensitive, highly specific and rapid technique of polymerase chain reaction (Saiki et al., 1988) is now finding wide application as a powerful tool for the diagnosis of latent and non-latent EHV in equine tissues. Using this technique, single-copy genomic sequences can be amplified by a factor of more than 10^7 with very high specificity, with DNA segments up to 2000bp in length being readily amplified. Due to the extremely high sensitivity of the technique, an exceptionally high standard of laboratory practice is essential to prevent amplification of contaminating material.

The rapidly growing discipline of molecular biology has now revolutionised scientists approach to the study of herpesviruses and attempts to control the diseases they cause. The continued application of this technology in this field promises to develop suitable vaccines and antiviral compounds which will severely curtail the incidence of herpesvirus-induced diseases and allow for improved treatment of infected individuals. The work presented in this thesis represents an initial step in the attempt to determine the genetic structure of EHV-4 and in the development of potentially effective recombinant vaccines against both EHV-1 and EHV-4.

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