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THE UNIVERSITY OF ALBERTA

MOLECULAR CHARACTERIZATION OF GLYCOPROTEINS OF HERPES SIMPLEX VIRUS

BY

© JOHN CLARENCE ZWAAGSTRA

A THESIS

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

Molecular Characterization of Herpes Simplex Virus Glycoproteins submitted by John Clarence Zwaagstra in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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To my precious daughter, Andrea Karolyn

ABSTRACT

HSV encodes several glycoproteins which are expressed on the surface of the virion and the infected cells. Glycoprotein gB plays a major role in virus infectivity. Both glycoproteins gB and gD have been implicated in the process of viral penetration into the host cell and are the major antigens responsible for eliciting virus neutralization antibodies. The purpose of this project was to characterize gB and gD. Two approaches were undertaken in order to achieve this aim. First, in the case of gB, the nucleotide sequence of the gB2 gene from HSV-2 (strain 333) was determined. The amino acid sequence of gB2 was deduced from the nucleotide sequence and analysed for potential antigenic regions. Secondly, glycoprotein gD1 from HSV-1 (KOS) was isolated and characterized directly by biochemical procedures.

Open reading frame analysis of the gB2 gene identified a gB2 polypeptide which consisted of 904 amino acids and showed extensive homology with gB1. Information gathered from comparison of the primary sequence of gB2 (333) with that of other HSV strains, secondary structure and surface profile analyses, was used to predict nine major antigenic regions at amino acid positions (1) 43-76, (2) 129-141, (3) 221-242, (4) 299-309, (5) 325-339, (6) 409-440, (7) 462-499, (8) 582-611 and (9) 689-712. Potential site specific epitopes were located between positions 59-68, 463-474 and 475-483.

Glycoprotein gD1 was synthesized in HSV-I infected Vero cells. Analysis of gD1 by SDS-polyacrylamide gel electrophoresis, showed that gD1 could be resolved into 3 to 4 distinct species ranging in size from 52 to 60K. Lectin affinity chromatography showed that gD1 did not bind to Wheat germ agglutinin, suggesting the absence of terminal sialic acid groups on the oligosaccharide moieties. A portion of the precursor and mature forms of gD1 bound to Lentil lectin and 100% of mature gD1 bound to Castor bean-120, suggesting the presence of high-mannose core sugars containing fucose on these molecules and the presence of terminal galactose on mature gD1. The conformation of gD1 was stable between pH4.0 - 9.0 and isoelectric focusing showed that it had a pI of 5.9 under non-denaturing conditions. Purification of the mature form(s) of gD1 was achieved by a combination of lectin affinity chromatography and gel filtration.

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LIST OF ABBREVIATIONS

HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
EBV	Epstein Barr virus
VZV	varicella-zoster virus
CMV	cytomegalovirus
TK	thymidine kinase
ICP	infected cell protein
mar	monoclonal antibody resistant
syn	syncytial
RER	rough endoplasmic reticulum
NK	natural killer
CTL	cytotoxic T lymphocyte
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate
c^7dGTP	7-deaza-deoxyguanosine triphosphate
SDS	sodium dodecyl sulfate
DEAE	diethylaminoethyl
BSA	bovine serum albumin
IEF	isoelectric focusing
pI	isoelectric point
LL	lentil lectin
WGA	Wheat Germ agglutinin

LIST OF ABBREVIATIONS

CB-120	Castor Bean-120
GlcNAc	N-acetyl-D-glucosamine
GalNAc	N-acetyl-D-galactosamine
Gal	Galactose
NeuAc	N-acetyl-neuraminic acid (sialic acid)
Meα-D-Man	methyl α-D-mannoside
CMP-NeuAc	CMP-N-acetyl-neuraminic acid

CHAPTER 1

LITERATURE REVIEW

I. The Herpesviruses

The viruses belonging to the family Herpesviridae share several common characteristics: linear, double-stranded DNA in the core of the virion, an icosadeltahedral capsid containing 162 capsomers assembled in the nucleus of the infected cell, and an envelope derived from the nuclear membrane.

Approximately 80 herpesviruses have been characterized. Out of this group, 5 herpesviruses were isolated from humans [herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), cytomegalovirus (CMV), varicella-zoster virus (VSV), and Epstein-Barr virus (EBV)], 4 from horses, at least 3 from cattle, 2 from pigs [pseduorabies virus (PSV) and porcine cytomegalovirus], and 2 from chickens [Marek's disease herpesvirus (MDV) and infectious laryngotracheitis virus] (Roizman and Batterson, 1985).

In spite of common features, it is difficult to classify members of the Herpesviridae into distinct genera due to extensive diversity in genomic arrangement and serological reactivity (Roizman, 1982; Honess, 1984). On the basis of their biological properties, the members of Herpesviridae are subdivided into three subfamilies: 1) Alphaherpesvirinae, 2) Betaherpesvirinae and 3) Gammaherpesvirinae (Matthews, 1982).

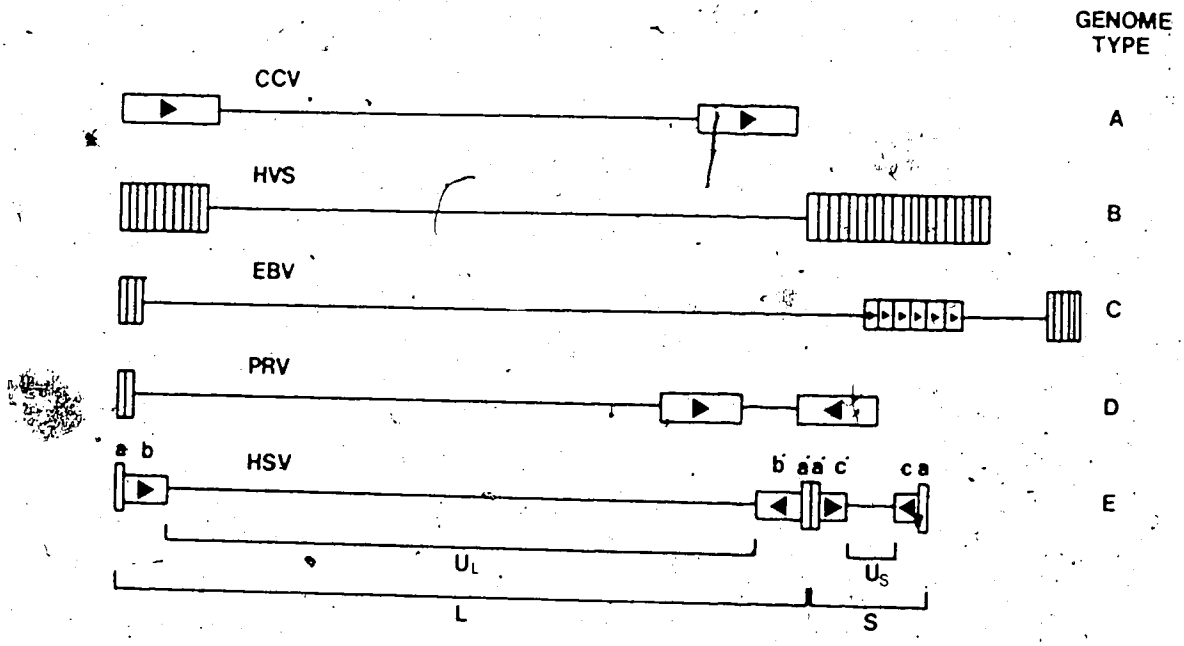
The members of Alphaherpesvirinae include the genera *simplex-virus* (HSV-1, HSV-2, bovine mamillitis virus) and *poikilovirus* [PSV, VZV, and equine herpes virus type 1 (EHV-1)]. These viruses infect a wide range of host cells, rapidly spread in cell culture, and efficiently destroy the infected cells. They are able to establish latent infections primarily in the ganglia. Members of the Betaherpesvirinae exhibit a restricted host range, a long replication cycle and slowly progressing infection in cell culture. Latency is established in secretory glands, lymphoreticular cells and the kidneys. This subfamily contains the genera CMV and *Muromegalovirus* (murine cytomegalovirus). *In vitro*, all members of the Gammaherpesvirinae replicate in lymphoblast cells and several viruses cause lytic infections in some epithelia and fibroblast cells. These viruses are specific for T or B lymphocytes and infection is frequently arrested in a prelytic or a lytic stage without production of infectious virus. Latency is often established in lymphoid tissue. This subfamily includes the genera *Lymphocryptovirus* (e.g. EBV), *Thetalymphocryptovirus* (e.g. HVV) and *Rhadinovirus* (e.g. herpesvirus saimiri). Recently a novel human herpes virus has been isolated from B cells of patients suffering from lymphoproliferative disorders (Salahuddin et al. 1986; Josephs et al. 1986). This virus has been tentatively named Human B lymphotrophic virus (HBLV) but is not fully characterized.

II. Genomic Structure of HSV

Herpes virus DNAs are variable in molecular weight (80-150 million), base composition [32-70 (G + C) mole %] and degree of sequence homology (Roizman and Batterson, 1985). All DNAs of the herpes virus group fall into 1 of 5 sequence arrangements (see Fig. 1). The sequence organization of HSV DNA is best understood (reviewed by Roizman, 1979).

The HSV genome is a linear, double-stranded molecule, approximately 100 x 10^6 in molecular weight (160 kilobases). The base compositions of HSV-1 and HSV-2 are 67 and 69 (G + C) moles %, respectively. The DNA consists of 2 covalently linked components L (long) and S (short), containing 82% and 18%, respectively, of the viral DNA sequence (see Fig. 1). Each component contains predominantly unique sequences, U_L (long unique sequence) and U_S (short unique sequence), flanked by reiterated repeats (Sheldrick et al., 1970; Wadsworth et al. 1975). The reiterated sequences flanking U_L were designated *ab* and *b'a'* and those flanking U_S were designated *a'c'* and *ca*. The size of the *a* sequence varies between the HSV strains and reflects the number of reiterations of tandemly repeated sequences comprising the *a* sequence (Roizman and Batterson, 1985). Most HSV-1 strains contain only one *a* sequence at the terminus of the S component. The number of *a* sequences at the junction between the L and S component and at the L terminus ranges from one to several.

Fig. 1.A schematic diagram of the sequence arrangements in the five types of genomes of viruses comprising the family Herpesviridae. The genome types A, B, C, D and E are exemplified by the channel catfish herpes virus (CCV), herpesvirus saimiri (HSV), Epstein-Barr virus (EBV), pseudorabies virus (PRV), and herpes simplex viruses (HSV), respectively. The horizontal lines represent unique regions. Reiterated sequences, larger than 1,000 base pairs in length, are shown as rectangles. The arrow head in the rectangle indicates the orientation of the repeated sequence (direct or inverted). The longer, vertical rectangles represent terminal sequences reported in the genome types B, C, D and E. All terminal reiterations are direct repeats. The reiterated sequences in HSV DNA (genome type E) are designated *b* and *c*, and the inverted sequences were designated *b'* and *c'*. The terminal reiterations, designated as the *a* sequence, are also repeated in inverted orientation internally. The long (L) and short (S) components, and the long unique (Ul) and short unique (Us) sequences of the HSV genome are indicated on the figure.


GENOME TYPE
CCV
A
HVS
B
EBV
C
PRV
D
a b
HSV
b' a a' c'
c a
E
U_L
U_S
L
S

The L and S components invert relative to each other, giving rise to 4 DNA isomers which differ with respect to the orientation of L and S (Delius and Clements, 1976; Hayward et al., 1975). Viral specific, trans-acting products mediate site-specific recombinations through the a sequence to produce the inversions (Mocarski and Roizman, 1981; Mocarski and Roizman, 1982a; Mocarski and Roizman, 1982b; Smiley et al., 1981) and these inversions may play a specific role in the biogenesis of linear packaged DNA (Roizman and Batterson, 1985).

III. Pathogenesis of HSV

A. HSV related diseases

Herpes virus infections are among the most common of all human infections and cause a broad spectrum of diseases. Of the five human herpesviruses, HSV-1 and HSV-2 are particularly important because of frequent recurrences and ready transmission of infectious virus. HSV-1 is usually isolated from lesions in or near the mouth and is spread by direct physical contact. HSV-2 is responsible for the majority of genital tract infections and the virus is transmitted venereally (Nahmias and Roizman, 1973). Both viral subtypes, however, can cause genital and oral-facial infections. Immunosuppressed patients, especially those with defects in cell-mediated immunity, have more frequent and more severe HSV infections (Whitley et al., 1984).

Gingiovastomatitis and pharyngitis are the most frequent clinical manifestations of primary HSV-1 infections, and

recurrent herpes labialis is the usual manifestation of reactivated HSV (Corey and Spear, 1986). HSV infection of the eye is a frequent cause of corneal blindness and HSV-1 encephalitis is a common viral infection of the central nervous system (Corey and Spear, 1986). Neonatal infections are severe with a mortality rate of up to 65% (Whitley et al., 1980).

B. HSV and oncogenesis

Numerous reports have associated HSV with the development of cancer, although not all the data have been definitive. Most of the studies are seroepidemiological in nature and demonstrate increased antibody titers to HSV-2 in women with cervical cancer (Nahmias and Norrild, 1980; Rawls and Adam, 1977). Molecular hybridization techniques have detected the presence of HSV-2 DNA and RNA in cancerous tissues (Frenkel et al., 1972; Jones et al., 1978) but other reports have been negative (Rapp, 1984). Moreover, there is evidence that portions of HSV DNA hybridize with normal human cellular DNA and RNA (Jones and Hyman, 1983; Jones et al., 1985) therefore, caution must be exercised in the interpretation of the DNA hybridization data.

HSV can transform cells *in vitro* (reviewed by Galloway and McDougall, 1983) and in some studies, the resulting transformants are oncogenic in mice (Duff and Rapp, 1973). Several DNA sequences from both HSV-1 and HSV-2 have transforming ability. The HSV-1 genomic region between map units 0.30 - 0.45 (BglII I fragment) transformed hamster

embryo cells (Camacho and Spear, 1978). Viral specific proteins, but not DNA sequences, could be detected in the transformants. Two HSV-2 sequences of map units, 0.58 - 0.62 (BglII N fragment) and 0.43 - 0.58 (BglII C fragment), were used to transform rat and hamster embryo cells, respectively (Galloway and McDougall, 1981; Jariwalla et al., 1980). Small amounts of viral DNA were detected in the transformed cells by dot hybridization.

The available data suggest that only a portion of the HSV genome is required for the initiation of transforming events and that no set of viral genes is consistently retained or expressed to maintain the transformed phenotype. Galloway and McDougall (1983) proposed that cell transformation by HSV can be explained by a "hit-and-run" mechanism based on the fact that there is little or no evidence of viral DNA or consistent viral protein in cells transformed by HSV DNA fragments.

C. Latency

Latency is a central feature of HSV infections and takes place in ganglion cells (reviewed by Hill, 1985). The most common feature of HSV latent infection is recurrence of lesions producing infectious virus in the peripheral body sites. Recurrence is frequently precipitated by a variety of stimuli such as light, trauma, stress, fever or menstruation (Wheeler, 1975) and in immuno-compromised individuals (Ho, 1977).

Co-cultivation of neural cells from the sensory or autonomic ganglia from cadavers with permissive cells, resulted in production of infectious virions and subsequent acute infection of susceptible cells (Warren et al., 1978). Viral replication and DNA were first detected in neurons during reactivation *in vitro* suggesting that the neuron harbors the latent virus *in vivo* (Cabrera et al., 1980; Fraser et al., 1981). In addition, HSV RNA was detected in a small percentage of the neurons which suggested that some HSV genes are expressed during latent infection (Galloway et al., 1979).

HSV DNA extracted from latently infected neural tissue differs from the HSV DNA of actively replicating virus. The HSV-1 genome present in latently infected brain and trigeminal ganglia lacked free ends (Rock and Fraser, 1983; Efstathiou et al., 1986). Southern blot analysis of latent HSV DNA, detected in mouse brain and digested with restriction enzymes, revealed two copies of the DNA joint fragment (Rock and Fraser, 1986). These results were consistent with the hypothesis that the absence of free ends was due to the joining of the HSV DNA termini, probably by concatemerization or circularization of the HSV genome, which existed as an episome or was integrated into the cell genome (Rock and Fraser, 1983). Support for this hypothesis came from studies of Epstein Barr virus, whose genome could be maintained as an episome in proliferating lymphoblastoid cells (Robinson and Miller, 1982).

Studies using HSV ts mutants suggested that specific viral genes are necessary for the establishment and maintenance of latency (Watson et al., 1980). One mutant (tsK) was defective in production of HSV α polypeptide ICP4 and could not establish latency (Preston, 1979). This same polypeptide was continually expressed in the nuclei of latently infected ganglionic neurons (Green et al., 1981). HSV mutants defective in thymidine kinase (TK^- mutants) produced a low incidence of latent infection in sensory and autonomic ganglia (Tenser et al., 1979; Price and Kahn, 1981). The results were not conclusive enough to suggest that TK is an absolute requirement for latency to occur since the "latency negative" phenotype of TK^- viruses may be related to their poor growth in skin tissue, thereby reducing the chance of entry into nerve endings (Hill, 1985).

In situ and northern blot hybridization of latently infected mouse ganglia with HSV-specific DNA probes detected an anti-sense ICP-0 transcript (Stevens et al., 1987). In latently infected cells this transcript was localized in the nucleus whereas in acutely infected neurons nuclear restriction was not observed. This RNA species may encode a protein required for the lytic cascade in infected cells. Nuclear retention of the mRNA for this protein may block its translation and the subsequent lytic cascade. Alternatively, the "anti-ICP-0" transcript may function as an anti-sense RNA and regulate expression of ICP-0 by lowering the pool of functional ICP-0 mRNA (Stevens et al., 1987).

The nature of the virus-cell interaction in latency and reactivation is not well understood. Roizman (1965; 1974) proposed two models: the *static model* proposes that the virus genome is not replicated but remains in the cell (probably integrated into the cellular genome), while the *dynamic model* proposes replication of virus at a reduced rate with gradual spread from neuron to neuron. The bulk of the evidence is in favor of the static state of latency in which only some of the early functions, such as ICP4, are expressed (Hill, 1985).

IV. Aspects of HSV Replication

A. Cell attachment and penetration

HSV initiates infection by attaching to the host cell and fusing its envelope with the plasma membrane (Morgan et al., 1968). The capsid is transported to the nucleus where DNA is released into the nucleoplasm. Internalization and transport of the capsid is probably mediated by the cellular cytoskeleton. Cytochalasins A and B, which interact with and inhibit cytoskeleton function, inhibited the internalization of HSV-1 virus in HEp-2 cells (Rosenthal et al., 1985) and in neurons (Lycke et al., 1984).

Virion components accompany the DNA into the nucleus and participate in viral replication (Fenwick and Walker, 1978; Read and Frenkel, 1983). Batterson and Roizman (1983) characterized a virion-associated protein, VP16, that induced expression of the α genes of HSV. HSV infection is followed by an early shut-off of host macromolecular synthesis.

Daksis and Chan (1987) have mapped two separate HSV-1 loci, defined by ts mutations ts 1-8 and ts 199, responsible for inhibition of host DNA synthesis.

B. Viral genes and their expression

Transcription of HSV DNA occurs in the nucleus. More than 50 polypeptides are encoded by the HSV genome (Wagner, 1984). The regulation of gene expression has three basic features (Roizman and Batterson, 1985); 1) HSV polypeptides can be classified into five main groups on the basis of their temporal order of expression and kinetics of synthesis, 2) the rate of synthesis and relative amounts of protein may vary and 3) the polypeptide groups are sequentially ordered in a cascade fashion.

The first genes expressed are the α genes which encode immediate-early polypeptides ICP0, 4, 22, 27 and 47. The rate of synthesis for these proteins reaches a peak between 2 to 4 hours post infection and then decreases. Functional α proteins are required for the accumulation of all subsequent classes of mRNAs (Honess and Roizman, 1975). Studies, using ts mutants, indicated that ICP4 plays an essential role throughout the replicative cycle and regulates the expression of early and late viral genes (Watson and Clements, 1980). ICP22 expression is required for viral replication in some cell types but not others (Post and Roizman, 1981). ICP0 and ICP27, like ICP4, are essential for the stimulation of early viral gene expression. ICP27 also plays a regulatory role in

virus replication and viral DNA synthesis (Sachs et al., 1985).

The second group of genes expressed are the β genes which encode the early polypeptides. This group can be subdivided into two categories, β_1 and β_2 (Roizman and Batterson, 1985). Examples of the β_1 category are ICP6 (a component of HSV ribonucleotide reductase) and ICP8 (the major DNA binding protein). The β_2 polypeptides include thymidine kinase and DNA polymerase. The β polypeptides reach peak rates of synthesis about 5 to 7 hours post infection (Honess and Roizman, 1974) and are distinguished from α polypeptides by their requirement for a functional ICP4 protein for their synthesis.

The γ genes encode the late polypeptides and can be divided into two categories, γ_1 and γ_2. The γ_1 genes, also called βγ or delayed-early genes, are transcribed in the absence of viral DNA synthesis, whereas the transcription of γ_2 genes strictly requires viral DNA synthesis (Honess and Roizman, 1974; Conley et al., 1981). The γ_1 proteins are distinguished from β polypeptides by the fact that the amounts of γ_1 proteins are greatly reduced in the presence of DNA synthesis inhibitors (Roizman and Batterson, 1985). Glycoproteins B and D and ICP5 (major capsid protein) are included in the γ_1 protein group, whereas glycoprotein C is a γ_2 protein.

HSV DNA is transcribed by the host cell RNA polymerase II (Constanzo et al.,1977). The transcripts are capped,

methylated and polyadenylated. They vary in their abundance and stability (Silverstein et al. 1976; Stringer et al. 1977). A limited extent of RNA splicing was observed for HSV transcripts. For example, the glycoprotein C transcript has the signals for alternate splicing but the major transcript from this gene is unspliced (Frink et al., 1983). A few genes that share 5' or 3' termini have been described (McLaughlan and Clements, 1982; Pellet et al., 1986). A transcript homologous to the genomic region between 0.185 and 0.225 map units contains a 4 kb intron (Costa et al., 1985a). Transcription of some HSV genes, e.g. thymidine kinase, occurs at multiple initiation sites (Sharp et al., 1983).

C. Transcriptional regulation: cis and transacting factors

Transcriptional regulation is an important mechanism for the control of HSV gene expression. Extensive investigations, including transient expression and *in vitro* transcription assays, have enhanced the understanding of the transcriptional regulatory sequences and the virus-encoding regulatory factors required for HSV gene expression.

The use of hybrid target genes, controlled by α promoter-regulatory regions to analyse virus transcription, indicated that α promoter activity was stimulated by a structural component of the virion (Batterson and Roizman, 1983). It was later identified as VP16 (also known as Vmw65) (Campbell et al., 1984; Dalrymple et al., 1985 and Pellet et al., 1985a). Sequence analysis revealed an

upstream consensus sequence, TAATGARAT (where R = a purine) present in two or more copies in the 5' regions of the genes which has been implicated in the trans-activation of gene transcription by VP16 (Mackem and Roizman, 1982; Whitton and Clements, 1984). The promoter regulatory region of ICP4 is also subject to auto-repression and stimulation by IE 110K polypeptide (O'Hare and Hayward, 1985). Gel electrophoresis of specific DNA fragments (derived from the ICP4 promoter region), after association with ICP4 protein *in vitro*, identified a defined DNA region to which ICP4 bound (Muller, 1987). This region contained a putative ICP4 consensus binding sequence, CGCCCCGATCGTC, which was shown by Faber and Wilcox (1986) to associate specifically with ICP4.

In vitro transcription of gD and gC ($\beta\alpha$ and γ genes, respectively) required the presence of a nuclear fraction from HSV infected cell extract which was enriched in ICP4 (Pizer et al. 1986). ICP4 was shown to bind 100 bp upstream from the transcription start site of gD and this region contained the ICP4 consensus sequence (Faber and Wilcox, 1986). Arsenakis et al (1986a) constructed a cell line which constitutively expressed gB1. Expression of gB1 in the primary cell line and after several serial passages was dependent on the expression of ICP4.

The major DNA binding protein, ICP8, plays a major role in HSV gene transcription. The negative regulation of ICP4 transcription required a functional ICP8 and defects in ICP8 resulted in increased levels of transcription of ICP4, ICP8,

ICP5 and gC (Godowski and Knipe, 1986). These results indicate that ICP8 may function to negatively regulate transcription of α, β and γ genes either in a direct or indirect manner.

Most β promoters are similar to other eukaryotic promoters recognized by RNA polymerase II (reviewed by Wagner, 1985). An identifiable "TATA" box occurs approximately 28-30 bases upstream from the transcription initiation site. Most β promoters also have a recognizable "CAT" sequence at about -60 and at -90 bases, and many contain an "AC-rich" sequence between -90 and -120 bases. HSV superinfection in cell lines containing HSV early genes indicated that sequences required for stimulating expression of several β genes were no greater than 200 bp upstream from the mRNA cap site (Sandri-Goldin et al., 1983). Everett (1983) studied the promoter of gD in a transient transcription assay and determined that only 83 bp 5' to the transcription cap site were required for induction of transcription during viral superinfection.

Homa et al. (1986) analyzed the expression of gC transcripts from cells infected by mutant HSV viruses which had variable deletions in the 5' r of the gC gene (a γ_2 gene). The results indicated that the DNA sequences required for regulating gC expression lie within bases -34 to +124 relative to the 5' terminus of the gC mRNA initation site and sequences between -34 and +14 contained signals essential for gC expression.

Transient expression assays, using the γ_2 gene VP5 and the β_2 gene TK fused to the promoter-regulatory domains of γ_2 genes, have shown that these genes were regulated as β genes when integrated into the host genome (Costa et al., 1985b; Silver and Roizman, 1985). Similarly, after transfecting and integration of the gC gene into LtK$^-$ cells, the resident gC gene was regulated as a β gene (Arsenakis et al., 1986b). These results suggested that, not only are the upstream sequences of the γ genes important, but the genomic environment of the gene also plays an important role in its proper regulation.

The underlying message derived from the above data is that both trans-acting proteins and cis-acting promoter sequences play a significant role in the regulation of HSV gene transcription. Elucidation of the precise mechanisms which are responsible for maintaining the highly ordered cascade of HSV gene expression will likely reveal a complex interaction between regulatory proteins encoded by the virus with specific DNA sequences. Additional factors such as genomic environment, cellular proteins, and post-transcriptional regulation must also be considered. One major question that remains unanswered is; what unique features determine whether a HSV gene will be transcribed as an α, β or γ gene? A more extensive compilation of promoter sequence data and generation of more mutations in the regulatory regions and proteins will assist in answering this question.

D. DNA synthesis

Viral DNA synthesis begins in the nucleus approximately 3 hours post infection and proceeds for 9-12 hours thereafter (Roizman et al., 1965). Herpes virus DNAs are thought to replicate by a rolling-circle mechanism (reviewed by Roizman, 1979). Electron microscopic studies have shown that, after the onset of replication, HSV DNA was in the form of full circles and circles with tails or lariats. Jacob et al. (1979) showed, by BUdR density shift experiments, that less than 5% of input parental HSV DNA entered the reproductive cycle. Pulse-chase experiments showed that the radio-label appeared first in rapidly sedimenting DNA and after a chase it appeared in unit length DNA. Restriction endonuclease analysis indicated that newly replicated DNA was linked head to tail. These results were consistent with a rolling circle mode of replication.

The HSV genome contains 3 origins of DNA synthesis. Two origins (ORI_S) map in the c repeats flanking the U_S component (Mocarski and Roizman, 1982) and the third origin (ORI_L) maps in the middle of the U_L component close to the genes encoding ICP8 and DNA polymerase (Weller et al., 1985). DNA sequencing of ORI_S revealed an A+T rich, 45bp, palindromic sequence. This sequence shared sequence homology with a 144bp palindromic sequence found in ORI_L (Stow, 1985; Weller et al., 1985). Insertional mutations in the ORI_S palindrome abolished its origin activity, demonstrating the essential

role of this sequence in the initiation of DNA synthesis (Stow, 1985).

Herpesviruses specify several enzymes involved in DNA synthesis. The HSV DNA-binding protein (ICP8) is essential for viral DNA replication (Godowski and Knipe, 1983). *In vitro*, ICP8 bound prefetentially to single-stranded DNA and lowered the melting temperature of poly(A)-poly(T) helices (Powell et al., 1981). Prior to DNA replication, ICP8 was located at pre-replicative sites in the cell nucleus and later was found bound to replicating viral DNA (Leinbach and Casto, 1983). Ruyechan and Weir (1984) reported that purified ICP8 could stimulate HSV DNA polymerase *in vitro*. Chiou et al. (1985) showed that mutations in ICP8 led to altered sensitivity to DNA polymerase inhibitors and that ICP8 interacted directly with HSV DNA polymerase.

Both the HSV-encoded DNA polymerase and the ribonucleotide reductase differ from the corresponding host enzymes. HSV DNA polymerase is highly sensitive to drugs such as phosphonoacetate (Purifoy and Powell, 1977) and nucleoside analogues (Crumpacker et al., 1980). HSV ribonucleotide reductase is insensitive to inhibiton by dTTP and dATP and requires Mg^{2+} for its activity (Husar and Bacchetti, 1981; Averett et al., 1984).

The HSV thymidine kinase (TK) gene is essential for virus growht *in vivo* (Tenser and Dunstan, 1979). The TK gen specified by HSV has a substrate range which is much greater than its host counterpart. HSV TK is composed of two

identical subunits and has binding sites for the nucleoside thymidine and deoxycytidine and also for ATP (Wagner et al., 1981). Darby et al. (1986) proposed a preliminary model for the active center of this enzyme. Their model suggests that 3 regions of the polypeptide cooperate in the generation of overlapping ATP- and nucleoside-binding sites, thus bringing the two substrates in close proximity on the surface of the enzyme.

Other HSV enzymes such as dUTPase (Preston and Fisher, 1984), and alkaline nuclease ICP18 (Banks et al., 1985; Preston and Cordingley, 1982) also play a role in HSV DNA replication.

E. DNA processing, virion assembly and egress

Newly synthesized HSV DNA is processed before packaging into capsules. Viral DNA, in circular or head-to-tail concatemeric form, is linearized by specific cleavage to produce unit length DNA molecules. ICP32 binds specific to viral DNA and is a possible candidate for the cleavage protein (Braun et al., 1984). The DNA a sequence is necessary for cleavage and/or packaging (Locker and Frenkel, 1979; Vlazny et al., 1982).

Empty viral capsids are assembled in the nucleus, followed by insertion of unit length viral DNA (Vlasny et al., 1982). Major capsid protein ICP5 was found to be strictly associated with the nuclear matrix, suggesting that capsid assembly occured at the nuclear matrix level (Bibor-Hardy et al., 1985). Nucleocapsid protein VP22a was required

for encapsulation of viral DNA (Preston et al. 1983). Nucleocapsids attach to modified patches on inner lamella of the nuclear membrane and acquire an envelope during passage through the inner membrane. Budding and envelopement may be mediated by the interaction between the cytoplasmic domains of HSV glycoproteins (extending from the modified nuclear envelope into the nucleoplasm) and the nucleocapsid proteins (Spear, 1985).

Electron microscopic studies suggested that the capsid may be stripped of the envelope derived from the nuclear membrane, acquire a new envelope from the endoplasmic reticulum and be released to the cell exterior, either by envelopement at the plasma membrane or by fusion of vesicles carrying enveloped virus at the plasma membrane (Roizman and Batterson, 1985). In contrast to this hypothesis, Spear (1985), favoured a "reverse phagocytosis" hypothesis which proposed that the capsid is enveloped once at the inner nuclear membrane and the egress of virions is via the cisternae of the RER, the Golgi apparatus, and transport vesicles. Evidence for this hypothesis came from experiments showing that monensin, which is known to block the transport of proteins from the Golgi to the cell surface, also blocked the transport of HSV virions to the cell surface (Johnson and Spear, 1982).

V. HSV Glycoproteins

A. An overview

Herpes simplex virus encodes several glycoproteins which are exposed on the surface of both the virion and the infected cell. Their expression is pertinent to a number of aspects of viral pathogenesis including: 1) mediation of virus entry and spread of infectious virus to adjacent cells, 2) affecting cellular morphology and host range, 3) viral budding from the nuclear membrane, and 4) they are the major elicitors of protective immune responses. Detailed characterisation of the HSV glycoproteins will increase the understanding of the mechanisms of HSV infection and assist in the development of more effective HSV vaccine.

By 1984, glycoproteins gB, gC, gD and gE from HSV-1 and HSV-2, and gG2 from HSV-2 had been identified (reviewed by Spear, 1985). Since then, two additional glycoproteins, gH1 (Buchmaster et al., 1984; Gompels and Minson, 1986) and the HSV-1 counterpart to gG2 (Richman et al., 1986), have been reported. Sequencing of the large S component of HSV-1 (strain 17) revealed the presence of three open-reading frames, US4, US5 and US7, which likely coded for additional glycoproteins (McGeoch et al., 1985).

Restriction analysis of intertypic recombinants between HSV-1 and HSV-2 determined the approximate genomic locations of gB, gC, gD and gE (Marsden et al. 1978; Ruyechan et al., 1974; Para et al., 1982). More precise map locations were obtained by the use of cloned viral DNA fragments for marker

transfer or rescue of mutations in gB1 (Little et al., 1981; DeLuca et al., 1982, Holland et al., 1983; Kousoulas et al., 1984); for selection of gC1, gD1 and gE1 transcripts identified by monoclonal antibody precipitation of their *in vitro* translation products (Lee et al., 1982a; Frink et al., 1983); and for insertion into the TK gene of appropriate HSV strains to detect expression of gC1 and gC2 or gD1 (Lee et al, 1982b; Gibson and Spear, 1983; Zezulak and Spear, 1984). Exact map locations for these genes were established by nucleotide sequence analyses.

Related counterparts of similar electrophoretic mobility for HSV-1 glycoproteins, gB, gD and gE have been identified in HSV-2 (Eisenberg et al., 1982; Pereira et al., 1981, Pereira et al., 1982; Showalter et al., 1981). Initially, gC2 was thought to be type-specific on the basis of its size and antigenic structure and was originally designated gF (Balachandran et al., 1981, 1982). The gF gene was mapped by inserting appropriate HSV-2 genomic DNA fragments into the TK gene of gC$^-$ strain HSV-1 (MP), which led to the expression of gF by the recombinant virus (Zezulak and Spear, 1984). This demonstrated that the gene boundary of gF was between 0.62-0.64 and was colinear with gC1. It also showed that gF and gC1 were antigenically related (Zweig et al., 1983; Zezulak and Spear, 1983). In the 8th International Herpesvirus Workshop (1983), the name gF was changed to gC2 (Spear, 1985). Nucleotide sequence analyses revealed extensive sequence homology between gC1 and gC2 and showed that gC1

contained 27 amino acids in the amino-terminal region that were missing from the gC2 protein (Dowbenko and Lasky, 1984). Glycoprotein gC1 and gC2 were structurally unique in terms of molecular weight (130K and 75K, respectively) and exhibited some antigenic differences (Balachandran et al., 1982; Zezulak and Spear, 1984; Zweig et al., 1983).

Glycoprotein gG2 was initially thought to be the HSV-2 counterpart to gC1 (Marsden et al., 1978) and was mapped between coordinates 0.65 - 0.70 map units. By a more careful analysis of intertypic recombinants (Roizman et al., 1984) the map position of this glycoprotein was changed to the S component between 0.85 - 0.97. The apparent molecular weight of gG2 (determined by polyacrylamide gel electrophoresis) reported by Roizman et al. (1984) and Marsden et al. (1978) was 124K and 92K, respectively. This discrepancy was attributed to the different cross-linking agents used in the gels by these investigators (Roizman et al., 1984).

Richman et al. (1986), using a type-specific monoclonal antibody LP10, precipitated a HSV-1 59K glycoprotein from purified HSV-1 virus. This glycoprotein was mapped within 0.892-0.924 map units by immunoprecipitation (using LP10) of *in vitro* translation products from transcripts corresponding to this region. This region corresponded to the US4 open-reading frame in the S component of HSV-1 DNA reported by McGeoch et al. (1985). Ackerman et al. (1986), using intertypic recombinant analyses and marker transfer experiments, determined that this glycoprotein was colinear

with the gG2 gene. Sequence comparisons of the S component of HSV-1 and HSV-2 genomes revealed that a HSV-2 gene in the region of gG2 was homologous but larger than the gene coded for by HSV-1 US4 (Frame et al., 1986), therefore indicating that US4 is likely the HSV-1 equivalent to gG2. Antiserum raised to a synthetic peptide, corresponding to a portion of the predicted polypeptide from the US4 sequence, precipitated three glycoprotein species having molecular weights; 37K, 48K and 56K from HSV-infected cell extracts.

Two additional open reading frames (US5 and US7), which potentially code for glycoproteins, in the S component of HSV DNA have been described (McGeoch et al., 1985). Further characterization is required to identify the existence and properties of the proteins corresponding to US5 and US7 in HSV infected cells. Transcript mapping data and nucleotide sequencing of the HSV-2 genome between gD and gE genes determined that HSV-2 has an HSV-1 equivalent to the US7 (Hodgman and Minson, 1986).

Showalter et al. (1981) prepared a series of monoclonal antibodies that precipitated a 110K glycoprotein from HSV-1 and HSV-2 infected cells. This glycoprotein was distinct from HSV-1 glycoprotein gB, gC, gD and gE and mapped between positions 0.28 to 0.31 on the HSV genome (Buckmaster et al., 1986). The gene for this glycoprotein, designated gH1, has been sequenced (Gompels and Minson, 1986).

B. Glycoprotein structure and antigenicity

A great deal of information has been derived from the nucleotide sequence and deduced amino acid sequence of HSV glycoproteins. Analyses of the primary structure of all the glycoproteins described to date have indicated that they are composed of at least four regions; 1) a hydrophobic signal sequence 2) a hydrophilic cell surface region containing potential glycosylation and antigenic sites 3) a hydrophobic membraning spanning sequence and 4) a cytoplasmic region.

1) The signal sequence

A signal sequence consisting of 15-30 amino acids is found at the amino terminus of the precursors of many membrane and secreted proteins. The signal sequence mediates the attachment of the nascent polypeptide to the rough endoplasmic reticulum (RER) and vectorial transport of the polypeptide across the membrane (reviewed by Kreil, 1981). The signal sequence is usually cleaved off during the export of the protein by an endoproteolytic "signal peptidase" contained on the inner surface of the RER (Perlman and Halvorson, 1983; von Heijne, 1984).

The hydrophobic amino-terminal ends of the precursors of gB1, gC1 and gD1, exhibit features that are characteristic of typical signal sequences (Spear, 1985). The first 25 amino acids of gD were cleaved off at some time after initiation of translation (Eisenberg et al., 1984). Amino acid sequencing of the amino-terminal end of the mature polypeptide and comparison with the primary sequence (deduced from the

nucleotide sequence) indicated that gB1 and gC1 also contained a cleavable signal sequence (Claesson-Welsh and Spear, 1987; Kikuchi et al., 1984).

DNA sequencing of the HSV-1 U_s component revealed 5 open reading frames which could encode polypeptides having structural features common to membrane proteins (McGeoch et al., 1985). Open-reading frames US6 and US8 corresponded to glycoproteins gD and gE, respectively, whereas US4, US5 and US7 encoded uncharacterized proteins. Comparison of the putative signal sequences of the proteins predicted from these regions with a reference set of amino-terminal regions of other membrane proteins indicated that US4, US5 and US7 gene products were also likely to possess amino-terminal signal peptides (McGeoch, 1985).

2) The cell surface region and antigenic regions

Analysis of the deduced amino acid sequence for several HSV glycoproteins indicated the presence of a large hydrophilic region beginning at the N-terminus of the mature polypeptide (Spear, 1985). The number of potential N-linked (where N=Asn) glycosylation sites that occur in this region varied between the different glycoproteins. All three N-linked glycosylation sites on gD1 and gD2 were in fact, glycosylated (Cohen et al., 1983). The potential sites that are actually glycosylated on the other HSV glycoproteins must still be determined.

Evidence that the major hydrophilic region is located on the cell exterior can be inferred from studies which show

that neutralizing monoclonal antibodies, raised against HSV glycoproteins, react with sites that map within this region of the protein (see below). Direct evidence for the external location of this domain on gB came from *in vitro* translation of gB in the presence and absence of pancreatic microsomal membranes (Claesson-Welsh and Spear, 1987). The pancreatic membrane protected a large portion of gB containing N-linked carbohydrate from proteolytic cleavage. *In vivo*, this portion would represent the region of gB which is inserted through the membrane into the cisternae of the RER and ultimately is expressed on the cell surface.

Several linear antigenic sites on gD were delineated by synthesizing oligopeptides corresponding to predicted antigenic regions of the amino acid sequence and comparison of their cross-reactivity with gD-specific monoclonal or polyclonal antibodies (Cohen et al., 1984; Dietzschold et al., 1984). A similar approach has been utilized for antigenic sites on gC (Zweig et al., 1984). Three continuous epitopes on gD were localized to residues 11-19, 272-279 and 340-356 (Cohen et al., 1986). These epitopes reacted with group VII, II and V monoclonal antibodies, respectively. Groups VII and II monoclonal antibodies exhibited type-common neutralizing activities. Two type-specific epitopes were localized to residues on either side of the type-common epitope (VII) by reacting monoclonal antibodies to a series of overlapping oligopeptides which consisted of amino acid sequences specific for gD1 or gD2

(Rietzschold et al., 1984). Synthetic peptides corresponding to residues 1-23 induced the production of neutralizing antibodies (Cohen et al., 1984) and protected mice against both HSV-1 and HSV-2 challenge (Eisenberg et al., 1985b). Reaction of truncated forms of gD with a panel of monoclonal antibodies defined four discontinuous epitopes within the first 250 N-terminal amino acids on the polypeptide (Cohen et al., 1986; Eisenberg et al., 1985). These epitopes were defined by groups I, III, IV and VI monoclonal antibodies. Groups I and III exhibited type-common neutralizing activities. Group IV neutralized only HSV-1. Group VI monoclonal antibodies inhibited HSV-1 adsorption (Fuller and Spear, 1985).

Antigenic sites on glycoproteins gB and gC have been determined, using antigenic variants of HSV selected in the presence of virus-neutralizing monoclonal antibodies (Holland et al., 1984). The patterns of resistance of *mar* mutants to neutralization with panels of monoclonal antibodies were analysed. Nine epitopes on gC1 were operationally defined and clustered in two distinct antigenic sites (Marlin et al., 1985). A similar analysis identified at least 5 discrete epitopes on gB (Marlin et al., 1986). Pellet et al. (1985) mapped and sequenced the gB regions responsible for the resistance of 3 *mar* mutants to neutralization and reactivity with gB-specific monoclonal antibodies. Determination of the amino acid substitutions in these regions, in combination with secondary structure analyses, determined that at least

two epitopes were within or near amino acid residues 273 and 305 on the mature gB1 polypeptide.

3) The membrane spanning sequence and the cytoplasmic region

A hydrophobic sequence of amino acids, followed by a strongly basic region, was found for the deduced primary structures of HSV-1 glycoproteins, gB, gC, gD, gE, gG and gH (Gompels and Minson, 1986; McGeoch et al., 1985; Richman et al., 1986; Spear, 1985).

The hydrophobic region spans the lipid bilayer and anchors the glycoprotein in the cellular membrane. *In vitro* translation of gB and gD in the presence of microsomal membranes resulted in glycosylation of the polypeptides and association of the polypeptides with the membranes such that the region containing carbohydrate was protected from proteolysis (Claesson-Welsh and Spear, 1987; Matthews et al., 1983). A nonglycosylated polypeptide fragment was cleaved off from the native protein. These results are consistent with the view that these glycoproteins span the microsomal membrane, leaving the remaining C-terminal end exposed on the outside of the membrane. Detailed hydropathicity, thermodynamic and secondary structure analyses of the proposed membrane spanning sequence of gB led to the hypothesis that this region contained three anti-parallel segments which traverse the membrane three times (Pellet et al., 1985c). Trypsin cleavage at an arginine residue exposed in the turn between the first and second membrane-spanning segment or at an arginine residue in the cytoplasmic tail

could account for the size of the protected gB1 fragments (Claesson-Welsh and Spear, 1987).

The presence of the hydrophobic transmembrane sequence affected the expression of HSV glycoproteins at least in some heterologous host systems. It was found that efficient expression of gD in E.coli required the removal of the transmembrane sequence (Weis et al., 1983). Expression of a secreted form of the gD polypeptide in mammalian cells was achieved by construction of an expression vector which encoded a truncated gD protein, lacking the transmembrane sequence (Lasky et al., 1984).

C. Synthesis and Processing of HSV Glycoproteins

1) Kinetics of expression

The kinetics of synthesis of HSV glycoproteins have been reviewed by Spear, (1985). The genes for the major glycoproteins are generally characterized as γ genes. The gene for gC is the only true late γ_2 gene, whereas gB, gD and gE are γ_1 genes. Between HSV-1 and HSV-2, the kinetics of synthesis was very similar but there were some minor differences. In infected cells, the rate of gD2 synthesis declined sharply after 5-7 hours, whereas the rate of gD1 synthesis declined after 4-6 hours post infection.

Jennings et al. (1986) studied the kinetics of appearance of the major glycoprotein species on the surface of HSV-1 infected murine, simian and human cells. Infected cells were removed from a monolayer culture at various times post infection and were stained for the presence of surface

glycoprotein using monoclonal antibodies in an immunofluorescence assay. Fluorescence flow cytometry was used to quantitate the levels of surface antigen expression and determined that the kinetics and levels of expression of the HSV glycoproteins varied between the cell lines tested. Only gB exhibited a consistent level of expression for all the cells investigated.

2) Glycoprotein processing

The intracellular processing of the major HSV glycoproteins, gB, gC, gD and gE has been extensively documented in the literature. The characteristic mobility of each protein was dependent on the cross-linking agent used in the SDS polyacrylamide gels (Eberle and Courtney, 1980) and the cell lines used in the infection studies. The estimated molecular weight of gB isolated from HSV-1 infected HEp-2 cells or Vero cells was 133K and 123K, respectively (Pereira et al., 1981). The apparent difference in molecular weight may have resulted from a differential post-translational processing and, to some extent, proteolytic cleavage during the extraction procedure from Vero cells (Zezulak and Spear, 1984b). Mature glycoproteins gC and gD had an estimated molecular weight of 130K and 59K, respectively, in HSV-1 infected BHK and KB cells (Cohen et al., 1980, Eisenberg et al., 1979). The mature form of gE had a molecular weight of approximately 80K (Baucke and Spear, 1979; Para et al., 1982b).

Pulse-label experiments identified precursor molecules to these glycoproteins which have been designated pgB, pgC, pgD and pgE (Spear, 1985). Studies using tunicamycin, which inhibits the dolichol dependent transfer of high-mannose core carbohydrate to asparagine residues in the polypeptide, indicated that each of the HSV precursor proteins contained N-linked oligosaccharides (Pizer et al., 1980). Experiments using endo-β-acetyl-glucosaminidase (Endo H), which cleaves the two proximal N-acetyl-glucosamine residues of high-mannose oligosaccharides, confirmed this conclusion.

The addition of core carbohydrate to HSV glycoproteins presumably takes place as the nascent polypeptide is transported (during translation) into the RER (Spear, 1985). The *in vitro* translation products of gB and gD associated with microsomal membranes only if the membranes were present during translation. The translation products contained N-linked carbohydrate (Claesson-Welsh and Spear, 1987; Matthews et al., 1983). These results suggested that addition of N-linked oligosaccharide to these HSV glycoproteins occurred co-translationally. However, it is of interest that after incubation of HSV-1 infected cells at 34°C, non-glycosylated precursors to gB and gC were detected in the nuclear fractions. This implied that N-glycosylation could be uncoupled from translation under these conditions (Compton and Courtney, 1984a). Compton and Courtney (1984b) demonstrated that high-mannose precursors of gB, gC and gD were major components of the nuclear fraction and suggested

that the nucleus may have the components necessary for at least core glycosylation of HSV glycoproteins. Electron microscopic radiography, using [^{3}H]-mannose to label core oligosaccharides and [^{3}H]-fucose to label terminal oligosaccharide chains, showed that precursor glycoproteins bearing mannose, but not fucose, accumulated in nuclear membranes (Poliquin et al., 1985).

Within 15-20 minutes after synthesis, the precursors were converted to the mature forms of the glycoproteins (Spear, 1985). This conversion involved processing of N-linked oligosaccharide from high-mannose to complex-type (which coincided with a decrease in sensitivity to Endo H) and acquisition of O-linked oligosaccharides (sensitivity to N-acetyl galactosamine oligosaccharidase was gained) (Johnson and Spear, 1983). The Golgi was shown to be necessary for this processing to occur. The ionophore monensin, which interferes with Golgi function, prevented conversion of the precursors to mature glycoproteins (Johnson and Spear, 1982).

Relative to the other glycoproteins, mature gC contained larger amounts of O-linked carbohydrate (Johnson and Spear, 1983). Tunicamycin prevented the expression of HSV glycoproteins on the cell surface, presumably due to the absence of carbohydrate moieties (Norrild and Pederson, 1982). However, a partially glycosylated gC was detected on the cell surface of tunicaymcin treated HSV-1 infected cells (Wenske and Courtney, 1983). Membrane bound gC from these cells likely contained O-linked oligosaccharide, a

carbohydrate linkage that is not sensitive to the tunicamycin block.

Fatty acylation and sulfation of glycoprotein gE has been demonstrated (Hope et al., 1982; Johnson and Spear, 1983). Fatty acylation occurred at an early stage of Golgi processing prior to conversion of the precursor to mature gE. Sulfation of the N-linked carbohydrate on gE, and to a lesser extent, on gB, gC and gD, occurred at a late stage in maturation.

3) Intracellular transport

HSV glycoproteins have a dual destination within the cell. First, they can be transported via cell organelle membranes and expressed on the cell surface. The second destination is the viral envelope which is acquired by budding of progeny nucleocapsids through the nuclear membrane (Spear, 1985).

Precursor glycoproteins, containing high-mannose core carbohydrate, accumulated rapidly in the nuclear membranes (Compton and Courtney, 1984; Poliquin et al., 1985). The results from electron microscopic radiography of HSV-infected cells were consistent with the hypothesis that newly enveloped nucleocapsids acquire these immature precursors and terminal glycosylation occurs at the surface of progeny virions as they depart from the cell via the Golgi apparatus (Poliquin et al., 1985). Johnson and Smiley (1985) demonstrated that intracellular transport of gD to the cell surface occurred much more slowly in HSV infected cells than

in transformed cells which constitutively express high levels of gD2. They attributed the slow transport of gD to the period of time required for gD to accumulate in the nuclear membranes and participate in the envelopment process when other HSV structural components were present. Immunofluorescent localization of HSV-2 glycoproteins indicated that glycoproteins gB, gC, gD, gE and gG were preferentially sorted to basolateral membranes in several different types of polarized epithelial cells (Srinivas et al., 1986), suggesting that these glycoproteins may contain putative sorting signals that determine their destination.

Changes in the structure of actin filaments and microtubules occurred in HSV infected cells (Norrild et al., 1986). Indirect immunofluorescent staining, using antibodies directed against actin, myosin, tubulin and HSV glycoproteins, revealed that all these molecules showed progressive association within juxtanuclear structures. The cytoskeleton-disrupting drug, demecolcine, led to accumulation of HSV glycoproteins in small cytoplasmic vesicles or focal adhesion areas, suggesting that intracellular transport of HSV glycoproteins was dependent on an intact cytoskeleton.

D. The Carbohydrate Moieties

Several steps of glycosylation occur before the final mature HSV glycopeptides are synthesized. Pulse-Chase experiments and two-dimensional gel analyses of HSV infected cell extracts suggested that synthesis of gB and gD

oligosaccharides was accomplished by at least 15 and 10 discrete steps, respectively (Haarr and Marsden, 1981). Similar analysis by Cohen et al. (1980), using specific antisera to identify the glycoproteins, detected at least 5 and 15 to 20 forms of gD and gC, respectively. Eisenberg et al. (1979) showed that processing from pgD to gD did not alter the polypeptide structure but involved carbohydrate addition. The results of gel filtration chromatography suggested that pgD contained an 1800 dalton oligomannosyl core moiety. Further processing of pgD was accompanied by an acidic shift in its isoelectric point. Mild acid treatment of mature gD lowered its molecular weight, suggesting that the acidic shift was partly due to the addition of sialic acid. Neuraminidase treatment of gB, gC and gD released sialic acid from these glycoproteins and decreased the size and acidic charge of the precursor forms (Cohen et al., 1980; Haarr and Marsden, 1981; Smiley and Friedman, 1985). Detailed structural studies of gD1 and gD2 indicated that all three potential N-linked glycosylation sites were utilized (Cohen et al., 1983). Treatment of pgD with trypsin generated three glycopeptides having molecular weights of 10K, 3.9K and 1.8K. Endo H cleavage of the glycan moieties and gel filtration chromatography of the digested products from each of the tryptic peptides established that the size of each N-linked oligosaccharide was approximately 1.4K to 1.6K.

The carbohydrate structures on gC have been characterized in great detail (reviewed by Campadelli-Fiume and Serafini-Cessi, 1985). Pronase-digested glycopeptides of pgC were isolated by gel filtration and analyzed by lectin-binding and SDS polyacrylamide gel electrophesis after treatment with Endo H. The results indicated that only high-mannose glycans were present, on pgC (Serafini-Cessi, 1984). Thin-layer chromatography of the high-mannose glycans from pgC showed that 5 glycan species were present, differing in the number of mannose residues (Campadelli-Fiume and Serafini-Cessi, 1985). Using a combination of ion-exchange chromatography and neuraminidase treatment, complex-type glycopeptides were resolved into three species, designated AI, AII and AIII, which corresponded to the elution position of mono-, di- and highly sialyated glycopeptides, respectively (Campadelli-Fiume et al., 1982). The results of analyses of binding of the glycopeptides to Con A-Sepharose suggested that AI and AII had a diantennary structure and AIII had a triantennary or more highly branched structure.

In addition to N-linked glycans, HSV glycoproteins also contained O-linked glycans. Evidence for this came from treatment of HSV glycoproteins with mild alkaline borohydride or N-acetylgalactosamine (GalNAc) oligosaccharidase which released O-linked oligosaccharide from glycoproteins gB, gC, gD and gE (Johnson and Spear, 1983). GalNAc occurs preferentially in O-linked carbohydrates (Montreuil, 1980). Olofsson et al. (1981a; 1981b), detected labeled

galactosamine after acid hydrolysis of (^{14}C)-glucosamine-labelled HSV glycoproteins and demonstrated that gC1 bound to *Helix pomatia* (HPA), a lectin with affinity for GalNAc. Serafini-Cessi (1983) identified an GalNAc-transferase activity in HSV-1 infected cell lysates which selectively added GalNAc onto the HSV precursors pgB, pgC, pgD but not onto the mature forms of these glycoproteins *in vitro*. Lectin binding studies with HSV-2 glycoproteins revealed that glycoprotein gG2 binds to (HPA) lectin indicating that at least some of the glycans are O-linked and contain GalNAc (Olofsson et al., 1986). Of all the HSV glycoproteins, only gC1 and gG2 bound to HPA lectin, suggesting that the addition of HPA-binding glycans requires special attachment sequences that are only present in gC1 and gG2.

Glycosylation and processing of HSV glycoproteins depends on the host glycosyltransferases and glycosidases. The electrophoretic profile of glycoproteins made in mutant cells Ric^{R}14 and Ric^{R}21 which are defective in specific glycosyl transferases responsible for late sugar addition in the Golgi, showed an accumulation of the precursor forms and the absence of mature glycoproteins (Campadelli-Fiume and Serafini-Cessi, 1985). Both sialyl- and galactosyl-transferases of HSV infected cells had kinetic properties different from those of uninfected cells, suggesting that HSV might influence glycosylation and somehow modifies the glycosyl transferases in the infected cell (Olofsson et al., 1980). Benzhydrazone (BD) is a specific inhibitor of high-

mannose glycan addition to HSV glycoproteins (Campadelli-Fiume et al., 1980). Togan et al., (1984) isolated a HSV mutant (HSV1(13)S11) which was resistant to BH. The BH resistance was shown to be encoded by the mutant virus and could be transferred by marker rescue to BH sensitive-wild type HSV-2. These results suggested that HSV encodes a product(s) responsible for HSV glycosylation which could be inhibited by BD.

E. Function

1) Viral infectivity and receptor activities

The various stages of HSV infection i.e. virion infectivity, virion envelopement and egress, and host responses are regulated and highly dependent on the expression of HSV glycoproteins.

The HSV component responsible for adsorption to the host cell receptors has not been identified but is likely to include at least some of the viral glycoproteins. HSV has a broad host range with respect to the cell type and species, therefore the viral attachment component may bind to a ubiquitious constituent of the cell surface. HSV-1 and HSV-2 appeared to bind to different cell surface receptors (Vahlne et al., 1980), suggesting differences in the attachment component. Fuller and Spear (1985) found that gD1 and gC1 specific antibodies most effectively inhibited adsorption of radio-labelled HSV-1 to HEp-2 cells. High concentrations of Fc domains of IgG partially inhibited adsorption, suggesting

that gE (the Fc-binding glycoprotein) may also be involved in adsorption.

Glycoprotein gB plays a major role in viral penentration. Three temperature sensitive gB1 mutants, tsB5, tsJ20 and tsJ12, could not synthesize mature gB1 at the non-permissive temperature (DeLuca et al., 1984). The virions produced in cells infected by these mutants were non-infectious but infectivity could be restored by treating virus-cell complexes with polyethylene glycol, suggesting that the defect in these mutants was in the membrane-fusing activity required for viral penetration (Sarmiento et al., 1979). DeLuca et al (1982) demonstrated that tsB5 virions entered host cells more rapidly than the parent HSV-1 strain KOS. Bzik et al.(1984a) showed by DNA sequencing that the location of the tsB5 mutation was separate from that of tsJ12 and tsJ20 which suggested that this mutation affects a region of gB which regulates the rate of viral penetration.

It has been proposed that gC negatively modulates viral penetration and cell fusion (Manservigi et al., 1977). This hypothesis was supported by the fact that cells infected by gC$^-$ viruses, for example, HSV-1 (MP) and HSV-2 (Gp), exhibited the *syn* (syncytium forming) phenotype (Manservigi et al., 1977; Zezulak and Spear, 1984). Certain HSV-1 strains were able to penetrate the restrictive cell line XC. A locus that affects this phenomenon was mapped to a location closely linked to the gC-regulating locus, Cr. Epstein et al. (1984) suggested that reduced levels of gC1 relative to

gB1 in these cells may be associated with enhanced ability to penetrate XC cells.

It is thought that the HSV glycoproteins are involved in the envelopment of nucleocapsids but as yet no conclusive evidence has been presented. Syncytial strains such as HSV-1 (MP) produced viable and enveloped virions despite the absence of gC1 (Manservigi et al., 1977; Spear, 1985). Glycoprotein gE was identified as an unessential gene since deletion of this gene did not affect viral replication (Longnecker and Roizman, 1986). DNA sequencing data suggested that gB has a long cytoplasmic tail (Bzik et al., 1984; Pellet et al., 1985) which could interact with viral nucleocapsids and promote envelopment. Hoffer, Weizhong et al. (1987) constructed a mutant HSV-1 strain that did not produce gB but could form virus particles, indicating that gB was not essential for nucleocapsid envelopment or viral assembly.

HSV-1 induces a receptor for the C3b complement component on the plasma membrane of infected cells (Friedman et al., 1984). This receptor was not detected on cells infected by HSV-2. Friedman et al. (1984) proposed that gC1 serves a function similar to C3b receptors on human blood cells in that it may protect the virus-infected cell from complement-mediated lysis. Evidence for this has come from studies which showed that gC1 was an inhibitor of the complement cascade (Fries et al., 1986). Neuraminidase treatment of HSV-1 infected cells greatly enhanced the

binding of C3b to gC suggesting that this binding was modulated by sialic acid on the carbohydrate moieties of gC (Smiley and Friedman, 1985).

Glycoprotein gE acted as a receptor for the Fc portion of human IgG on the surface of HSV infected cells (Nucke and Spear, 1979). Examination of the IgG subclasses showed that IgG3 did not bind to HSV-1 infected cells and that binding affinity increased in the order: IgG2; IgG1; IgG4 (Johansson et al., 1984). The binding of IgG to gE on the surface of infected cells may hinder immune cytolysis of HSV infected cells and may influence the expression of viral gene products (Lehner et al., 1975) but a precise role for gE has not been identified.

2) Role of oligosaccharides

In the presence of deoxyglucose, tunicamycin, or ammonium ions, HSV infected cells produced virions with markedly reduced infectivity (Spear, 1985; Campdallei-Fiume and Serafini-Cessi, 1985), indicating that addition of N-linked core carbohydrate was a minimal requirement for infectivity. Monensin, which inhibits further processing of N-linked core sugars and addition of O-linked oligosaccharides, has little adverse effect on the infectivity of the progeny virions.

In mutant cell lines deficient in specific glycosyl transferases, infectious virus, containing immature glycoproteins, was produced but transport to the cell surface was impaired (Campadelli-Fiume et al., 1982; Serafini-Cessi

et al., 1983b). Johnson and Spear (1982) showed that the monensin block in maturation of virion glycoproteins was associated with a block in the transport of the virions to the cell surface. Therefore, virion egress was hampered under conditions where further carbohydrate processing was impaired.

3) Structural similarities of the glycoproteins within the herpes viridae family.

The overall organization of Varicella-zoster virus (VZV) DNA was similar to HSV and encoded at least some proteins which were functional analogues of the HSV proteins (Felser et al., 1987). DNA sequence analysis has shown that several VZV glycoproteins share sequence homology with HSV glycoproteins (Davidson et al., 1986). The VZV gpII gene, in particular, had extensive sequence homology with HSV gB (Keller et al., 1986) and displayed serological cross-reactivity (Kitamura et al., 1986). Open-reading frame analysis, of the U_L region of VZV predicted a protein having partial sequence homology to HSV gC (Kinchington et al., 1986).

DNA sequence analysis of Epstein Barr virus (EBV) predicted a protein encoded by open-reading frame BALF4 which showed extensive sequence homology with HSV gB (Pellet et al., 1985b). Expression of BALF4 in mammalian cells showed that it encoded a 110K glycoprotein that localized to intracellular membranes (Gong et al., 1987). Pseudorabies virus encoded a glycoprotein (gp50) homologous to HSV gD

(Petrovskis et al., 1986) and tryptic peptide analysis showed that several glycoproteins from HSV, bovine mammillitis virus and equine herpesvirus type 1 had considerable structural conservation of the peptide backbone (Snowden et al., 1985). Further characterization of these glycoproteins will likely reveal many functional similarities.

F. Immune responses to HSV

The HSV glycoproteins are the major viral antigens exposed on the surface of HSV infected cells. The glycoproteins are highly immunogenic and are the principal inducers of viral-specific antibodies in the host (Vestergaard, 1980). Antibodies against the glycoproteins participate in antibody-dependent, complement-mediated and cell-mediated immunolysis of infected cells (Glorioso et al., 1983; Norrild et al., 1979). The HSV glycoproteins were essential to recognition of infected cells by cytotoxic T cells (Carter et al., 1981) and contained epitopes recognized by human natural killer (NK) cells (Bishop et al., 1983). The humoral and cell-mediated immune responses to HSV have been reviewed by Norrild (1985) and Nash et al. (1985), respectively.

Preparations of gB, gC, gD and gE, which had been solubilized from polyacrylamide gels, induced the production of neutralization antibodies in animals (Spear, 1985). Studies using cells infected with HSV-1 mutants, altered in the ability to express gC and gB, demonstrated that gC and gB were dominant immunogens (Glorioso et al., 1984).

Comparison of the immunogenic potency of gB1, gC1 and gD1 revealed that gD1 induced the highest titer of neutralizing antibodies (Norrild and Vestergaard, 1977). Glycoprotein gD1, purified by monoclonal affinity column, provided strong protection in mice against lethal HSV-1 and HSV-2 challenge ong et al., 1984). The protective effect was correlated to ne presence of high titers of gD-specific antibodies.

Monoclonal antibodies specific for gB, gC and gD were able to neutralize HSV infectivity (Balachandran et al. 1982a). The highest neutralizing activities, in the absence of complement, have been associated with anti-gD monoclonal antibody (Spear, 1985). Passive transfer of monoclonal antibodies directed against gB2, gC2, gD2, gE2 and gG2 provided protection in BALB/c mice to subsequent lethal HSV challenge (Balachandran et al., 1982b). The mechanism of immune protection is unclear but may involve virus neutralization and monoclonal antibody-dependent complement- or cell-mediated lysis of HSV virions and HSV infected cells. *In vitro* incubation of infected trigeminal ganglia, in the presence of monoclonal antibody directed against gB and gE, suppressed intracellular virus replication suggesting that interaction of antibody with the infected cell was sufficient to suppress viral replication (Oakes and Lausch, 1984). Kumel et al. (1985) showed that passive transfer of monoclonal antibodies recognizing different epitopes on a single glycoprotein were not equally protective despite the fact that the antibody doses were functionally equivalent on

the basis of *in vitro* neutralization tests. These results imply that monoclonal immune protection is not only due to virus neutralization.

Recurrent HSV infections occur in the presence of circulating HSV specific antibody therefore the precise role of the humoral response *in vivo* remains uncertain (Norrild, 1985). In an animal model, specifically designed to study the effect of maternal antibodies on neonatal HSV infection, both immunization of the mother and oral-feeding of newborn mice with HSV specific IgG gave protection (Hayashi et al., 1983). However, in humans, there was no apparent correlation between the presence of maternal HSV antibodies and the development of neonatal HSV infection (Norrild, 1985). In a mouse model, for recurrent HSV infection, injection of HSV in the flank leads to the development of ganglionic infection, subsequent transport of the virus to the skin via nerve fibers and development of a zosteriform rash. Simmons and Nash (1985) found that neutralizing antibodies prevented the development of this rash and concluded that a heightened humoral response to HSV could interrupt recurrent epidermal infection.

The HSV glycoproteins expressed on the surface of infected cells also serve as target antigens for HSV-specific cytotoxic T lymphocytes (CTL) (Carter et al., 1981; Nash et al., 1985). The CTL response is restricted by the association of these antigens with the class I and, in some cases, class II MHC antigens (Jennings et al., 1985; Yasukawa

and Zarling, 1984). CTL lysis of HSV infected cells required the presence of helper T cells (Schmid and Rouse, 1983). Relative to HSV-1, HSV-2 infected cells exhibited a reduced susceptibility to lysis by CTL which may result from a reduction in class I H-2 antigen expression on these cells (Jennings et al., 1985). Human CTL clones generated with HSV-1 or purified gD1 were able to lyse autologous cells infected with a recombinant vaccinia virus that expressed gD1 (Zarling et al., 1986). This result demonstrated that gD served as a target antigen for HSV-specific CTL. Immunization with the recombinant vaccinia virus protected mice from lethal HSV challenge (Cremer et al., 1985).

Martin et al. (1987) evaluated the nature of the gD-specific T cell response using the recombinant vaccinia gD1 virus, VgD52. T-cell proliferation and interleukin-2 production was detected when HSV-stimulated immune cells (derived from mice immunized with HSV) were cultured with syngeneic spleen cells infected with HSV-1 or Vgd52. However, when VgD52 infected target cells were incubated in the presence of HSV-stimulated immune cells, gD-specific cell lysis (i.e. gD-specific CTL) was not observed. Further investigation revealed the presence of suppressor cells and factors capable of inhibiting HSV-specific CTL induction. Horohov et al. (1985) have also demonstrated that HSV-specific suppressor cells regulate T-cell responses to HSV antigens *in vitro*. There is some evidence that helper-to-suppressor T lymphocyte ratios change during recurrence of

[illegible] et al., 1982), suggesting that suppressor cells and their products are involved in modulating immunity to HSV *in vivo*.

The susceptibility of HSV-infected target cells to human NK cell activity correlated with the presence of gB and gC on the infected cell surface (Bishop et al., 1983). Monoclonal antibodies specific for gB and gC blocked NK activity against HSV-infected cells, indicating that NK cells recognized these glycoproteins. Experiments, using cells infected by glycoprotein-*mar* mutant viruses showed that these cells exhibited reduced sensitivity to NK-mediated cytotoxicity and defined some of the antigenic sites on the glycoproteins that are recognized by NK cells (Bishop et al., 1984). Bishop et al. (1986) demonstrated that cells mediating NK activity showed clonally restricted recognition of HSV-1 infected target cells and that precursor NK cells, specific for determinants of gB and gC glycoproteins, exist in normal human-peripheral blood mononuclear cells.

In vivo, interferon-alpha (IFN-α) was produced during systemic or localized HSV infections from peripheral blood mononuclear cells (Kirchner et al., 1979). *In vitro*, monoclonal antibodies specific for gD neutralized the IFNα-inducing capacity of infected fibroblasts indicating that gD plays a key role in IFNα-induction (Lebon, 1985). Chatterjee et al. (1985) reported that cloned IFN-β or IFN-α did not impair synthesis of major nucleocapsid proteins in HSV infected cells but the synthesis of glycoproteins gB and gD

as inadequately reduced. These results suggested that human IFN blocked HSV morphogenesis at a late stage and inhibited the release of virus particles from IFN treated cells.

In order to further understand the involvement of the glycoproteins of HSV in the humoral and cell-mediated immune responses to HSV, it will be necessary to fully define their antigenicity. Although some of the antigenic sites on glycoproteins gC and gD have been located, little was known about gB. Glycoprotein gB is a strong immunogen and its expression is required for the production of infectious virus. Therefore, characterization of the antigenic strucuture of gB is essential to the understanding of the immunobiology of HSV infection. The extent of glycosylation on HSV glycoproteins affects their immunogenicity (Glorioso et al., 1983). Therefore, it will also be necessary to purify the different glycosylated forms (precursor and mature) of each glycoprotein, to characterize their carbohydrate moieties and examine their contribution to immunogenicity. Glycoprotein gD is an important immunogen and has been purified by immuno-affinity chromatography (Eisenberg et al. 1982). Structural analysis of gD determined that it contained three N-linked carbohydrate moieties (Cohen et al., 1983) and possibly several O-linked carbohydrate moieties (Johnson and Spear, 1983). However, neither their composition nor structure were known. The development of an efficient purification scheme, which achieves fractionation of the precursor and mature forms of

gD, is a prerequisite to the biochemical analysis of the oligosaccharides attached to gD.

CHAPTER 2

MATERIALS AND METHODS

I. Cells, Viruses and Vectors

A. Tissue culture

a) Cells

Vero cells were used as host cells for the propagation of HSV. They were grown in Dulbecco-modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum. Sodium penicillin G and streptomycin sulfate (P/S) (Gibco Canada, Burlington, Ontario) were added to the medium to control bacterial contamination. The pH of the medium was adjusted to approximately 7 by the addition of 7.5% (w/v) sodium bicarbonate.

b) Viruses

HSV-1 (strain KOS) and HSV-2 (strain 333) were obtained from Dr. William Rawls (McMaster University, Hamilton, Ontario) and propagated in Vero cells. HSV-infected cells were prepared by infecting confluent monolayers of Vero cells in 490cm^2 roller bottles or 150 cm^2 flasks at a multiplicity of infection of 10 pfu/cell. After 1hr. adsorption period, the unbound virus suspension was removed and replaced with fresh medium. After incubation at 37°C for 16 hrs., the cells were harvested for the purpose of RNA or protein extraction.

B. Bacterial host cells

E.coli HB101 cells were used to propagate the plasmid vectors. The cells were grown in Luria broth containing

2μg/ml of ampicillin (Sigma Chemical Co., St. Louis, Mo., USA) to select for the host cells containing the transfecting plasmids utilized in this study. *E.coli* JM101 and JM103 hosts were obtained from New England Biolabs (Beverly, MA, USA) and used to propagate the M13 phage vectors. These host strains carry a chromosomal *pro* deletion which ensures selection of the episome on glucose/minimal medium. The bacteria were transferred from a glucose/minimal plate and grown for one generation in Luria broth just prior to transfection by M13 phage.

C. Plasmids and M13 vectors

HSV-1 (KOS) and HSV-2 (333) BglII genomic libraries were constructed in our laboratory by cloning the genomic BglII restriction fragments into the BglII site of plasmid pKC7. Plasmids p1BI1 and p2BJ1 contain HSV-1 BglII DNA fragment I (0.314 - 0.416) and HSV-2 BglII DNA fragment J (0.314 - 0.395), respectively, and were used as sources of DNA for the determination of restriction enzyme maps, northern blot analysis and sub-cloning. Clone 12 is a recombinant clone of the HSV-1 7.8kb BamH1 DNA fragment (0.345 - 0.397) inserted into the BamH1 site of pBR322. Clone 75 is a recombinant clone of the HSV-2 BamH1-BglII DNA fragment (0.345 - 0.395) inserted into the BamH1 site of the mammalian expression vector pSV_2-gpt. Clone 12 and Clone 75 contain the gB1 and gB2 genes, respectively. M13 vectors mp8, mp9, mp10 and mp11 were obtained from New England Biolabs and used to subclone gB2 DNA subfragments for the purpose of DNA sequencing.

II. Restriction Endonuclease Analysis of DNA

A. Restriction enzymes

The restriction enzymes were obtained from Pharmacia P-L Biochemicals (Dorval, Quebec), Bethesda Research Laboratories (Burlington, Ontario) and New England Biolabs (Beverly, MA., USA); and were used according to the instructions of the suppliers.

B. Agarose gel electrophoresis

Electrophoresis of DNA was carried out in agarose gels ranging from 0.5 to 1.5% agarose (Bio-Rad Laboratories, Richmond, Calif. USA) in 1XTBE buffer [1mMEDTA, 50mM Tris-borate (pH8.3)] at a constant current. Low melting point agarose (Mandel Sci. Co., Rockwood, Ontario) was used for recovery and purification of DNA fragments. DNA fragments were visualized by staining with 0.5µg/ml ethidium bromide (Sigma Chemical Co.). The sizes of the fragments were determined by comparing their migrational positions with that of the molecular size DNA markers: lambda phage DNA cut with HindIII or pBR322 plasmid DNA cut with AvaI.

C. Terminal labeling of DNA fragments

a) Labeling procedure using T4 DNA polymerase

The 20µl reaction mixture consisted of 1.25 units T4 DNA polymerase (Bethesda Research Laboratories) per µg of DNA sample in exonuclease buffer [6.7 mM potassium acetate, 3.3 mM Tris-acetate (pH7.8), 1.0 mM magnesium acetate, 0.05 mM DTT, 100µg/ml BSA] and was incubated at 37°C for 2 min. The 3'-> 5' exonuclease activity of T4 DNA polymerase removed

nucleotides sequentially from the 3' terminal ends at a rate of approximately 20 nucleotides per min. The exonuclease activity was then terminated by transfer of the reaction mixture onto a dried mixture of 1.5 mM dATP, dTTP, dGTP, (Pharmacia P-L Biochemicals Inc.) and 100 µCi α^{32}P-dCTP (~800 Ci/mmole)(Amersham Canada Limited, Oakville, Ontario). The deoxyribonucleotides acted as substrates and promoted the 5'-> 3' polymerase activity of T4 DNA polymerase. The polymerase reaction was carried out at 37°C for 15 min. and it was terminated by the addition of 2.0 µl of 1.0% (w/v) SDS, 0.1 M EDTA and incubation at 60°C for 5 min.

b) Labeling procedure using *E.coli* DNA polymerase 1 (Klenow fragment)

The 25 µl reaction mixture consisted of 1 unit of Klenow fragment (Bethesda Research Laboratories) per µg of DNA, in 6 mM Tris-HCl (pH7.5), 6 mM $MgCl_2$, 6 mM NaCl, 0.1M KCl. The mixture was added to a tube containing 25 µCi of dried α^{32}P-dNTP and incubated at room temperature for 12.5 min. The 5'-> 3' polymerase activity of the Klenow fragment adds the radiolabeled nucleotide to the recessed 3' hydroxyl terminus of the restriction site. The radioactive nucleotides, α^{32}P-dGTP and α^{32}P-dATP, were used as substrates to radio-label the restriction endonuclease sites, BglII and EcoRl, respectively. The reaction was terminated by addition of 5.0µl 25 mM EDTA, 25 mM Tris-HCl (pH8.0), 0.1% (w/v) SDS.

D. Mapping of restriction endonuclease sites by partial digest

The procedure of Smith and Birnstiel (1976) was used. The HSV-1 BglII fragment I (2 µg) was labeled at a single terminus (either the BglII terminus or the EcoR1 restriction site close to the opposite terminus) by the T4 DNA polymerase labeling procedure. The radiolabeled fragment was purified by sequential extraction with phenol and ether (in order to remove contaminating agarose), and followed by alcohol precipitation. The DNA sample was dried and suspended in 60 µl 10 mM Tris-HCl(pH7.5), 1mM EDTA. An aliquot containing approximately $2x10^5$ cpm was removed and diluted to a final volume of 40 µl with the appropriate restriction buffer. Partial digestion was performed by adding 0.5 units of restriction enzyme and the mixture was incubated at 37°C. Aliquots of 6 µl were removed at 0, 5, 10, 30, 60 and 120 min. and added to 2 µl stop buffer [25 mM Tris-HCl (pH8.0), 25 mM EDTA, 1.0% SDS, 0.4% (w/v) bromphenol blue, 25% (v/v) glycerol]. Samples were loaded onto 0.5% agarose gel and subjected to electrophoresis at 35 mA for 16 hrs. The gel was then dried and autoradiography was performed using Kodak XAR-5 film in a par-speed intensifying screen (Cronex) cassette. Film exposures required 4-8 hrs.

III. DNA Hybridization Analyses

A. DNA hybridization

DNA restriction fragments were subjected to electrophoresis in agarose gel and transferred onto

nitrocellulose paper (Schleicher and Schuell BA85 0.4 mm), using the Southern transfer method described by Maniatis et al. (1982). The DNA fragments which were to be used as probes were nick-translated using the nick-translation kit and α^{32}P-dCTP (>3000 Ci/mmole) supplied by Amersham Canada Limited. The hybridization procedure used was a modification of the protocol described by Maniatis et al. (1982). The final concentration of the radiolabeled probe was approximately $1-2x10^6$ cpm/ml in the hybridization buffer. Hybridization was carried out at 42°C for 16-20 hrs. The blots were washed 4 times with 2xSSC, 0.1% (w/v) SDS at room temperature for 5 min. (20xSSC = 3M NaCl, 0.3M sodium citrate) and then 3 times with 0.1xSSC, 0.1% (w/v) SDS at 60°C for 15 min. The blots were dried at 50°C for 30 min. Autoradiography was performed using Kodak XAR-5 film for 1 to 4 hrs.

B. Dot blot analysis

This procedure was utilized to identify positive M13 clones. DNA samples (approximately 0.1 µg) were applied onto nitrocellulose paper and allowed to dry at room temperature for 15 min. The blot was baked at 80°C for 2 hrs. The hybridization procedure was identical to that outlined for DNA hybridization (see above). Blots were washed 4 times with 2xSSC, 0.1% (w/v) SDS at room temperature for 5 min. and then 2 times with 0.1xSSC, 0.1% (w/v) SDS at 60°C for 45 min.

IV. Procedures for RNA Extraction and Hybridization Analysis

A. Isolation of RNA from Vero cells

RNA was extracted from HSV-1 or HSV-2 infected-Vero cells by a modification of the procedure outlined by Kumar and Lindberg (1972). Infected cells were harvested by scraping the cells from the flask surface, followed by rinsing with phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The cells were centrifuged at 3000g for 10 min. and then washed twice with cold PBS. Approximately $1x10^8$ cells were suspended in 3 ml of isotonic buffer [150 mM NaCl, 10 mM Tris-HCl (pH7.8), 1.5 mM MgCl]. An equal volume of isotonic/NP_{40} buffer (150 mM NaCl, 10 mM Tris-HCl (pH7.8), 1.5 mM MgCl, 1.3% (v/v) NP_{40}) was then added. This mixture was incubated at 4°C for 10 min. and then centrifuged at 3000g at 4°C for 5 min. The supernatant solution was transferred to a 30 ml Clorex tube and an equal volume of a solution consisting of 7M urea, 35 mM NaCl, 10 mM Tris-HCl (pH7.4), 10 mM EDTA was added. Protein was removed by 2 extractions with an equal volume of 50% (v/v) chloroform (saturated with sodium acetate, NaCl, EDTA), 50% (v/v) phenol. The aqueous phase was separated by centrifugation at 1200g at 4°C for 10 min. and transferred to a new tube. RNA was precipitated by adding 1/10 volume of 3M sodium acetate (pH7.8) and 2 volumes of cold 95% ethanol and incubated at -20°C overnight. RNA was sedimented by centrifugation at 16000g at 4°C for 15 min. The RNA pellet

was washed twice with 1.0 ml of cold 70% (v/v) ethanol, 125 mM sodium acetate (pH7.0) and stored at -70°C. The concentration of RNA was determined by its absorbance at 260 nm. The total yield of cellular RNA ranged from 200-300 µg per $1x10^6$ cells (150 cm^2 flask).

B. Selection of polyadenylated RNA

Poly(A)$^+$ RNA was selected from total cellular RNA by chromatography on oligo(dT)-cellulose (Pharmacia P-L Biochemicals) using a protocol modified from that of Maniatis et al.(1982). The RNA sample was dried in a Speed Vac concentrator (Savant) and suspended in 1.0 ml sterile H_2O. After heating at 70°C for 2 min. an equal amount of 2x loading buffer was added (1x loading buffer = 2 mM Tris-HCl (pH7.5), 0.5 M NaCl, 1mM EDTA, 0.1% (w/v) SDS). The mixture was passed 3 times through the oligo (dT)-cellulose column which had been equilibriated with 1x loading buffer. The column was washed with 20 volumes 1x loading buffer. The poly(A)$^-$ RNA which bound to the column was eluted with 4 volumes of poly(A)$^-$ elution buffer [0.1 M NaCl, 10 mM Tris-HCl (pH7.5), 1mM EDTA, 0.05% (w/v) SDS]. The poly(A)$^+$ RNA was eluted with 4 volumes of poly(A)$^+$ elution buffer [10mM Tris-HCl(pH7.5), 1mM EDTA, 0.05% (w/v) SDS]. The poly(A)$^+$ RNA was precipitated by adding 1/10 volume of 3M sodium acetate and 2.2 volumes of 95% ethanol at -20°C overnight and then centrifugated at 16000g for 10 min. The RNA pellet was resuspended in 70% ethanol, 125 mM sodium acetate (pH7.0) and

stored at -70°C. Typical yields from 100 µg of total RNA were 1-2 µg poly(A)$^+$ RNA.

C. RNA electrophoresis

RNA samples were subjected to electrophoresis in agarose gels in the presence of glyoxal, according to the procedure of McMaster and Carmichael (1977). The sample was dried in a Speed Vac concentrator and suspended in 32 µl of denaturing solution (1M deionized glyoxal (BDH Chemicals Ltd., Poole, England), 50% (v/v) dimethyl sulfoxide (BDH Chemicals Ltd.), 10mM sodium phosphate (pH7.0)]. After the mixture was incubated at 50°C for 1 hr, followed by cooling on ice for 3 min., 8 µl of RNA sample buffer [50% (v/v) glycerol, 10mM sodium phosphate (pH7.0), 0.5% (w/v) bromphenol blue] was added. Denatured RNA samples were subjected to electrophoresis in 0.8% agarose gel containing 10 mM sodium phosphate (pH7.0) at 70 mA for 5 hrs. Circulation of the electrophoresis buffer [10 mM sodium phosphate (pH7.0)] was required to maintain a constant pH.

D. RNA transfer to nitrocellulose and hybridization procedure

The procedure was modified from Thomas (1980). After electrophoresis the RNA was transferred onto nitrocellulose paper pre-soaked in 20x SSC. The RNA blot was baked at 80°C for 2 hrs. The blot was prehybridized at 42°C for 7 hrs. in the presence of prehybridization solution [50% (v/v) deionized formamide (BDH Chemicals Ltd.), 5xSSC, 50 mM sodium phosphate (pH6.5), 250 µg/ml salmon sperm DNA, 0.02% (w/v)

BSA, 0.02% (w/v) Ficoll (Sigma Chem. Co.) 0.02% (w/v) polyvinyl pyrrolidone (PVP-360)(Sigma Chem.Co.)]. After prehybridization this solution was replaced with hybridization solution [prehybridization solution, 50% (w/v) dextran sulfate (Pharmacia)(4:1)] containing 1-2x10^7 cpm/ml of the DNA probe and incubated at 42°C for 20 hrs. The blot was then washed 4 times with 2xSSC, 0.1% (w/v) SDS at room temperature for 5 min, and 2 times with 0.01xSSC, 0.1% (w/v) SDS at 50°C for 15 min. The blot was dried at 50°C for 30 min and autoradiography was performed. The molecular sizes of the bands detected were determined by comparison with the migration distance of denatured DNA marker fragments (pIBI1 DNA cut with BglII and SalI) which were included in the agarose gel.

V M13 Cloning and Sequencing

A. M13 cloning

1. Purification of the DNA fragment

The DNA fragment to be cloned was fractionated by electrophoresis in low melting point agarose gel. The agarose slice containing the DNA was excised. After adding 5 volumes of TE buffer [10 mM Tris-HCl(pH7.5), 1 mM EDTA], the agarose was melted by incubation at 65°C for 10 min. After adding 25 µg of yeast tRNA carrier, the DNA was purified by 2 phenol extractions and 1 ether extraction. The DNA was alcohol precipitated in the presence of 2 volumes of 95% ethanol and 1/10 volume 3M NaAcetate pH 7.0, collected by centrifugation and suspended in 20 µl TE buffer.

2. Preparation of the M13 vector

The replicative form (RF) of the M13 vector (1µg) was digested with the appropriate enzyme(s). The reaction mixture was extracted twice with 1/2 volume of phenol and once with 1 volume of ether. The final volume was made up to 30 µl with sterile dH_2O.

3. Ligation reaction, transformation and plating

The total volume of the ligation reaction mixture was 20µl and consisted of the following:

DNA restriction fragment (0.5 - 1.0µg)

M13 vector (~150ng)

66mM Tris-HCl(pH7.6), 10 mM MgCl

0.5 mM ATP

15 mM DTT

T4 DNA ligase (2.5 units) (Pharmacia-PL Biochem. Inc.)

The ligation mixture was incubated at 4°C for 16-20 hrs.

For transformation, the ligation mixture was added to 200 µl of competent JM101 or JM103 cells. After incubation at 4°C for 1-2 hrs. the transformed cells were mixed with 200 µl of log-phase host cells, 10 µl of 100 mM isopropyl-ß-D-thiogalactopyranoside (Sigma Chem. Co.), 50 µl of 2% (w/v) Xgal (5-Bromo-4-chloro-3-indolyl-ß-D-galactoside) in N-N dimethyl formamide and 3.0 ml of liquid soft agar. The mixture was poured onto an L agar plate and allowed to solidify. The plate was incubated at 37°C overnight. Recombinant phage were identified by formation of white plaques.

4. Growth of recombinant phage and purification of single strand DNA

A single white plaque was inoculated into 2 ml of Luria broth containing 20 µl of an overnight JM101 or JM103 culture. The culture was incubated at 37°C for 8 hrs. with shaking in a G10 Gyrotary shaker (New Brunswick Scientific Co., Edison, N.J., U.S.A.). The cells were removed by centrifugation and 250 µl 20% (w/v) polyethylene glycol-6000 (Sigma Chem. Co.), 2.5 M NaCl was added to 1.0 ml of the supernatant solution. After incubation at room temperature for 30 min. the phage were sedimented by centrifugation for 10 min. in an Eppendorf microfuge. The phage were resuspended in 100 µl TES buffer (10 mM NaCl, 20 mM Tris-HCl (pH7.5), 0.1 mM EDTA). Protein was removed by 1 phenol extraction and the phage DNA was further purified by 3 ether extractions. The phage DNA was precipitated in the presence of ethanol, washed with 70% ethanol, 125 mM sodium acetate (pH7.0), dried and resuspended in 50 µl TES buffer. Recombinant phage DNA was screened for the presence of inserts by dot blot analysis.

B. M13 sequencing

1. Primer-template annealing

The final volume of the primer-template annealing mixture was 10µl and consisted of the following reagents: M13 recombinant DNA (~2-4µg), DNA primer (~2-4ng) and 20 mM Tris-HCl (pH8.5), and 10 mM $MgCl_2$.

The mixture was incubated at 65°C for 30 min. and then cooled slowly to room temperature for 30 min. The M13 universal primer (15mer; Pharmacia PL-Biochem., Inc.) and synthetic primers were used in this study. Generally, a slightly higher amount of synthetic primer (~10ng) was required to efficiently prime the sequence reaction.

2. Sequencing reactions

Aliquots of 2 µl from the annealing reaction mixture were distributed into 4 separate Eppendorf tubes containing 2 µl of the dideoxy/deoxy nucleotide mixes; ddA/dA, ddC/dC, ddG/dG and ddT/dT and 2 µl of enzyme-label mix. The mixes consisted of the following reagents:

ddA/dA		ddC/dC	
ddATP	0.016mM	ddCTP	0.45mM
dCTP	0.15mM	dCTP	0.006mM
dGTP	0.15mM	dGTP	0.12mM
dTTP	0.15mM	dTTP	0.12mM
ddG/dG		**ddT/dT**	
ddGTP	0.1mM	ddTTP	0.35mM
dCTP	0.12mM	dCTP	0.16mM
dGTP	0.006mM	dGTP	0.16mM
dTTP	0.12mM	dTTP	0.008mM

All nucleotide reagents were obtained from Pharmacia PL-Biochem.Inc.

Enzyme-label mix

9mM DTT

~0.8µCi/ml ^{35}S-dATP (650 Ci/mmole, Amersham)

8 mM Tris-HCl (pH8.5)

0.1 unit/µl Klenow fragment (Pharmacia PL-Biochem.)

The sequencing mixture was incubated at 37°C. After 20 min., 2µl of dNTP chase solution (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP) was added and the reaction was incubated for an additional 20 min. The reaction was terminated by adding 5 µl formamide dye mix [100% (v/v) deionized formamide, 0.013% (w/v) bromphenol blue, 0.013% (w/v) xylene cyanol]. The sequencing reaction samples were boiled for 3 min. and loaded onto 30x50 cm gels containing 8% (w/v) acrylamide and 7M urea. Electrophoresis was carried out in a Bio-Rad Sequi-Gen™ Nucleic Acid Sequencing Cell at a constant voltage of 2000 volts, 55°C. Double loading of the samples permitted a practical sequence resolution of 300-350 base pairs.

In some cases the resolution of band compressions on the sequencing gel required the use of deoxy-7-deazaguanosine triphosphate (c^7dGTP; Boehringer Mannheim, Dorval, Quebec) in place of dGTP. The final concentrations of c^7dGTP in the ddA/dA, ddC/dC, ddG/dG and ddT/dT mixes were 0.43 mM, 0.43 mM, 0.075 mM and 0.43 mM, respectively. The labeled nucleotide used in conjunction with c^7dGTP was α^{32}P-dATP (3000 Ci/mmole) and the samples were subjected to electrophoresis in 100 cm gels containing 6% (w/v) acrylamide and 8M urea.

C. Synthetic primers

Synthetic primers were synthesized in order to prime the sequence reaction at specific locations to confirm the DNA

sequence. These primers were synthesized by a phosphite triester method using ß-cyanoethel N,N-diisopropyl phosphoramidites purchased from Pharmacia PL-Biochem. Inc. All solvents and chemicals for DNA synthesis were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA) and BDH Chemicals. Synthesis was performed on a solid support in Pharmacia's Gene Assembler. The primers were purified by gel filtration and acrylamide gel electropheresis according to the protocol of Jing et al.(1986).

VI. Computer Analyses

A. BIONET

DNA sequence management, translation reading frame and deduced primary sequences, hydropathicity, and Chou and Fasman analyses were performed with the CORE library programs of BIONET (IntelliGenetics, Inc., Mountain View, CA., USA). The core programs, GEL and GENED, were used to assemble and edit sequencing data. SEQ analysed the sequence for open reading frames and corresponding polypeptides. IFIND aligned the gB DNA and amino acid sequences from different HSV strains. PEP determined the hydropathicity along the gB polypeptide. The program used the values chosen by Kyte and Doolittle (1982), which reflect the water-vapour transfer free energies and the interior-exterior distribution of amino acids. The program uses a frame size of 6 residues and plots the average hydropathicity along the length of the polypeptide. The Chou and Fasman (1978) algorithm scanned the gB polypeptide chain sequentially, looking for local

groups of amino acids whose average propensity and former/indifferent/breaker characteristics allow for the nucleation of an alpha helix, or beta sheet, or the formation of a turn.

B. Composite surface profile

Prediction of antigenic regions on HSV-2 gB2 was performed using a computer program developed by Parker et al (1986) and run on an Apple-Macintosh microcomputer. The program incorporates 3 different sets of parameters: 1) hydrophilicity values, derived from the retention time of 20 model synthetic peptides in high-performance liquid chromatography (HPLC), 2) accessibility and 3) flexibility. The program first calculated hydrophilicity, accessibility and flexibility profiles and then combined regions having values greater than 25% above the mean parameter value to construct a composite surface profile graph.

VII. Preparation of HSV-1 Infected-Cell Extract

a) Preparation of ^{35}S-methionine-labeled infected cells.

Confluent Vero cells were infected with HSV. After 1 hr. adsorption the unbound virus suspension was replaced with methionine-free media which contained the following:

1 volume: 1xMinimal essential Eagle's medium (modified)
(Flow Laboratories, McLean, Virginia, USA).

1/50 volume: 2% dialysed fetal bovine serum

1/20 volume: modified Eagle's medium

1/100 volume: P/S [Penicillin G (5 x 10^6 units) 0.5% (w/v)/ Streptomycin sulfate]

1/100 volume: 30% (w/v) L-glutamine

30µCi/ml: ^{35}S-methionine (>800 Ci/mmole) (Amersham Canada Ltd., Oakville, Ontario)

The cells were harvested after incubation at 37°C for 16 - 18 hrs.

b) Extract preparation

^{35}S-labelled HSV-1 infected-Vero cells were scraped from 150 cm^2 culture dishes using a rubber policeman and washed 3 times in cold PBS containing 0.1 mM of the protease inhibitor phenylmethyl-sulfonyl fluoride (PMSF) (Sigma Chem. Co.). The cells were lysed in 0.15 M NaCl, 0.01 M Tris-HCL (pH7.5), 0.5% (v/v) nonidet P-40 (Bethesda Research Lab.), 0.5% (w/v) deoxycholic acid (Sigma Chem. Co.). Cells were lysed using 100 µl of this lysis buffer per ~1x10^6 cells (150 cm^2 dish) at 4°C for 10 min. PMSF was added to a final molarity of 10 mM and the mixture was centrifuged at 4,000g for 10 min. to remove cellular debris and nuclei. DNA was precipitated from the supernatant solution by addition of 1/10 volume of 30% (w/v) streptomycin sulfate (Allen and Hanburrys, Glaxo Canada Ltd. Toronto, Ont.) and cetrifuged at 100,000g at 4°C for 1 hr. The supernatant solution was stored at -70°C.

Certain procedures required dialysis of the lysate or lysate fractions. Dialysis was performed using #4 cellulose dialysis tubing (molecular weight exclusion: >14,000,

Spectrum Medical Industries, Inc. Los Angeles, CA., USA) against 2 changes of 10 mM NH_4HCO_3 (pH7.0) buffer.

VIII. Immunoprecipitation and SDS-polyacrylamide Gel Electrophoresis

A. SDS-polyacrylmide gel electrophoresis

Protein samples were dissolved in protein sample buffer [125 mM Tris-HCl (pH6.8), 6% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol] and loaded onto 7.5-15% or 10% polyacrylamide gels containing 0.2% (w/v) SDS. Electrophoresis was carried out in electrophoresis buffer [50 mM Tris-HCl (pH8.6), 380 mM glycine, 0.1% (w/v) SDS] at 150 volts for 5-6 hrs. Electrophoresis in 10% gel at 150 volts for 8 hrs improved the resolution of peptides in the 40-80,000 range. Proteins were stained (Paul et al., 1972) with 0.25% (w/v) Coomassie brilliant blue in 50% (v/v) methanol and 10% (v/v) glacial acetic acid at room temperature for 30 min. Gels were destained in 25% (v/v) methanol and 10% (v/v) glacial acetic acid overnight. Radio-labeled polypeptides were visualized by a film (Kodak XAR-5) detection method described by Bonner and Laskey (1974). Molecular weight protein standards were obtained from Bio-Rad Lab. Ltd. and Pharmacia Fine Chem.

B. Immunoprecipitation

Protein samples were diluted to 0.5 ml with RIPA buffer [50 mM Tris-HCl (pH7.2), 150 mM NaCl, 0.1% (w/v), SDS, 0.1% (w/v) deoxycholic acid, 1.0% (v/v) Triton X-100 and 1mM PMSF]. Proteins binding non-specifically to the agarose

beads were removed by adding 100 µl of a 1:1 dilution of protein A-Sepharose CL-4B beads (Pharmacia Fine Chem.) in RIPA buffer, rotating at 4°C for 30 min., and sedimenting the beads. To the supernatant solution, 3 µl of monoclonal antibody was added and the mixture was rotated at 4°C for 2 hrs. Antibody-antigen complexes were precipitated by adding 100 µl of the Sepharose-bead solution, rotating at 4°C for 1 hr., and sedimenting the beads. The beads were washed 5 times with RIPA buffer at 4°C and then resuspended in protein sample buffer. Samples were heated at 100°C for 3 min. to elute and denature immune complexes. The monoclonal antibody, 18ßB3, used to precipitate gD of HSV was obtained from Dr: William Rawls (McMaster University, Hamilton, Ont.).

IX. Flat-bed Isoelectric Focusing (IEF)

A 30 ml flat-bed IEF gel was prepared and consisted of the following reagents: 50% (w/v) acrylamide, 0.15% (w/v) N,N'-methylene-bis-acrylamide, 3.3% (v/v) glycerol, 15.8% (v/v) 3-10 Pharmalyte (Pharmacia). The gel was prefocused at a constant power of 50 watts at 15°C. The anode and cathode buffers consisted of 0.04 M aspartic acid (Sigma Chemical Co.) and 1 M NaOH, respectively. Protein samples were loaded on the gel using 3MM Whatmann filter paper and focused for 3500 volt-hours. The pH gradient was determined by cutting 1 cm^2 strips from the gel and placing them in 1 ml ddH_2O and measuring the pH using a pH meter. The gel was fixed by 5% (w/v) sulphosalicylic acid, 10% (w/v) trichloracetic acid for 60 min. and then the gel was treated with 30% (v/v) methanol,

10% (v/v) glacial acetic acid (destain). The gel was stained in 30% (v/v) methanol, 10% (v/v) glacial acetic acid containing 0.2% (w/v) Coomassie brilliant blue and then destained overnight. The gels were dried and the radio labeled proteins were detected by autoradiography.

X. Ion-exchange Chromatography

A. DEAE-Sephadex chromatography

DEAE-Sephadex columns were prepared by packing a 2 ml bed volume of DEAE-Sephadex A50 (Pharmacia Fine Chem.) in pasteur pipettes and equilibriated with one of the following buffers:

pH	buffer
3	50 mM sodium acetate
4	50 mM sodium acetate
5	50 mM sodium phosphate
6	50 mM sodium phosphate
7	20 mM Tris-HCl
8	20 mM Tris-HCl
9	20 mM Tris-HCl

The equilibrating buffer was used to dilute 50 µl (approximately 2.5 x 10^6cpm) HSV-1 infected-cell lysate to a final volume of 500 µl. This mixture was passed through the column at a flow rate of approximately 0.05 ml/min. The flow rate was controlled by a C-clamp. Bound proteins were eluted by 1 ml of 1 M NaCl in the appropriate buffer. The flow-through and elution fractions were dialysed against 10 mM NH_4HCO_3(pH7.0) overnight and then lyophilized.

B. DEAE-Sepharose chromatography

The DEAE-Sepharose column was prepared by packing a 10 ml bed volume of DEAE-Sepharose into an HR10/10 column (Pharmacia). The column was equilibriated with 20 mM Tris-HCl (pH6.5). An aliquot of 300 µl from the HSV-infected cell lysate was first dialysed against 20 mM Tris-HCl (pH6.5) and then injected onto the column. Bound proteins were eluted in 30 min. by a linear NaCl gradient (from 0 to 0.5 M) in 20 mM Tris-HCl (pH6.5) buffer. The gradient was rate was 16.7 mM NaCl/min and was generated by Pharmacia's FPLC machine. A constant flow rate of 1 ml/min. was used and 2 ml fractions were collected. The fractions were dialysed and lyophilized overnight. About 70% of each sample was analysed by immunoprecipitation, followed by SDS-gel electrophoresis to detect the gD1 polypeptide. The remaining volume of each sample was analysed by SDS-gel electrophoresis to visualize the total protein profile.

XI. Ammonium Sulfate Precipitation

^{35}S-labeled HSV-1 infected-cell extract (300 µl) was diluted to 5 ml in 10 mM Tris-HCl (pH6.0). Solid ammonium sulfate, was added to precipitate the proteins at 0, 0-30, 30-50 and 50-70% saturation. The protein precipitate was collected by centrifugation at 8000g, for 15 min. at 4°C and then the protein pellet was resuspended in 1.0 ml of 10 mM Tris-HCl (pH7.5), dialysed overnight and lyophilized. The dried samples were resuspended in 100 µl of RIPA buffer. About 30% of each sample was analysed by SDS-gel

electrophoresis and the remainder of each sample was analysed by immunoprecipitation, followed by SDS-gel electrophoresis.

XII. Lectin Affinity Chromatography

Each lectin affinity column was prepared by packing a 1-1.5 ml bed volume of lectin (coupled to Sepharose 4B) into a siliconized pasteur pipet. A list of the lectins utilized in the study and the corresponding eluting agent is given as follows:

Lectin	Elution Agent
Wheat germ agglutinin (WGA: PL-Biochem. Inc.)	0.2M N-acetyl-D-glucosamine (GlcNAc)
Castor Bean-120 (CB-120: PL-Biochem Inc.)	2.5%(w/v) N-acetyl-D-galactosamine (GalNAc) or 2.5%(w/v) D(+) galactose (Gal)
Lentil lectin (LL: Sigma Chem. Co.)	0.2M methyl α-D-mannoside (Me αDMan)

All eluting agents were purchased from Sigma Chem. Co.

^{35}S-labeled HSV-1 infected-cell extract (100µl = approximately 5-6 x 10^6cpm) was diluted to 1 ml in lectin start buffer [50 mM Tris-HCl (pH7.5), 0.15 mM NaCl, 0.1% (w/v) deoxycholic acid] and passed throught the lectin column at a flow rate of 0.1 ml/min. The sample was then reapplied onto the column to enhance protein binding. The column was washed with 5 ml start buffer. Bound glycoproteins were eluted with 4 ml start buffer containing the eluting component. The size of the fractions collected was 1 ml. Fractions 1-4 (flow-through) and 7-10 (elution) were pooled. The pooled fractions were dialysed against 10 mM NH_4HCO_3 (pH7.0)

overnight, lyophilized and analysed by SDS-gel electrophoresis or immunoprecipitation.

XIII. Iodination of Fetuin and Asialofetuin

Fetuin and asialofetuin were obtained from Dr. Glen Armstrong (University of Alberta, Edmonton, Alberta). The fetuin or asialofetuin protein (10 µg/10 µl) was added to 100 µl 0.33 M sodium phosphate (pH7.5) and 5 µl sodium 125-iodide [20 MBq (0.5 mCi)] in an Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Pierce Chemical Co.)-coated glass tube. After 5 min. at room temperature the reaction mixture was filtered through a glass wool-plugged pasteur pipet to remove any flakes of the Iodo-Gen which may have come off the surface of the glass tube. The iodination reaction was quenched by the addition of 200 µl of cysteine (1mg/ml in PBS). Carrier protein [200µl of 0.1% (w/v) BSA in PBS] was then added and the mixture was passed through a 10 ml Sephadex G-25 gel filtration column in order to fractionate the unincorporated radiolabel from the labeled protein. The void volume fractions, containing radiolabeled protein, were identified by counting an aliquot of each fraction in a Rackgamma 1271 gamma counter. Peak fractions were pooled and a portion of this material was precipitated by addition of trichloroacetic acid to determine the amount of radioactivity which was covalently bound to protein.

XIV. Sialyltransferase Reaction and Lectin Binding

The α2,6-Sialyltransferase enzyme from rat liver catalyses the addition of terminal sialic acid to the

structural unit galactose ß1-4 N-acetyl-D-glucosamine (Gal ß1-4GlcNAc) of oligosaccharides at the 6-position of the galactose. Gal ß1-3GlcNAc units are not sialylated (Weinstein et al. 1982a; 1982b). The sialyltransferase reaction mixture contained the following reagents:

Acceptor: ^{125}I-asialofetuin cpm)	1µl(20-25,000
50mM sodium cacodylate (pH6.0), 0.5% (w/v) Triton CF-54, 0.1% (w/v) BSA	150µl
Donor: CMP-N-acetyl-neuraminic acid (CMP-NeuAc)	1µl (100µg)
α2,6-Sialyltransferase	1µl (2 munits)

[Sodium cacodylic acid (Polysciences Inc., Warrington, PA, USA), Triton CF-54 and CMP-NeuAc (Sigma Chem. Co.), α2,6-sialytransferase (Boehringer Mannheim)]. The reaction mixture was rotated at 37^{0}C overnight. Prior to loading onto WGA columns the sample was diluted to 0.6 ml in lectin start buffer.

XV. Gel Filtration of Lectin-Bound Glycoproteins

^{35}S-labeled HSV-1 infected-cell proteins which bound to CB-120 lectin were eluted with 2.5% GalNAc, dialysed against 10mM NH_4HCO_3(pH7.0) and lyophilized. The sample was dissolved in 250µl filtration buffer [0.05M sodium phosphate (pH7.0), 0.15M NaCl, 10% (v/v) 2-mercaptoethanol, 2% (w/v) SDS] containing 2mg/ml BSA and ovalbumin (molecular weight standards). An aliquot of this mixture (approximately 2x10^5 cpm) was passed through a Superose 12 HR10/30 column (Pharmacia) at a flow rate of 0.2 ml/min. The flow rate was

maintained using an FPLC machine (Pharmacia). The eluent buffer consisted of 0.05 sodium phosphate (pH7.0), 0.15M NaCl, 0.2% (w/v) SDS. The column was run at room temperature and 0.5ml fractions were collected. After removing 50µl for scintillation counting the fractions were dialysed against 10mM NH_4HCO_3 (pH7.0) overnight, lyophilized, and dissolved in 25µl of protein sample buffer [125mM Tris-HCl (pH6.8), 6% (w/v) SDS, 20% (w/v) glycerol, 10% (w/v) 2-mercaptoethanol]. The samples were subjected to electrophoresis in 10% polyacrylamide gels containing SDS. The molecular weight standards were detected by Coomasie blue stain. The ^{35}S-labeled proteins were detected by autoradiography.

CHAPTER 3

FINE MAPPING THE GENOMIC LOCATION OF THE gB1 GENE FROM HSV-1

I. Introduction

At the onset of this study it was necessary to determine the location of the gB2 gene on the viral genome and obtain a molecular clone of the gene. Marsden et al. (1978) and Ruyechan et al. (1979) determined a low resolution genetic map for the HSV genes encoding glycoproteins B, C and D by restriction endonuclease site analyses of genomes of intratypic recombinants between HSV-1 and HSV-2. Glycoprotein B was mapped to the 15,000 base pair region between map coordinates 0.3 to 0.4. The length of the DNA sequence required to encode the 120-125K glycoprotein gB should be no more than 4 kilobases. Therefore, it was expedient to determine more precisely the gB gene location. In order to achieve this aim, this study made use of plasmid plBI1 which is a molecular clone of the BglII fragment 1 (0.314 - 0.416 map units) of HSV-1 (strain KOS) inserted into the BglII site of plasmid vector pKC7.

A restriction endonuclease map of the BglII fragment I was constructed. DNA subfragments were used as probes in Northern blot analyses in order to fine map the transcript which encodes gB1. Northern blot analysis also identified a 6.6 kb BamH1 - BglII DNA fragment from the HSV-2 genome containing the gB2 gene.

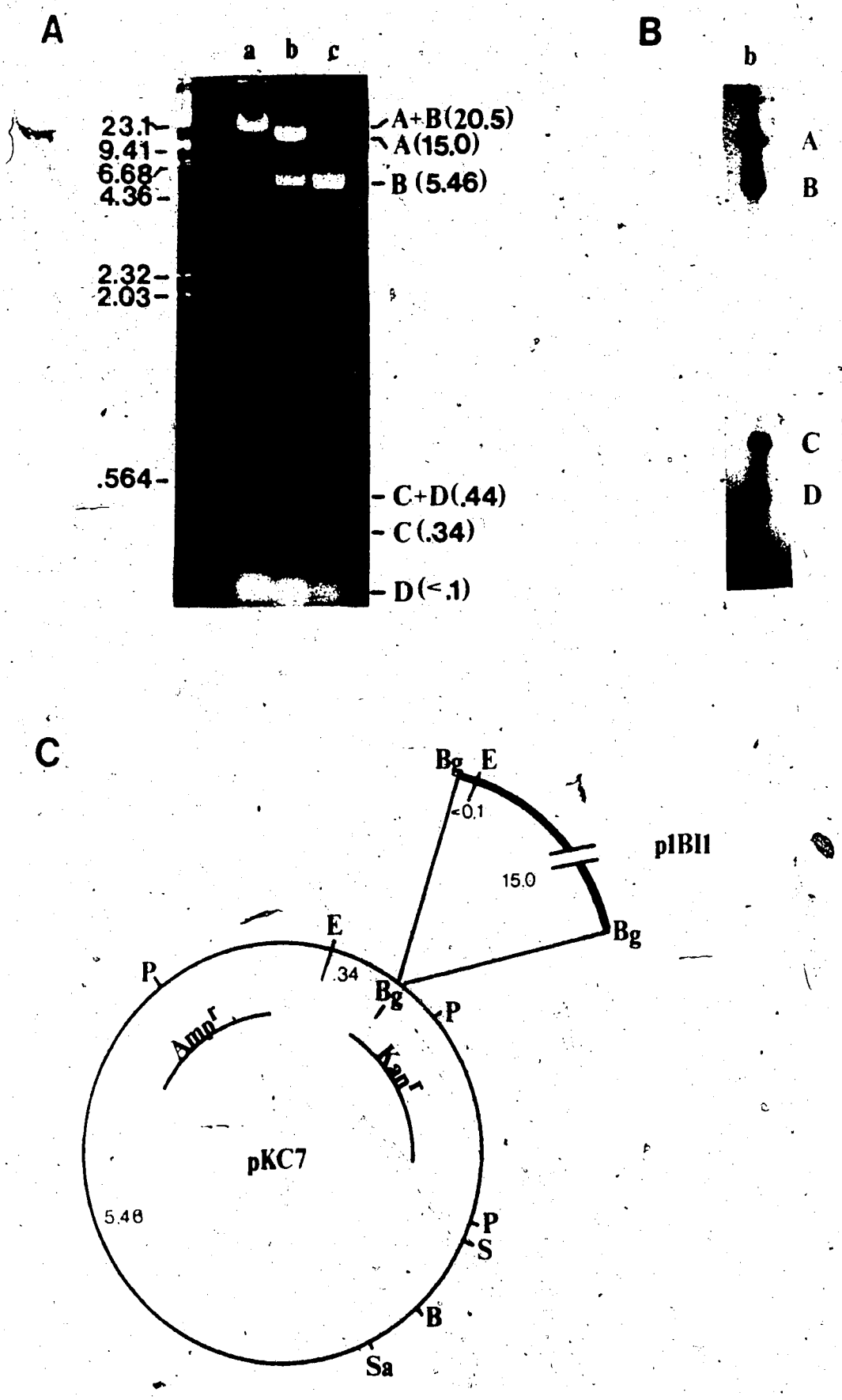
II. Results

A. The orientation of the HSV-1 BglII fragment I in plasmid plBIl.

Digestion of plBIl with EcoRl and BglII produced 4 DNA fragments A, B, C and D as shown in the autoradiogram of terminally labeled DNA fragments (Fig. 2B). Comparison with the DNA fragment profiles of parental plasmid pKC7 indicated that fragments B (5460) and C (340) were derived from the pKC7 vector (Fig. 2A, lane c). Fragments A (approximately 15 kb) and D (less than 100 bp) were then derived from the BglII fragment I insert. These results indicated that a single EcoRl site was located on fragment I within 100 bases from one of the BglII termini. In ethidium bromide stained gels fragment D was obscured by the residual RNA present in the DNA plasmid preparation (Fig. 2A, lane b) and hence was best visualized by autoradiography (Fig. 2B).

Digestion of the plBIl DNA with EcoRl alone produced two DNA fragments; A + B and C + D, having molecular sizes of 20500 and 440 bases respectively (Fig. 2A, lane a). The size of these fragments demonstrates that the EcoRl site on the BglII fragment I must be adjacent to the single EcoRl site of the pKC7 vector [i.e. C(340) + D(less than 100)]. This allowed the assignment of the orientation of the HSV-1 BglII fragment I in plasmid plBIl as shown in Fig. 2C.

Fig.2.Determination of the orientation of the HSV-1 BglII fragment I insert in p1BI1 by digestion with EcoR1 and BglII. A) Agarose gel electrophoretic profile of p1BI1 cut with EcoR1 (lane a), EcoR1 and BglII (lane b) and pKC7 cut with EcoR1 and BglII (lane c). The sizes of the restriction fragments are given in kilobases. The molecular sizes of the DNA marker fragments are indicated on the left of the figure. B) Autoradiogram of terminally labeled fragments generated by digestion of p1BI1 with EcoR1 and BglII (lane b). C) Orientation of the BglII fragment I in p1BI1. Restriction enzyme sites are PstI(P), EcoR1(E), BglII(Bg), BamH1(B) and SalI(Sa). The numbers correspond to the sizes of the fragments in kilobases [i.e. A(15.0), B(5.46), C(0.34) and D(<0.1)].

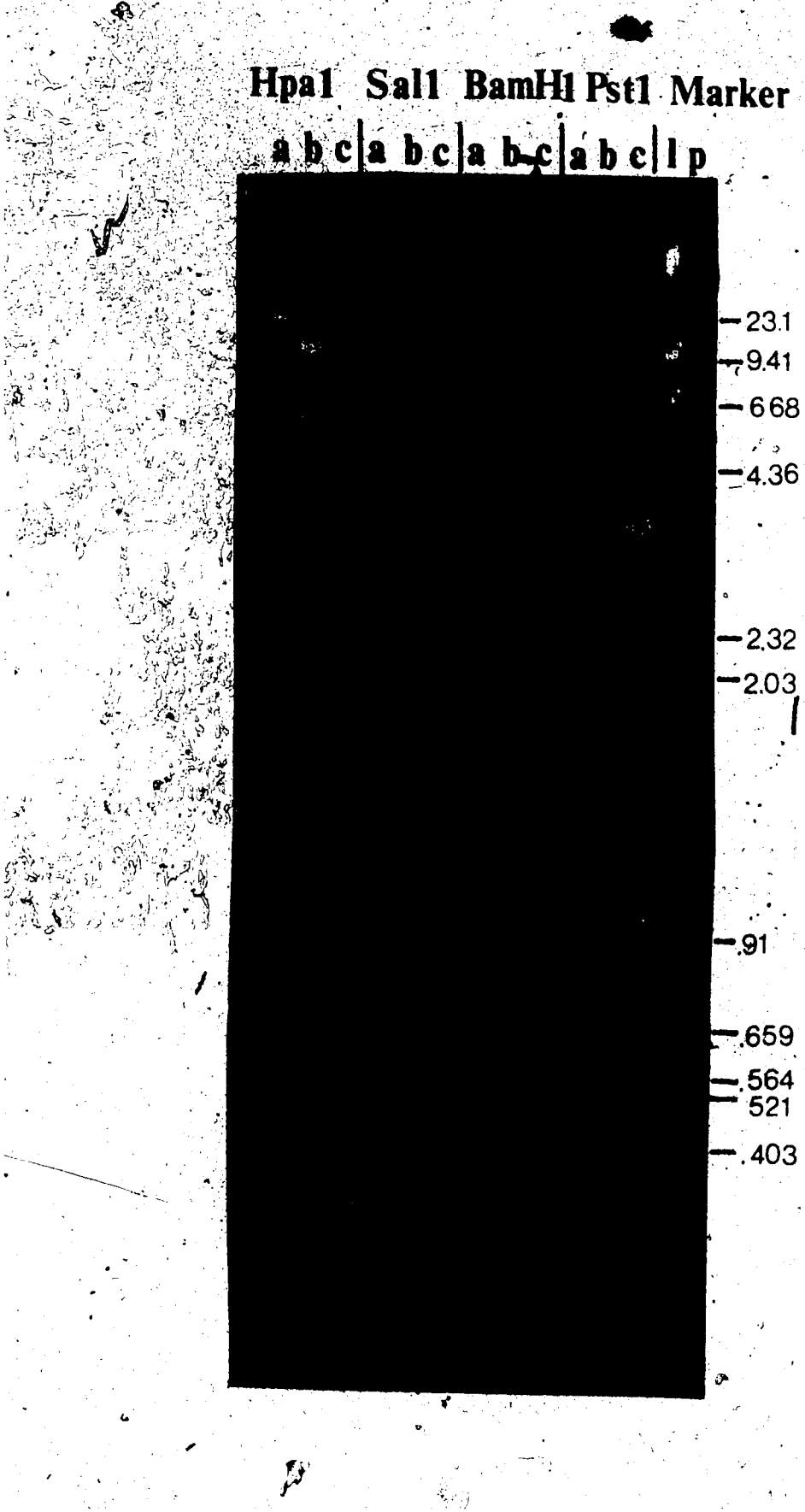

A
a
b
c
23.1
9.41
6.68
4.36
2.32
2.03
.564
A+B(20.5)
A(15.0)
B (5.46)
C+D(.44)
C (.34)
D (<.1)
B
b
A
B
C
D
C
Bg
E
<0.1
15.0
pIBI1
Bg
E
.34
Bg
P
P
Ampr
Kanr
pKC7
5.46
P
S
B
Sa

B. Determination of the size and number of restriction endonuclease DNA fragments produced when the BglII fragment I was digested with the enzymes HpaI, SalI, BamHI and PstI.

Plasmid plBIl was digested with each restriction enzyme alone or in combination with BglII and the resulting restriction fragments were subjected to electrophoresis in 0.8% agarose gel. Fig. 3, lane a, shows plBII cut with the designated enzyme, lane b shows plBIl cut with both the designated enzyme and BglII. In lane c plasmid pKC7 was cut with the designated enzyme and BglII to determine which fragments were derived from the pKC7 vector. The size of each fragment was calculated by comparison with a plot of log molecular size versus distance migrated of the DNA marker fragments. The terminal subfragments of the BglII fragment I were identified by their differential migration rate after being released from the pKC7 vector by double digestion with BglII and the designated enzyme.

The identity of the terminal fragments was further confirmed by end labelling the BglII fragment I using T4 DNA polymerase and subsequent digestion with the appropriate enzyme. Plasmid plBIl was first cut with BglII to release the DNA insert. The linearized pKC7 vector and fragment I were end-labelled with T4 DNA polymerase, cut with the second enzyme and the resulting fragments were subjected to electrophoresis in 0.8% agarose gel. Subsequent autoradiography allowed the detection of the labelled

Fig.3.Agarose gel electrophoretic profile of pIBI1 DNA digested with HpaI, SalI, BamHI and PstI. In lane a, pIBI1 was digested with the designated enzyme. In lane b, pIBI1 was digested with the designated enzyme and BglII. In lane c, pKC7 was digested with the designated enzyme and BglII. Marker fragments are: lambda phage DNA c[illegible] with HindIII (lane l) and pB322 cut with AvaI (lane p). Their molecular sizes are given in kilobases.


Hpa1 Sal1 BamH1 Pst1 Marker
a b c a b c a b c a b c l p
23.1
9.41
6.68
4.36
2.32
2.03
.91
.659
.564
.521
.403

terminal fragments (Fig. 4). This procedure end labels both the pKC7 vector and the fragment I terminal subfragments. Taken together with Fig. 3, these results unambigiously identified the BglII fragment I terminal subfragments. A summary of the sizes of internal and terminal subfragments is given in Table I.

C. Determination of the order of restriction endonuclease DNA fragments

1. Partial digest mapping

The BglII fragment was radioactively labeled at one of its termini. This was accomplished by first cutting plBI1 with EcoRI, labeling the EcoRI site by the DNA polymerase I (Klenow fragment) labeling procedure and then cutting the DNA with BglII. This procedure produced an I fragment which was less than 100 base pairs shorter than the original size (15,000 base pairs) with a labeled EcoRI terminus and unlabeled BglII terminus. This fragment was designated *EcoRI/BglII (or *E/B) fragment A. Alternatively, the I fragment was labeled at the opposite terminus by first cutting plBI1 with BglII, labeling the BglII terminus and then cutting with EcoRI. This fragment was designated E/B* fragment A. In each case, fragment A was purified by agarose gel electrophoresis and eluted from the gel. It was then cut with the appropriately diluted enzyme to obtain a partial digest. Subsequent gel electrophoresis and autoradiography detected a series of overlapping subfragments which were labeled at a common terminus. Comparison with molecular size

Table I

Subfragments of the HS -1 BglII DNA fragment I

HpaI	SalI	BamHI	PstI
A 11.2*	A 4.4*	A 7.8	A 2.8*
B 2.1*	B 3.8	B 2.2	B 2.5
C 1.7	C 2.6	C 2.1	C 2.1
	D 2.3*	D 0.80	D 1.7
	E 1.1	E .76*	E 1.4
	F 0.8	F 0.75	F 1.1
		G 0.73*	F 0.82
			H 0.78
			I 0.51
			J 0.48
			K<0.20*

The sizes of the fragments (in kilobases) represent average values obtained from duplicate gel electrophoresis experiments. Terminal fragments are indicated by an asterisk.

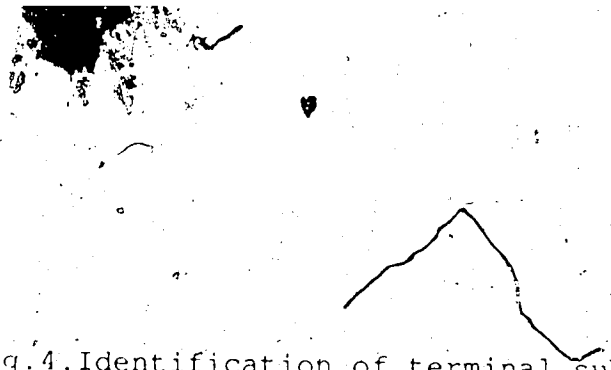
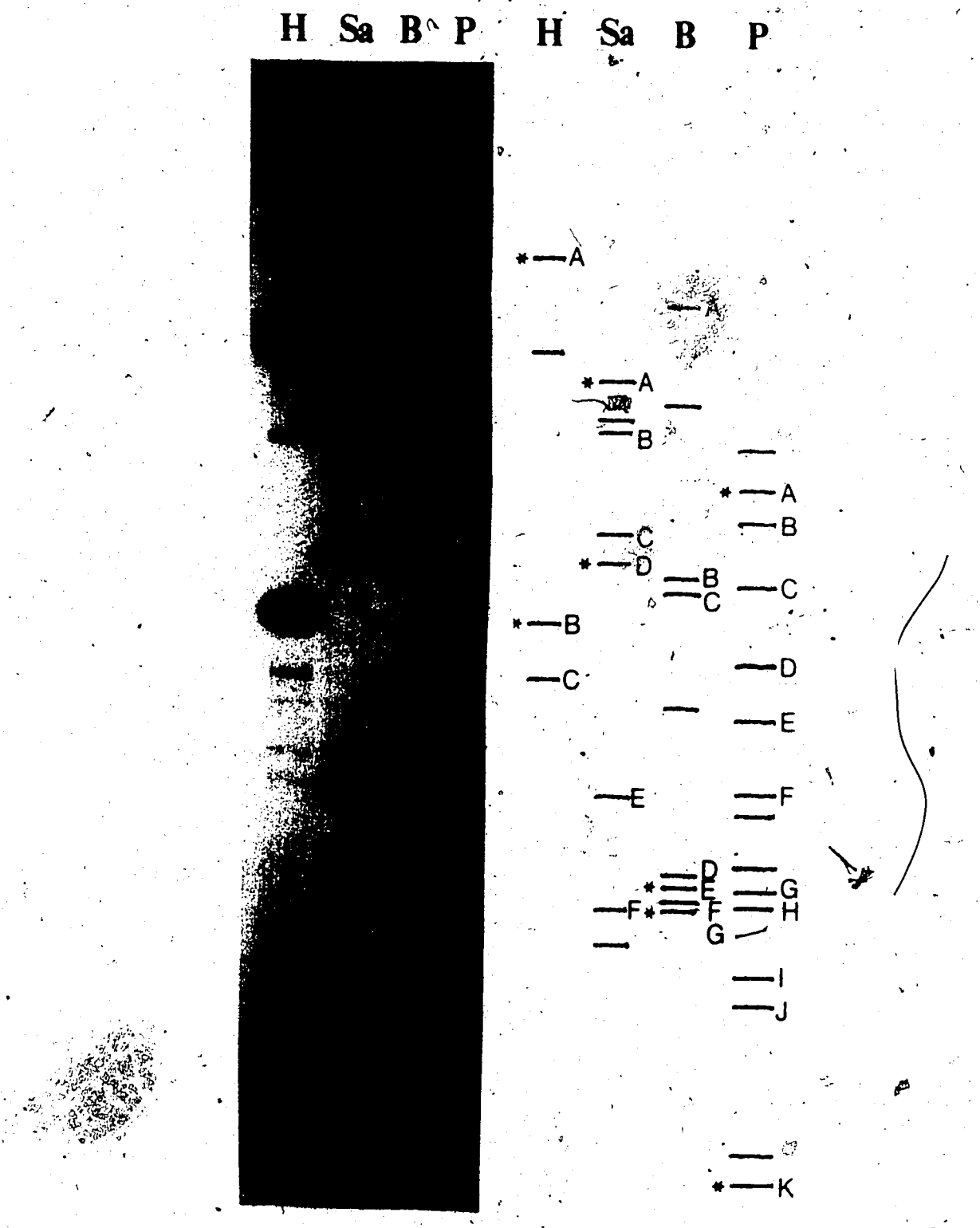


Fig.4. Identification of terminal subfragments of the HSV-1 BglII fragment I. The plBI1 plasmid was cut with BglII, endlabeled by the T4 DNA ligase end labeling procedure and digested with HpaI(H), Sal1(Sa), BamH1(B) or Pst1(P). After electrophoresis in 0.8% agarose gel the terminal fragments were detected by autoradiography. The position of each subfragment in the gel is indicated by a line drawing of the elctrophoretic profile. Terminal fragments are denoted by an asterisk. Fragments derived from the pKC7 vector are also detected by autoradiography (not given letter designation).


H Sa B P H Sa B P
A
A
A
B
A
B
C
D
B
C
B
C
D
E
E
F
D
E
G
F
F
H
G
I
J
K

standards allowed the determination of the size of each fragment and therefore the distance between each of the restriction sites and the labeled terminus could be estimated.

Fig. 5A shows the autoradiograph of the *E/B fragment A after incubation from 0 to 120 minutes at 37°C in the absence of enzyme. Several contaminating fragments were present in the *E/B but not in the E/B* fragment A sample. These fragments were also present in the HpaI partial digest profile of *E/B fragment A (Figure 5B). From the data obtained from figure 4B it was evident that the BglII site had also become labeled resulting in two overlapping partial digest profiles. The restriction profiles obtained for the E/B* fragment were used to construct a restriction endonuclease map for the enzymes HpaI (Fig. 5B) and SalI (Fig. 5C) in order to eliminate the confusion presented by these problems.

2. Hybridization of SalI and BamHl subfragments

The BamHl partial digest profile was difficult to interpret because several BamHl subfragments were similar in size. Therefore, a map for this enzyme was constructed by using SalI subfragments to probe for related DNA sequences in the BamHl fragments.

Plasmid plBIl DNA was digested with BglII and SalI or BamHl and subjected to electrophoresis in a 0.75% agarose gel. Figure 6A shows a typical ethidium bromide stained gel profile of plBIl cut with BglII and SalI (lanes s) or BglII

Fig.5 Partial digest mapping of HpaI and SalI restriction sites on the HSV-1 BglII fragment I. A) 0.5% agarose gel electrophoresis of the endlabeled BglII fragment I after incubation at 37°C in the absence of restriction enzyme. The *EcoR1/BglII (*E/B) and EcoR1/BglII* (E/B*) fragment A (described in text) were incubated at 37°C for 0,5,10,30, 60 and 120 min. (lanes 0, 1, 2, 3, 4, and 5, respectively). B) HpaI partial digest profile of *E/B and E/B* fragment A. The DNA fragments are diagramatically represented below. The size of each subfragment is given in kilobases. C) SalI partial digest profile of EB* fragment A.

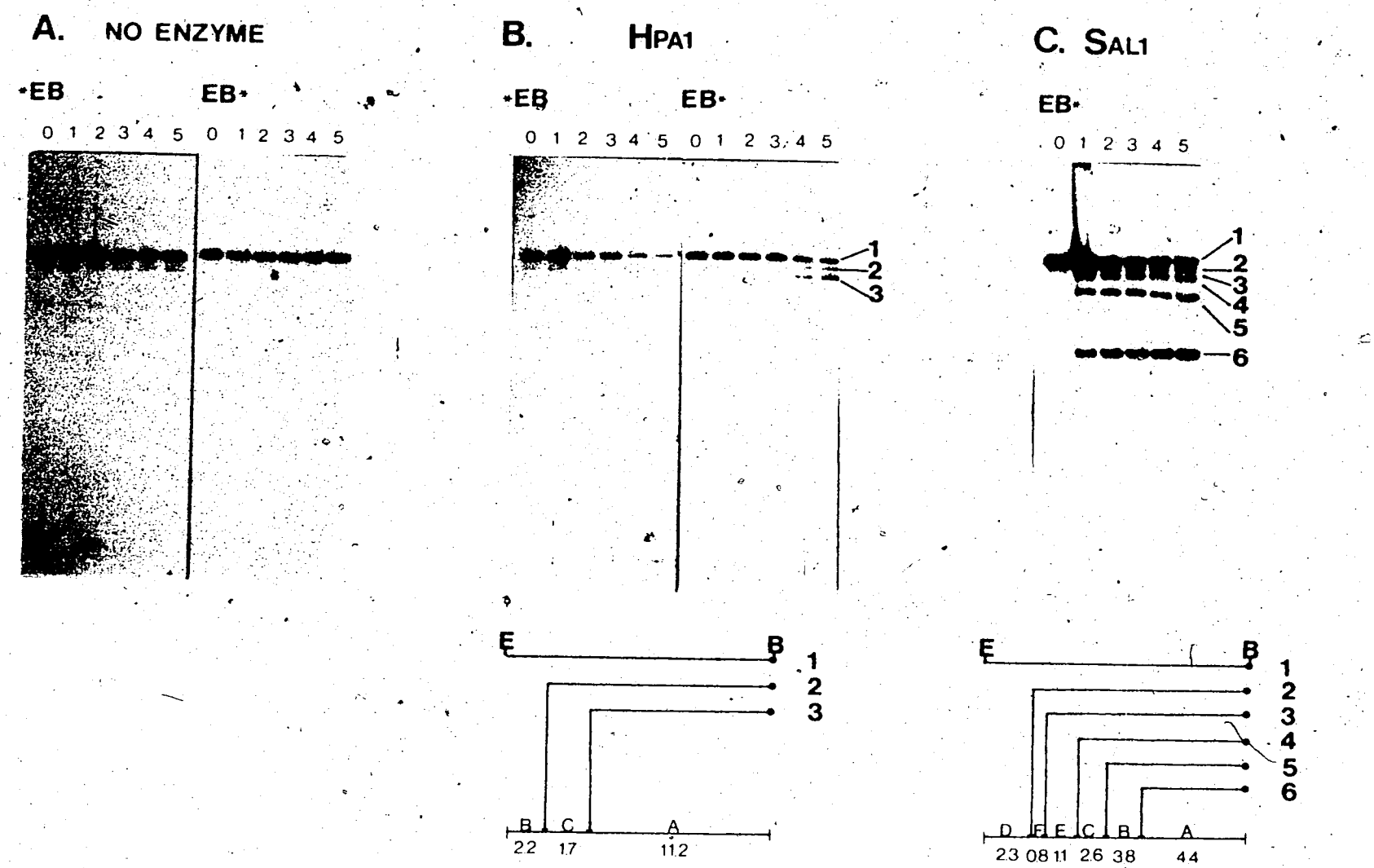
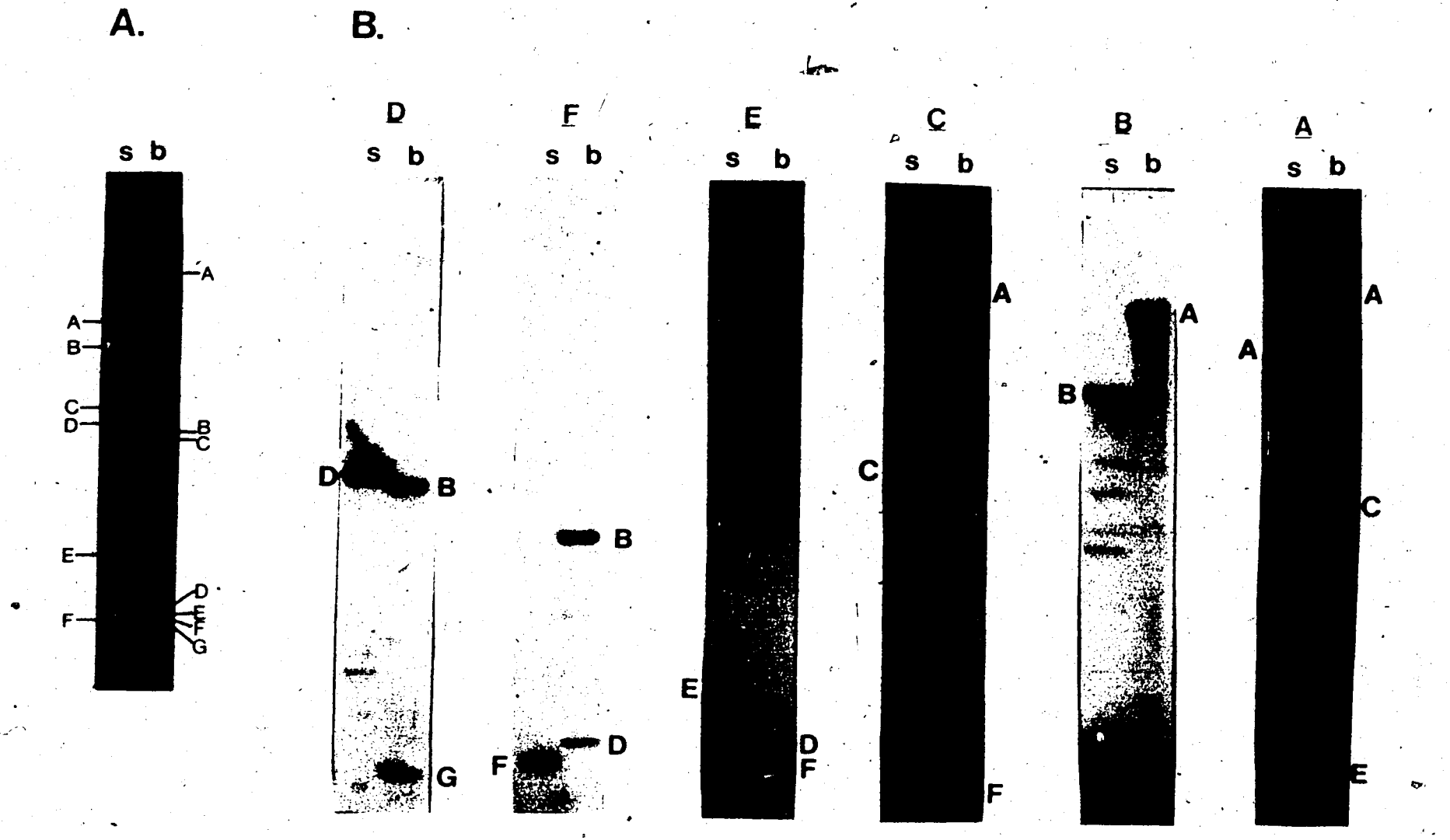

A. NO ENZYME
*EB
EB*
0 1 2 3 4 5 0 1 2 3 4 5
B. HPA1
*EB
EB*
0 1 2 3 4 5 0 1 2 3 4 5
1
2
3
E
B
1
2
3
B
C
A
2.2
1.7
11.2
C. SAL1
EB*
0 1 2 3 4 5
1
2
3
4
5
6
E
B
1
2
3
4
5
6
D
F
E
C
B
A
2.3 0.8 1.1 2.6 3.8 4.4

Fig.6.Hybridization of Sal1 and BamH1 subfragments from the HSV-1 BglII fragment I. A) Agarose gel profile of p1BI1 cut with BglII and Sal1 (lane s) or BglII and BamH1 (lane b). B) Cross hybridization of Sal1 DNA subfragments D,F,E,C,B, or A with Sal1 and BamH1 fragments by Southern blot analysis. The position and identity of the fragment(s) detected by the Sal1 probe is indicated by the letters beside each autoradiograph.


A.
B.
s b
A
B
C
D
E
F
G
D
F
E
C
B
A

and BamHl (lanes b). Six duplicate gels were blotted onto nitrocellulose paper. Figure 6B shows the autoradiograph of each blot after hybridization to the nick translated Sall probe. Lane s in each case demonstrates the probe specificity. Lane b shows the BamHl fragments which cross hybridize with the Sall probe.

Fig. 7 shows the restriction endonuclease map of the BglII I fragment which is consistent with all the above data obtained for the enzymes EcoRl, Hpal, Sall and BamHl.

D. Northern blot analysis for the HSV genomic region between 0.314 and 0.416 using HSV-1 and HSV-2 specific DNA probes

The autoradiograph in Fig. 8 shows a northern blot of poly $(A)^+$mRNA from HSV-1 infected Vero cells after hybridization to radioactively labeled plBIl plasmid. At least five mRNA species were detected ranging in size from 4.4 to 2.0 kilobases. On the basis of protein coding capacity and in vitro translation data, the 3.3 kb mRNA species was most likely the gB message (see discussion).

Northern blot analysis, using individual DNA subfragments from BglII fragment I, refined the map locations of these mRNA species. Clone 12 contains the 7.8 kb BamHl A restriction fragment (map coordinates 0.345 - 0.396). When used as a hybridization probe in Northern blot analysis, Clone 12 detected only the 3.3 kb message (Fig.9A). Sall C and B fragment probes also detected this message, Sall E and A did not (Fig. 9B). These data localized the 3.3 kb gB

Fig.7. Restriction endonuclease map of the HSV-1 BglII fragment I for the enzymes EcoR1, Hpa1, Sal1 and BamH1. The lower portion of the figure shows a schematic diagram of the HSV genome. The long (L) and short (S) components of the genome, and the map coordinates of the BglII fragment I, are indicated.

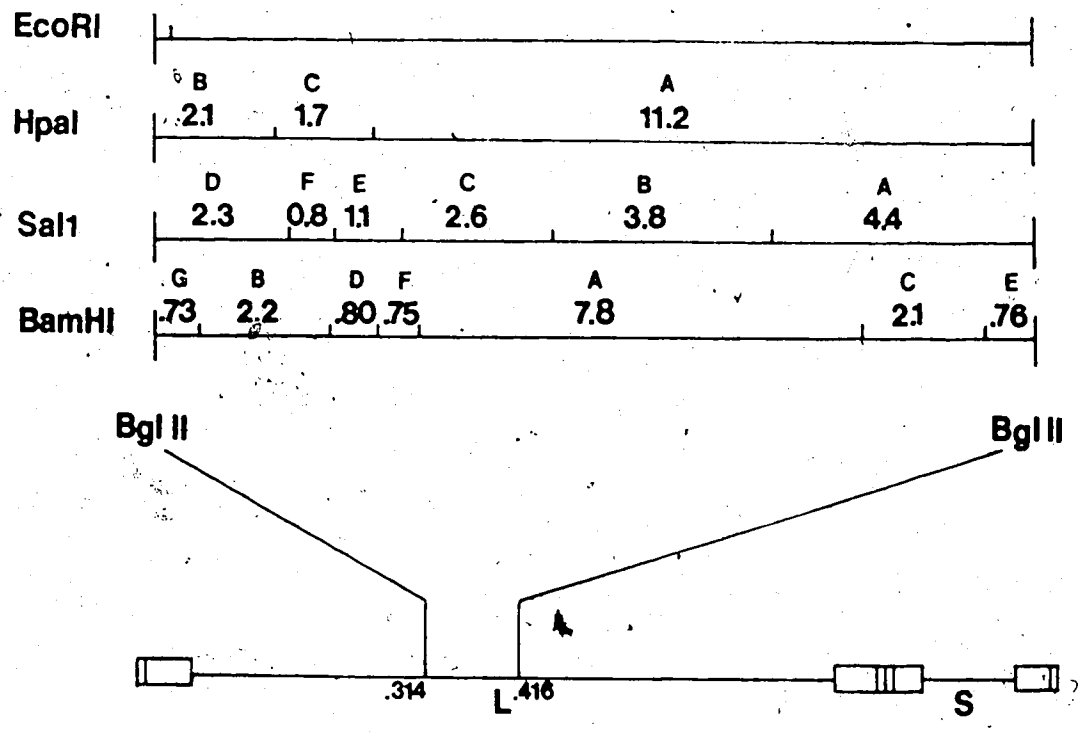

EcoRI
HpaI
B
2.1
C
1.7
A
11.2
Sal1
D
2.3
F
0.8
E
1.1
C
2.6
B
3.8
A
4.4
BamHI
G
.73
B
2.2
D
.80
F
.75
A
7.8
C
2.1
E
.76
Bgl II
Bgl II
.314
L
.416
S

Fig.8.HSV-1 infected-cell RNA transcripts detected by the HSV-1 BglII fragment I. Lanes 1,2, and 3 contain 0.2, 1.0 and 2.0 µg of polyadenylated RNA. The sizes of the transcripts are given in kilobases and were calculated by comparing their migration rate with DNA size-marker fragments (plBI1 digested with BglII and SalI).

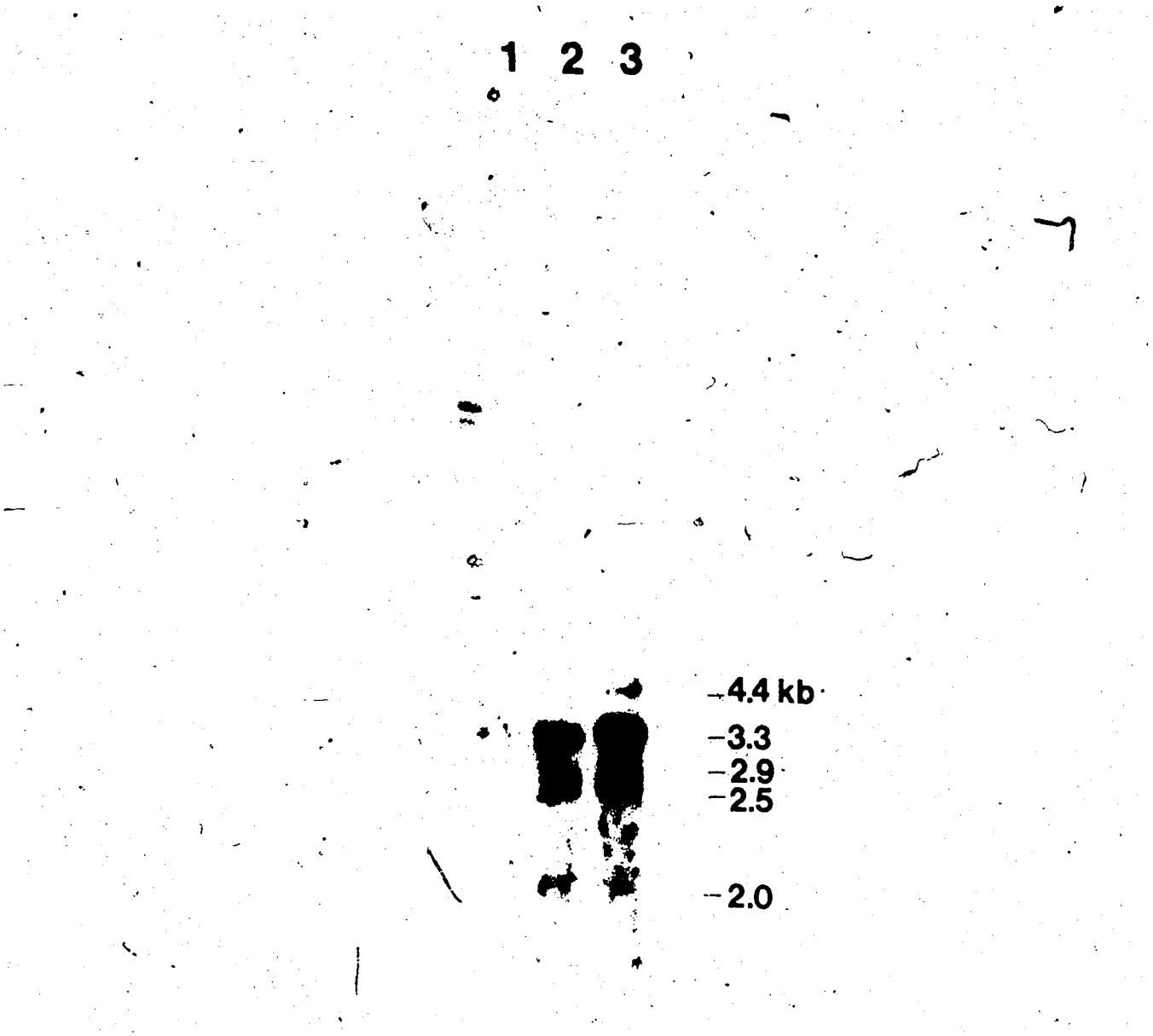
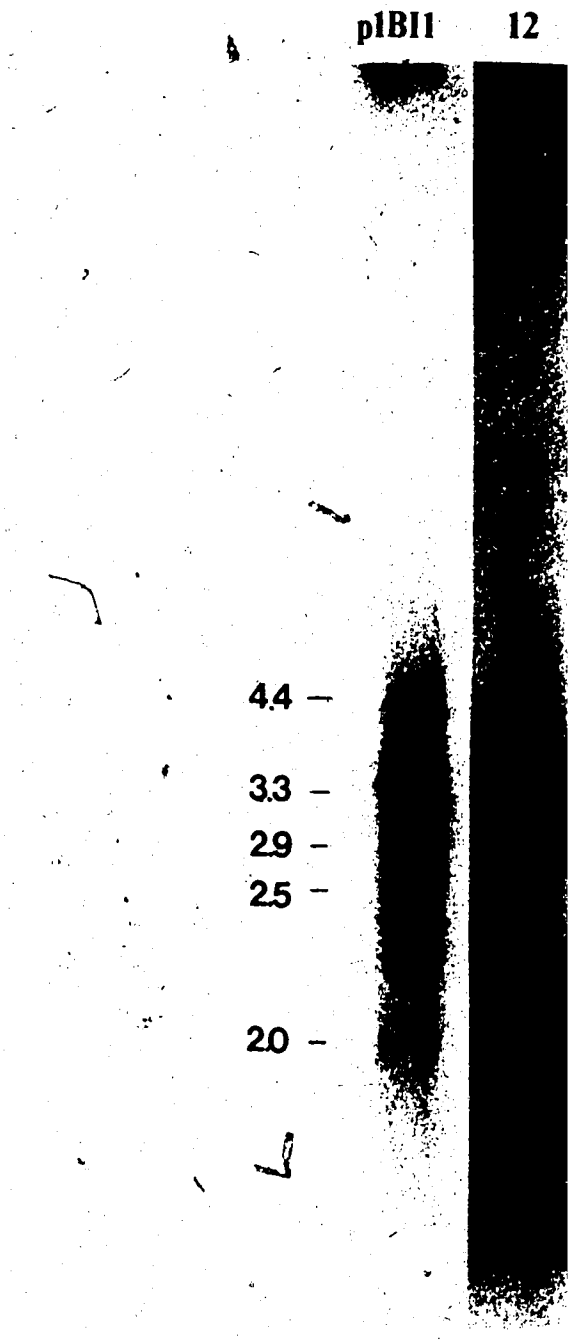

1 2 3
4.4 kb
3.3
2.9
2.5
2.0

Fig.9 RNA transcripts in HSV-1 infected-cells detected by hybridization with BamH1 and Sal1 subfragments from BglII fragment I. A) Transcripts detected by BglII fragment I (p1BI1) and the 7.8 Kb BamH1 (0.345-0.396 map units) fragment (Clone 12). B) Transcripts detected by Sal1 fragments E(0.337-0.342), C(0.342-0.361), B(0.361-0.386) and A(0.386-0.416).

A.



message to within map coordinates 0.345 and 0.386 on the HSV-1 genome.

The gB genes from HSV-1 and HSV-2 were presumed to be colinear based on restriction endonuclease site analyses of intertypic recombinants and marker rescue experiments (Marsden et al. 1978 and Ruyechan et al. 1979). Alignment of the BglII genomic maps for HSV-1 and HSV-2 (see Fig. 16, Chapter 4), predicted that HSV-2 BglII fragment J (0.314 - 0.395) contained the gB2 gene. The region containing gB1 (0.345 - 0.386) corresponded to HSV-2 6.6kb BamHl - BglII subfragment (0.345 - 0.395). A molecular clone of this fragment (Clone 75: described in detail in chapter 4) hybridized to a 3.3 kb mRNA from HSV-1 and HSV-2 infected cells (Fig. 10). This result shows that these 3.3 kb messages share sequence homology and that the HSV-2 6.6 kb BamHl-BglII fragment probably contains the gB2 gene. Nucleotide sequencing (see Chapter 5) confirmed this prediction.

III. Discussion

At the onset of this project, a detailed restriction endonuclease map of the HSV-1 genome (strain KOS) between map coordinates 0.3 and 0.4 units was not available. Genomic maps of a number of other strains for the restriction enzymes BglII, Hpa I and EcoR1 were published but their resolution was poor and was not sufficient to define the limits of the glycoprotein gB gene. In addition, restriction enzyme maps vary between HSV virus strains, therefore it would be unwise

Fig.10 RNA transcripts in HSV-1 and HSV-2 infected-cells detected by HSV-1 BglII fragment I and HSV-2 6.6Kb BamH1-BglII fragment (Q.345-0.395). Polyadenylated mRNA from uninfected (lane u) and HSV-1 (lane 1) or HSV-2 (lane 2) infected cells, was hybridized to the BglII fragment I (p1BI1) or the 6.6Kb BamH1-BglII fragment (Clone 75).


p1Bl1
75
U
1
2
1
2
4.4 kb
3.3
2.9
2.5
2.0

to strictly infer genomic map data from one strain to another. A more precise map of the genomic region that contained the gB gene was required. Plasmid plBIl, which is a molecular clone of the HSV-1 (KOS) BglII fragment I (0.314 - 0.416) was utilized as a molecular tool to achieve this aim.

The restriction enzymes Hpal, Sall, BamHl and Pstl were chosen because they cut fragment I into a manageable number of fragments for restriction analysis. Figure 3 shows an 0.8% agarose gel profile of plBIl cut with each enzyme, which gave the best resolution of both large and smaller sized DNA fragments. Variable size estimates were obtained for the larger fragments, such as the 7.8 kb BamHl A and 11.2 kb Hpal A. Actual sizes are probably accurate to within 200 base pairs. A more accurate size estimation of DNA fragments smaller than 5 kilobases was possible due to their better resolution in agarose gel.

Agarose gel electrophoresis analysis does not absolutely rule out the existence of additional restriction fragments in the low molecular weight range which migrate with the same mobility. However, in the case of each enzyme, summation of the estimated sizes of all the fragments yielded a value which was approximately equal to the expected 15 kilobase size of the BglII fragment I. DNA fragments smaller than 200 bp were difficult to visualize due to reduced staining intensity and the presence of residual RNA in the plasmid preparation. These fragments were best visualized by radio-

labeling of DNA termini. Radioactive endlabeling detected the EcoRl D fragment (less than 100 base pairs) (Fig. 2A) and the Pstl K fragment (less than 200 base pairs) (Fig. 4, lane p).

The restriction maps of the BglII fragment I for the enzymes, Hpal and Sall were determined by the partial digest technique described by Smith and Birnsteil (1976). Radioactive labeling of a single terminus was achieved by taking advantage of the fact that a single EcoRl site exists within 100 nucleotides of one BglII terminus. This made possible the end labeling of either this EcoRl site or the opposite BglII terminus (see Results: section II C.1). Attempts to label the EcoRl site also resulted in the incorporation of label at the BglII terminus. The reason for this is unclear. In contrast, end labeling the BglII terminus did not produce this double labeling problem. Partial digest fragments were subjected to electrophoresis in 0.5% agarose gels in order to increase the resolution of the larger DNA fragments and therefore improve their size estimation.

Hybridization of Sall fragments with the BamHl fragments allowed the BamHl map to be constructed. Occasionally, partially digested BamHl fragments were detected by the Sall probes. However, these bands were faint and migrated to different positions than the BamHl fragments visualized by ethidium bromide staining.

A summary of the restriction endonuclease map of the BglII fragment I which is consistent with all the data

obtained for the enzymes EcoR1, Hpal, Sall and BamH1 is given in Fig. 7. The single EcoR1 site defines the orientation of the map which is shown in relation to the entire prototype HSV genome. It was unnecessary to complete the Pst1 map since the information provided by the map and northern blot analyses proved sufficient to determine the approximate location of the glycoprotein B1 gene. The map closely agrees with the restriction map of the EcoR1 fragment F region (map units 0.315 - 0.421) of HSV-1 (KOS) published by Holland et al. (1984a).

The glycoprotein B gene was fine mapped by northern blot analysis using HSV specific DNA probes to hybridize to HSV infected cell RNA. RNA samples were enriched for polyadenylated mRNA species by selection on oligo (dT) columns. This procedure also reduced the problem of high radioactive background in the autoradiograph of the northern blot. The optimal RNA concentration for the detection of mRNA species which hybridized to the HSV1 BglII fragment I was between 1.0 - 2.0 micrograms (Fig. 8). At least five HSV-1 specific transcripts were identified: 4.4-, 3.3-, 2.9-, 2.5- and 2.0- kb mRNAs.

The BamH1 A (0.345 - 0.396), Sall C (0.342 - 0.361) and Sall B (0.361 - 0.386) probes detected the 3.3 kb gB mRNA (Fig. 9). These results mapped this message to the genomic region within map coordinates 0.345 and 0.386. This location is consistent with the results of Rafield and Knipe (1984) and Holland et al. (1984a), who used northern blot analysis

to map a major HSV1 3.3 - 3.4 kb transcript to the region between 0.346 and 0.385. In vitro translation of the transcripts homologous to this region directed the synthesis of two major polypeptides: 103K and 99K, which were precipitable by gB specific monoclonal antibody (Rafield and Knipe, 1984). Marker rescue experiments have localized mutations in the gB gene to the region 0.346 to 0.368 (Deluca et al., 1982 and Holland et al., 1983). Taken together, these findings indicate that the 3.3 kb mRNA encodes glycoprotein gB.

The SalI A probe (0.386 - 0.416) identified a 4.4 kb mRNA. This message most likely encodes HSV DNA polymerase (ICP8). Holland et al, (1983) determined that mRNA homologous to this region of the genome directed the synthesis of ICP8 specific polypeptides. Temperature sensitive ICP8 mutants were found to have mutations that map within this region (Carley et al., 1981 and Lee and Knipe, 1983). The SalI E (0.337 - 0.342), C (0.342 - 0.361) and B(0.361 - 0.386) probes also detected one or more minor transcripts in the 4.4 kb size range. Rafield and Knipe (1983) detected a 4.4 kb and 4.2 kb RNA transcribed from the regions 0.320 - 0.345 and 0.380 - 0.417 respectively. Therefore, the minor transcript(s) identified by these SalI probes may be distinct from the 4.4 kb transcript identified by SalI A. The BamHI A probe (0.345 - 0.396), however, did not detect a 4.4 kb transcript. Figure 9A shows that the band intensities of all five transcripts detected by the

Bc II fragment I probe (plBIl) were reduced, implying that, in this case, the concentration of poly (A)$^+$mRNA may have been less than the optimum 1-2 micrograms. The Sall E and A probes also detected minor transcripts in the 2.0 - 3.0 kb range. Holland et al. (1984a) identified similar mRNAs homologous to these regions. To date, these messages have not been assigned unambiguously to any known HSV specific polypeptides.

Alignment of the HSV-1 and HSV-2 BglII genomic maps predicted that the HSV-2 6.6 kb BamHl - BglII restriction fragment (0.345 - 0.395) contained the entire gB2 gene. Northern blot analysis, using a molecular clone of this fragment (Clone 75) as a probe, detected a HSV-2 transcript having exactly the same size as the gBl transcript (Fig. 10). The strong hybridization of the probe with the 3.3 kb transcript from both HSV-1 and HSV-2 suggests extensive sequence homology. The HSVl BglII fragment I (plBIl) probe hybridized less strongly to the HSV-2 4.4 and 3.3 Kb mRNA and did not detect the smaller HSV-2 transcripts. This was most likely due to the lower specific radioactivity of this probe and a greater homology with HSV-1 transcripts.

CHAPTER 4

FINE MAPPING THE GENOMIC LOCATION OF THE gB2 GENE FROM HSV-2

I. Introduction

Glycoprotein B has epitopes common to both HSV-1 and HSV-2 (Showalter et al., 1981) and the 3.3 kb gB transcript from both viruses hybridized to the same gB2 DNA probe, hence the gB1 and gB2 genes must share DNA sequence homology. Therefore, it was reasonable to map the gB2 gene using HSV-1 DNA restriction fragments, containing gB1 sequences, as hybridization probes for the corresponding gene in HSV-2.

Clone 75 is a molecular clone of the HSV-2 6.6 kb BamHl - Bgl[illegible] fragment (0.345 - 0.395) inserted into the BamHl site of [illegible]malian expression vector pSV2-gpt. This chapter describes the construction of a restriction map of Clone 75 and the mapping of the gB2 gene by Southern blot analyses.

II. Results

A. Orientation of the HSV-2 6.6 kb BamHl - BglII fragment in Clone 75

Upon ligation of the BglII terminus of the 6.6 kb BamHl - BglII fragment into the BamHl terminus of the pSV2-gpt vector a hybrid site was formed. Consequently, this fragment could not be excised from the vector by digestion with either BamHl or BglII. Its orientation was determined by locating the SalI sites on the HSV-2 insert with respect to the BamHl and BglII site present on the pSV2-gpt vector portion of Clone 75.

Fig. 11 shows the agarose gel profile of Clone 75 cut with SalI, BglII or BamHI. Clone 75 was cut by SalI into four restriction fragments; SalI A (8.0 kb), SalI B (3.7 kb) and SalI C (0.26 kb) (lane a). Since the pSV2-gpt vector portion of this plasmid contains no SalI sites, this result indicated that the insert contains 3 SalI sites. BglII cuts SalI A (8.0 kb) into SalI A1 (5.6 kb) and SalI A2 (2.3 kb) (lane b). BamHI cut SalI A (8.0 kb) into SalI X1 (6.1 kb) and SalI X2 (1.85 kb) (lane c). A restriction map of Clone 75 for the enzymes BamHI, BglII and SalI that is consistent with these results is shown in Fig. 12. The results in section II.B confirmed the location of the fragment SalI C (0.26 kb).

B. SalI, PstI and SstI restriction endonuclease maps of Clone 75

1. Restriction enzyme analyses and cross hybridization.

Fig. 11 shows the agarose gel profile of Clone 75 cut with PstI (lane a), PstI and SalI (lane b), SstI (lane c), SstI and SalI (lane d), and SalI (lane e). Fig. 13 shows the Southern blot of Clone 75 cut with PstI and SalI (lane a) and SstI and SalI (lane b) after hybridization with fragment SalI A (8.0 kb) from Clone 75. Fig. 13 also shows the result when these fragments were hybridized with another probe: a 5.0 kb KpnI fragment containing the gB1 gene of HSV-1 and 5' flanking sequences (see Fig. 16).

Fig.11 Restriction endonuclease site analysis of Clone 75. A) Agarose gel elctrophoretic profile of Clone 75 cut with Sal1 (lane a); Sal 1 and BglII (lane b), and Sal1 and BamH1 (lane c). The sizes of the DNA fragments were determined by comparison of their migration rate with marker DNA fragments: lambda phage DNA cut with HindIII (lane 1). A diagramatic representation of the restriction profile is also given which identifies the fragments and their sizes. New restriction fragments generated by the second enzyme are assigned the same alphabetic letter as the parent fragment. B) Agarose gel profile of Clone 75 cut with Pst1 (lane a), Pst1 and Sal1 (lane b), Sst1 (lane c), Sst1 and Sal1 (lane d), and Sal1 (lane e).

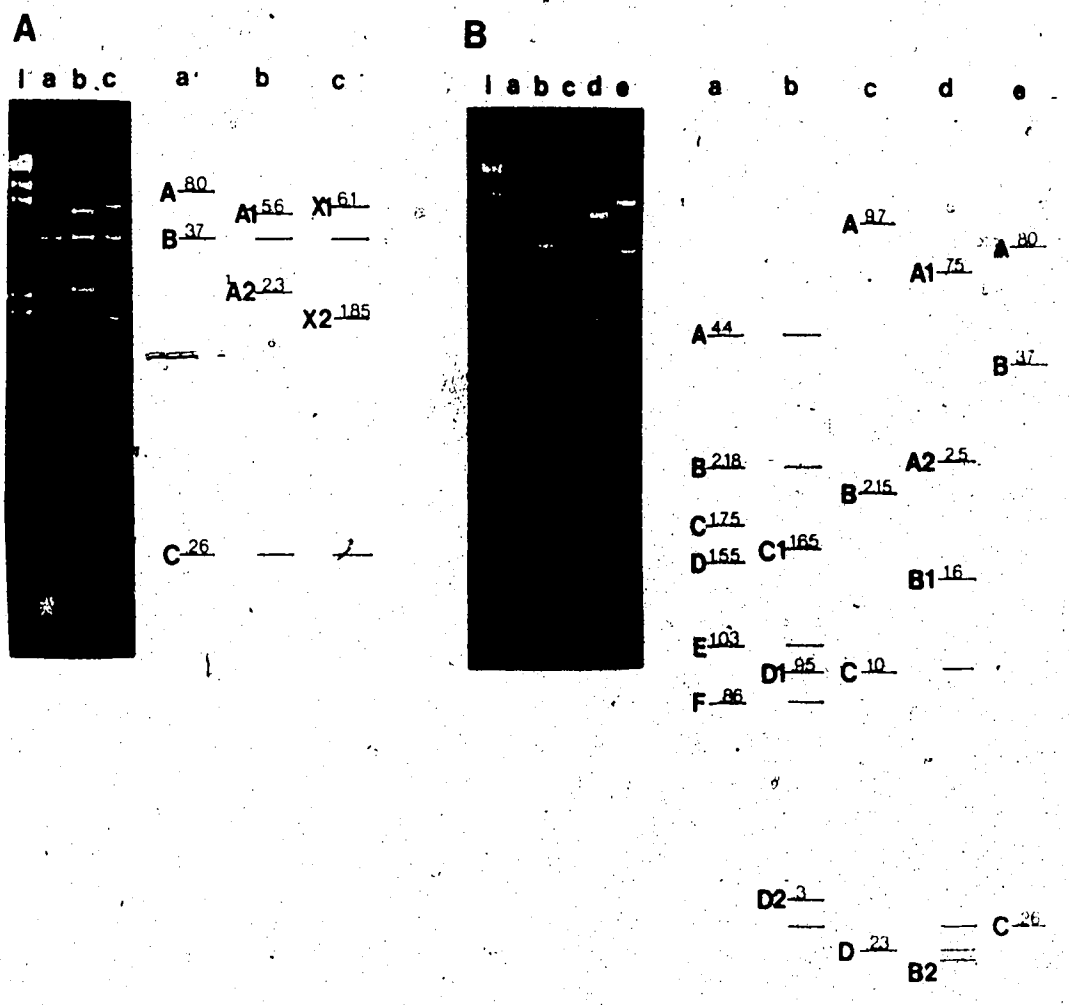
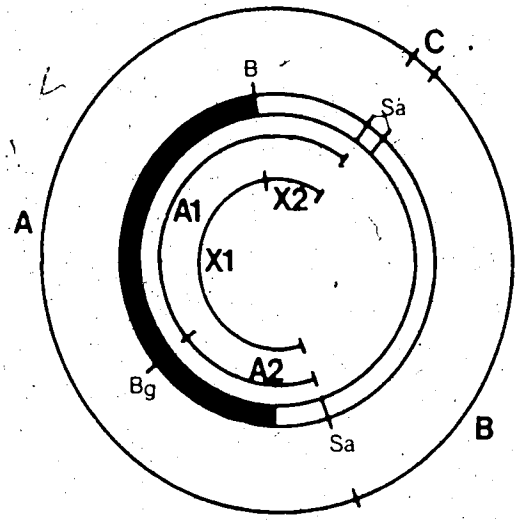
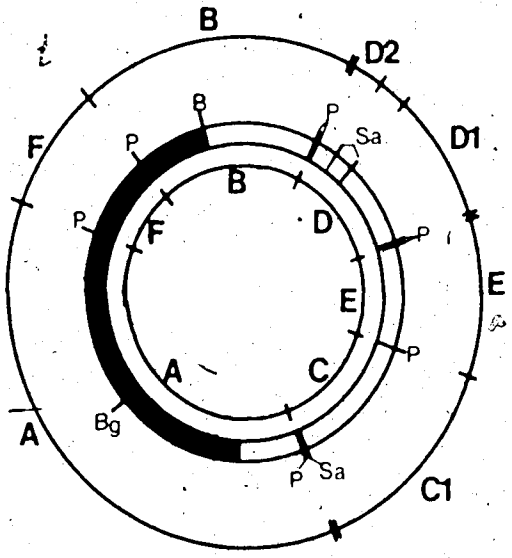

A
l a b c
a
b
c
A 80
A1 56
X1 61
B 37
A2 23
X2 185
C 26
B
l a b c d e
a
b
c
d
e
A 97
A 80
A1 75
A 44
B 37
B 218
A2 25
B 215
C 175
D 155
C1 165
B1 16
E 103
D1 95
C 10
F 86
D2 3
C 26
D 23
B2

Fig.12. Sal1, Pst1 and Sst1 restriction endonuclease maps of Clone 75 determined by restriction endonuclease and Southern blot analyses. A) Sal1, BamH1, BglII map. B) Pst1, Sal1 map. C) Sst1, Sal1 map. The shaded region represents the pSV_2-gpt vector and the remaining region represents the HSV-2 6.6Kb BamH1-BglII fragment (0.345-0.395) insert. Designations for the restriction enzymes are :BglII (Bg), BamH1(B), Sal1(Sa), Sst1(S), and Pst1(P).

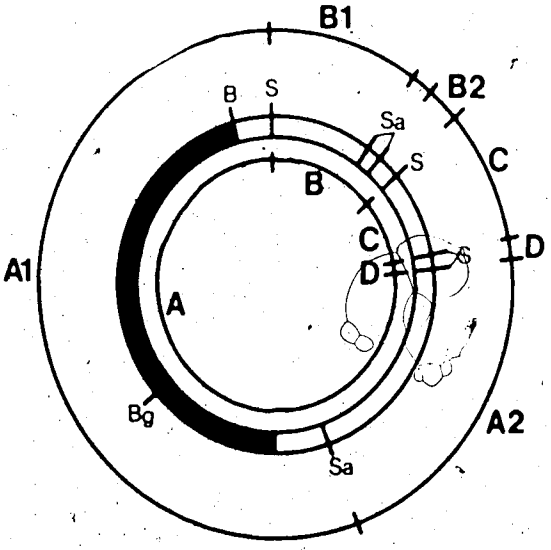
A. Sal 1



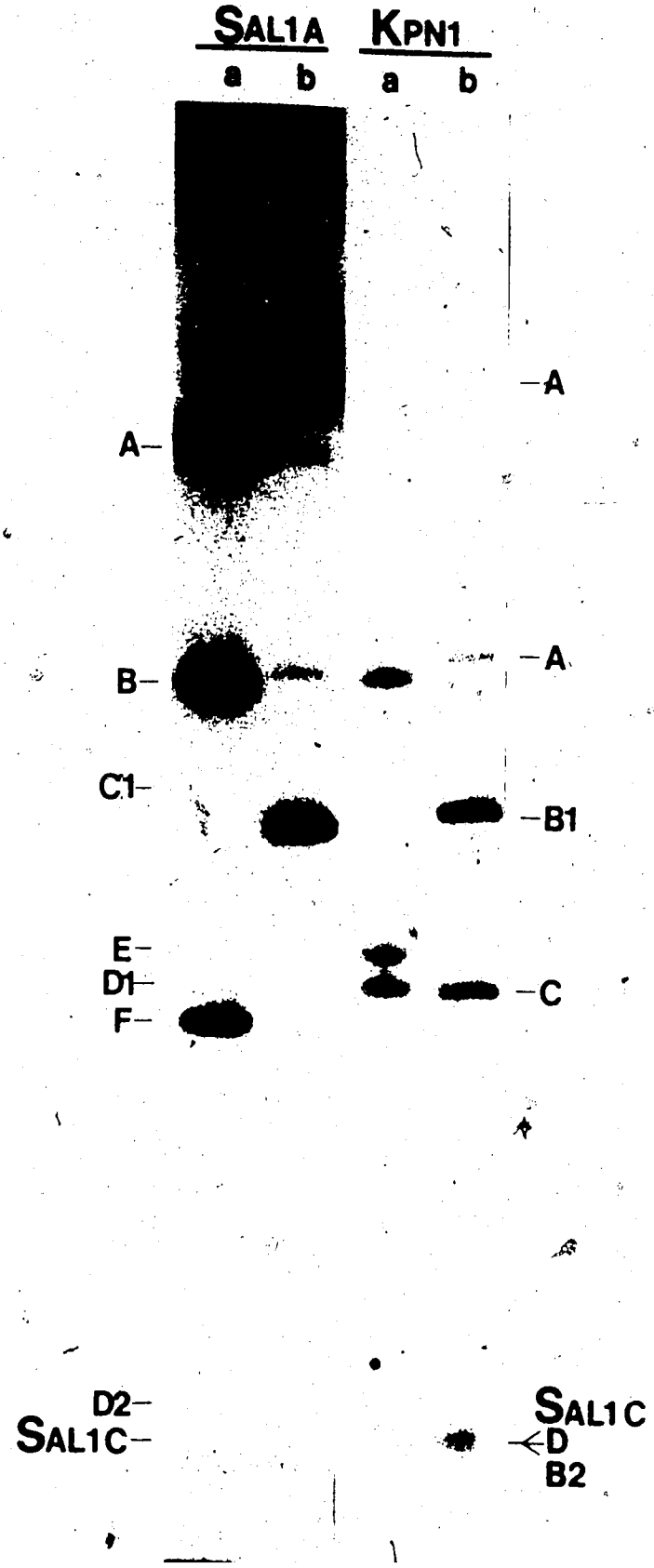
B. Pst 1



C. Sst 1



ig.13. Hybridization of Sal1 DNA fragment A from Clone 75 and 5.0Kb Kpn1 fragment (gB1 probe) from HSV-1 with Clone 75 subfragments. Clone 75 was cut with Pst1 and Sal1 (lane a) or Sst1 and Sal1 (lane b). The fragments were subjected to electrophoresis in agarose gel, blotted onto nitrocellulose and hybridized with either Clone 75 Sal1 fragment A (Sal1A) or HSV-1 5.0 Kb Kpn1 fragment (Kpn1). The identity of the fragments which hybridize with the probe are indicated.


SAL1A
KPN1
a
b
a
b
A
A
B
A
C1
B1
E
D1
F
C
D2
SAL1C
SAL1C
D
B2

The information obtained from these data together with the SalI map (Fig. 12) were used to establish a restriction map of Clone 75. The rationale for construction of the PstI (Fig. 12) and SstI (Fig. 12) maps, was as follows:

PstI Map

(i) The SalI A (8.0 kb) probe hybridized to the PstI - SalI fragments A, B, F, and D2. PstI fragment F (0.86 kb) is the internal PstI fragment derived from the vector pSV2-gpt (Fig. 12). PstI fragments A and B contain vector sequences plus HSV-2 sequences. The 5.0 kb KpnI (gBl) probe hybridized to PstI - SalI fragments D2 and B. These data suggested the following clockwise order of PstI fragments; A, F, B and D2, and that B and D2 contain DNA sequences homologous to the gBl probe.

(ii) PstI fragment E (1.55 kb) was cut twice by SalI generating DNA fragments D2 (0.95 kb), SalI C (0.26 kb) and D1 (0.3 kb). These results placed the SalI C fragment in the middle of PstI fragment D. These fragments and PstI fragment E hybridized with the gBl probe.

(iii) PstI fragment C (1.75 kb) was cut once by SalI and generated fragment C1 (1.745 kb) and a fragment which was presumably too small to be seen by ethidium bromide staining. PstI fragment C does not hybridize to the gBl probe.

The above analysis suggests the PstI - SalI restriction endonuclease map shown in figure 12.

SstI Map

(i) The SalI A (8.0 kb) probe hybridized with SstI - SalI fragments A1 and B1. SstI fragment B (2.15 kb) was cut twice by SalI into SstI - SalI fragments B1 (1.65 kb), B2 (0.24 kb) and SalI fragment C (0.26 kb). The location of SalI fragment C was already determined for the PstI - SalI map (see the above and Fig. 12). These data suggested the following clockwise order of fragments; A2, A1, B1, SalI C and B2.

(ii) SstI - SalI fragments B1, B2, C, D, A2 and SalI C hybridized to the 5.0 kb KpnI (gB1) probe.

(iii) SstI fragments C and D must be located between fragments A and B. The correct order of the former two fragments was deduced from the fact that PstI fragments D and E cross hybridized with SstI fragment C and D, respectively (see section II.B.2).

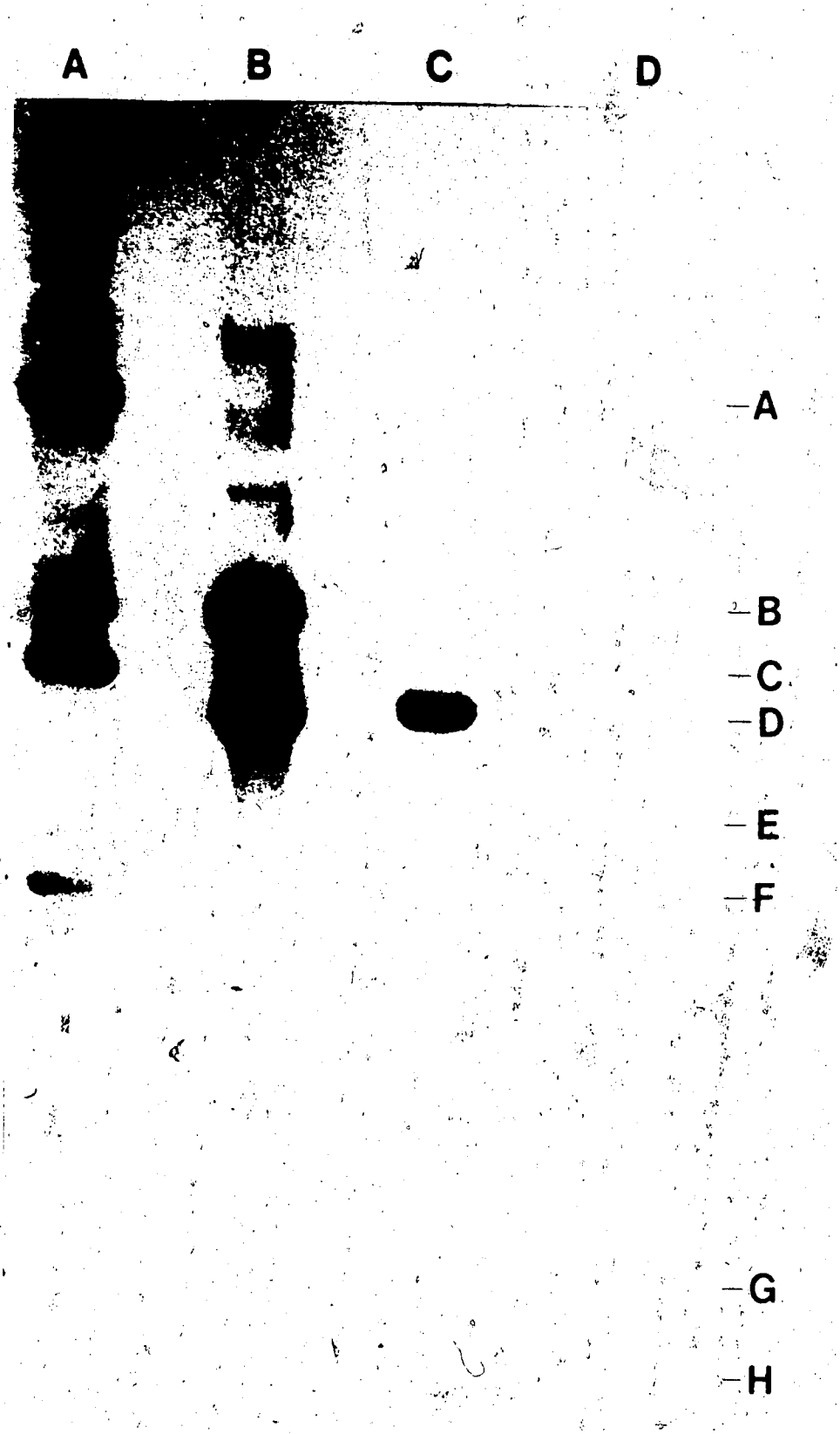
The above analysis suggests the SstI - SalI restriction endonuclease map shown in Fig. 12.

2. Hybridization between SstI and PstI DNA fragments of Clone 75.

Fig. 14 shows the composite restriction endonuclease map of Clone 75 constructed by superimposing the PstI - SalI (Fig. 12) and SstI - SalI (Fig. 12) maps. This map was confirmed by hybridization of SstI and PstI fragments (Fig. 15). Two additional PstI fragments, G and H, were identified by SstI fragment probes C and B, respectively. These

Fig.14 Restriction endonuclease map of Clone 75. The location and direction of the gB2 gene, determined by Southern blot and DNA sequence analyses, is indicated by the arrow.

Fig.15. Hybridization of Sst1 and Pst1 DNA fragments of Clone 75. Sst1 fragments A, B, C and D were used to probe a Southern blot of Pst1 DNA fragments. The positions and identities of the Pst1 DNA fragments are indicated.


A
B
C
D
A
B
C
D
E
F
G
H

fragments are less than 100 bases in length and therefore were not readily detected by ethidium bromide staining of the agarose gel. Their position on the map was further confirmed by DNA sequencing (see Chapter 5).

C. Fine mapping the gB2 gene by Southern blot analysis

Fig. 16 shows the alignment of the BglII physical maps for the HSV-1 and HSV-2 genomes and alignment of the BglII fragment I (0.314 - 0.416) of HSV-1 with the BglII fragment J (0.314 - 0.395) of HSV-2. Three HSV-1 DNA fragments were used as hybridization probes to identify the restriction fragments, from the HSV-2 6.6 kb BamHl-BglII (0.345 - 0.395) insert of Clone 75, which contain gB2 DNA sequences.

The Kpnl DNA probe (0.346 - 0,377) of HSV-1, containing the entire gB1 gene and flanking sequences, hybridized with Clone 75 fragments Pstl - Sall B, D1, D2 and E, Sstl - Sall fragments A2, B1, B2, C and D and Sall C (Fig. 13). The HSV-1 Xhol (0.55 kb) fragment (0.367 - 0.71) contains the 5' end of the HSV-1 gB gene. This probe detected HSV-2 Sstl, fragment D (256 bp) (Fig. 17). The HSV-1, Sst 1 (2.4 kb) fragment (0.352 - 0.367), containing a major portion of the gB-1 5' coding sequence, detected HSV-2 fragments Pstl B, D and G; Sall A, B and C; and Sstl B and C. These results, in combination with DNA sequencing data, defined the location of the gB2 gene shown in Fig. 14 and 16.

Fig.16. Alignment of the BglII restriction endonuclease maps for HSV-1 and HSV-2 genomes (from fig.3 of Preston et al.(1978)) and alignment of HSV-1 BglII fragment I (0.314-0.416) and HSV-2 BglII fragment J (0.314-0.395). Restriction maps of the HSV-1 BglII fragment I and HSV-2 BamH1-BglII fragment (0.345-0.395) are shown. The positions and transcriptional direction of the gB2 gene (determined by southern blot analysis and DNA sequencing) is indicated by the arrow. HSV-1 DNA fragments KpnI (5.0Kb) and Sst1 (2.4Kb) and XhoI (0.55Kb) contain gB1 sequences (described in the text) and were used as probes for gB2 sequences. Restriction enzymes used were BglII (Bg), BamH1(B), Sal1(sa), Sst1 (S), Xho1(X), Kpn1(K) and Pst1 (P).

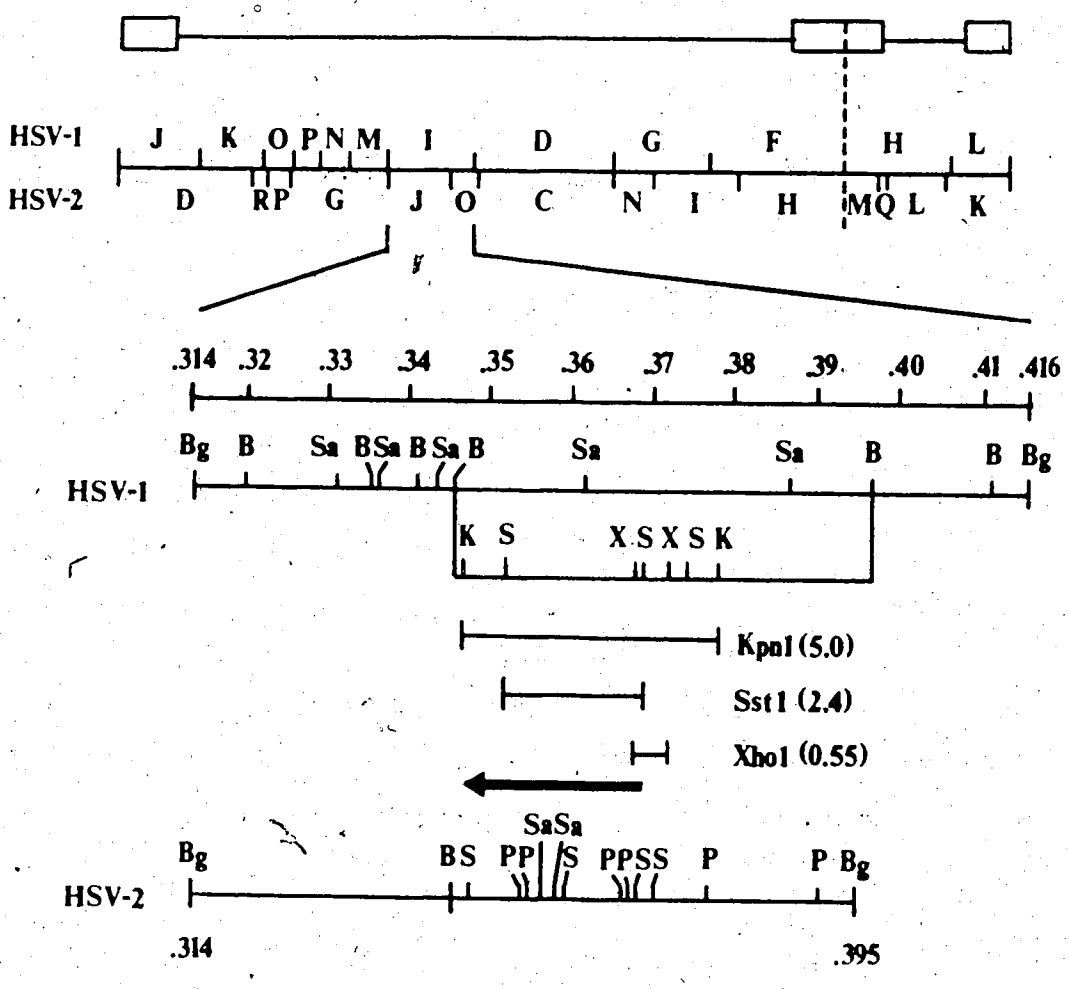
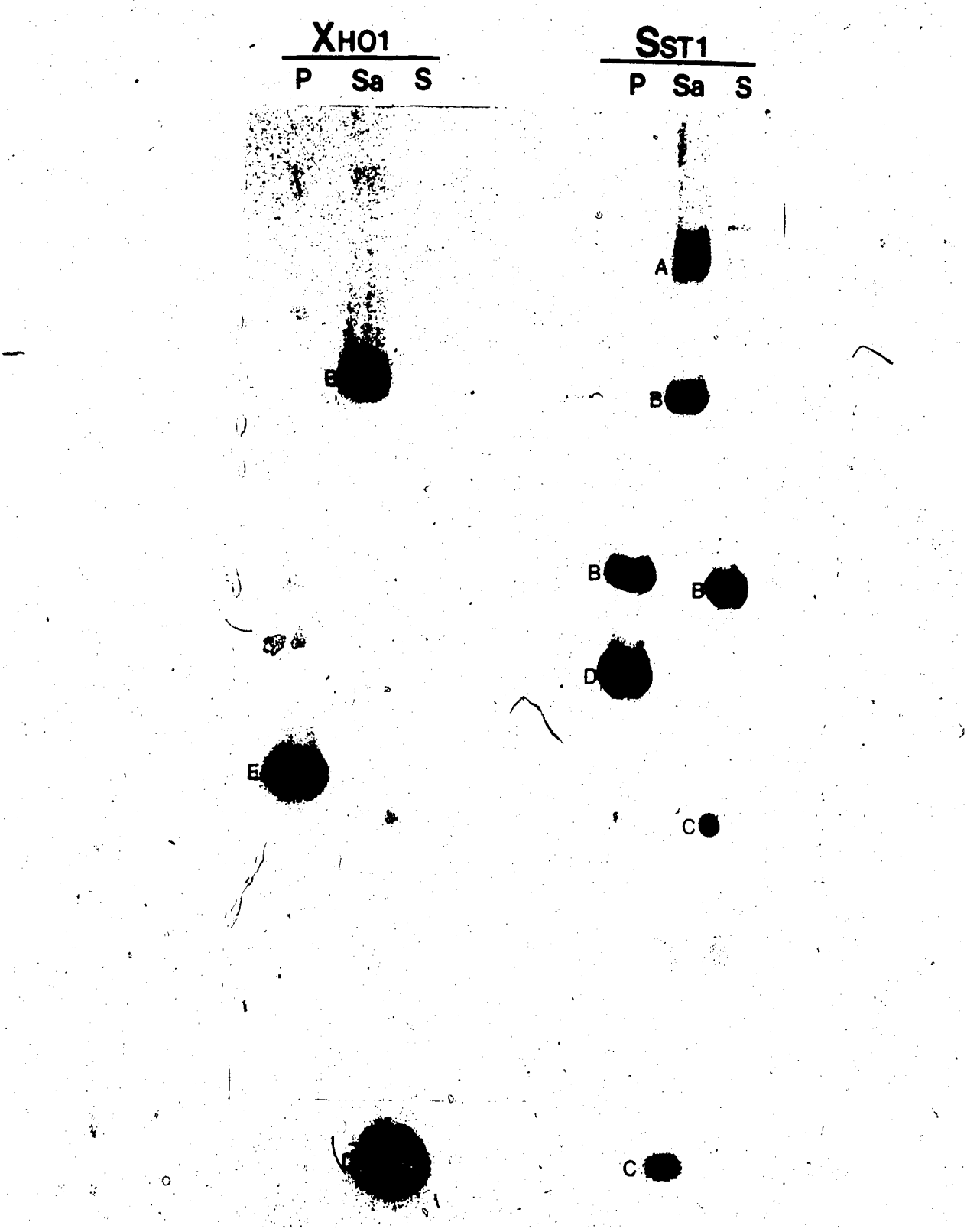

HSV-1
J K O P N M I D G F H L
HSV-2
D R P G J O C N I H M Q L K
.314 .32 .33 .34 .35 .36 .37 .38 .39 .40 .41 .416
HSV-1
Bg B Sa B Sa B Sa B Sa B Sa B Bg
K S X S X S K
Kpn1 (5.0)
Sst1 (2.4)
Xho1 (0.55)
HSV-2
Bg Sa Sa B S P P S S P P Bg
.314
.395

Fig.17. Hybridization of HSV-1 gB1 probes with Clone 75 Pst1, Sal1 and Sst1 DNA fragments. The 2.4 Kb Sst1 fragment (0.351-0.368) of HSV-1 (Sst1) contains a major portion of the gB1 coding sequence and the 0.55 Kb Xho1 fragment (0.367-0.371) (Xho1) contains the 5' end of the gB1 gene. The Pst1 (P), Sal1 (Sa) and Sst1(S) DNA fragments which were detected by the probe are identified.


XHO1
P Sa S
SST1
P Sa S
A
B
B
B
B
D
E
E
C
C

III. Discussion

In Chapter 3, gB1 was mapped by northern blot analyses to within map units 0.345 and 0.386 on the HSV-1 genome. The gB2 gene was likely to be colinear and share DNA sequence homology with gB1. This assumption was based on the fact that 1) the 6.6 kb BamHl - BglII fragment (0.345 - 0.395) of HSV-2 hybridized to both gB1 and gB2 transcripts (Fig. 10), 2) restriction analyses of HSV intertypic recombinants and marker rescue experiments locate the two genes to the same region (Marsden et al., 1978; Ruyechan et al., 1979) and 3) gB has epitopes common to both HSV-1 and HSV-2 (Showalter et al., 1981). Functional mutations in gB1 have been localized within map units 0.346 to 0.368 (Deluca et al., 1982; Holland et al., 1983). Therefore, HSV1 DNA probes Kpnl (0.346 - 0.377), Sstl (0.352 - 0.367) and Xhol (0.367 - 0.371) (see Fig. 16) were chosen to probe for gB2 sequences on the HSV2 6.6 kb BamHl - BglII fragment (0.345 - 0.395). DNA sequencing of the HSV-2 genomic region between 0.345 to 0.371 map units revealed an open reading frame having sufficient length to encode the gB2 glycoprotein and having extensive homology with the gB1 (strain KOS) sequence (see Chapter 5).

The HSV-2 6.6 Kb BamHl - BglII fragment (0.345 - 0.395) insert could not be excised from Clone 75 due to the hybrid BglII/BamHl cloning site. Therefore, hybridization, rather than partial digest analyses, was a more convenient method to determine the restriction map of this fragment. In fact, two additional DNA fragments, Pstl G and H, were detected by

hybridization to SstI and PstI fragments from Clone 75 (Fig. 15 and 17) which contained gB2 sequences.

In most cases, the gB1 DNA probes clearly identified the HSV-2 fragments containing gB2 sequences. The HSV-1 2.4 kb SstI fragment did not hybridize as strongly to the Clone 75 SalI 8.0 kb fragment A (Fig. 17), probably as a result of the less efficient transfer of larger DNA fragments to nitrocellulose membrane.

CHAPTER 5

DETERMINATION OF THE NUCLEOTIDE SEQUENCE AND AMINO ACID SEQUENCE OF GLYCOPROTEIN gB2 AND PREDICTION OF MAJOR ANITGENIC REGIONS

I. Introduction

The characterization of the antigenic structure of glycoprotein gB is essential to the understanding of the immunobiology of HSV infection There may be a correlation between the antigenic and biological properties of HSV (Frankel et al., 1985). This chapter describes the M13 cloning of Clone 75 restriction fragments which contain gB2 (Strain 333) sequences and the subsequent determination of the entire nucleotide sequence of this gene by the Sanger dideoxy chain-termination method. The amino acid sequence was deduced and compared with the primary sequence of gB from other HSV strains. Secondary structural features were predicted by the Chou and Fasman (1978) algorithm. Antigenic sites were predicted from the primary sequence on the basis of three different parameters: 1) a hydrophilicity scale derived from high performance liquid chromatography (HPLC) peptide retention data, 2) accessibility and 3) flexibility. The computer program which was used combined these three parameters and sucessfully predicted many of the known antigenic sites for myoglobin, lyzozyme, cytochrome c and influenza hemagglutinin (Parker et al., 1986). Extensive amino acid diversities were found in certain regions among

the several gB sequences compared and these regions correlated well with the predicted antigenic determinants.

II. Results

A. M13 cloning and sequencing of the gB2 (strain 333) gene

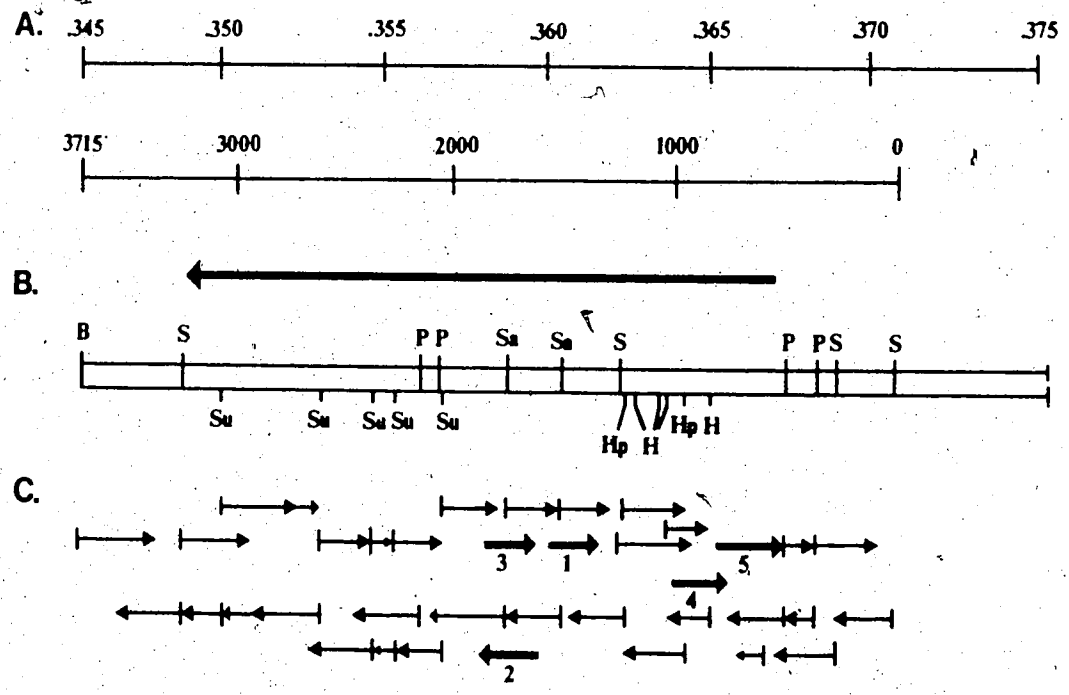
1. Construction and identification of M13 clones.

Fig.18 shows the overall strategy used to obtain the sequence of the gB2 gene. Several Pst1, Sst1, and Sal1 DNA fragments, between the BamH1 and the Pst1 sites (map units 0.345-0.377), were cloned into M13 vectors. Sau3A1 subfragments from the Sst1 fragment B (0.348-0.362) were shot-gun cloned into vectors mp10 and mp11. The Sal-Pst1 fragment (0.360-0.367), from Clone 75, was isolated from agarose gel and found to be resistant to digestion by HpaII and HaeIII. Therefore this fragment was cloned into plasmid vector pUC8 and the resulting recombinant was cut with Pst1, Sal1 and HaeIII or HpaII. Fragments derived from the Sal-Pst1 fragment were separated by agarose gel electrophoresis and cloned directly into M13 vectors. M13 phages carrying DNA inserts were identified by their reduced migration rate in 0.5% agarose gel relative to the M13 vector and also by dot blot analysis. Fig.19 shows an example of a dot blot which identified M13 clones containing Clone 75 Pst1 fragments B, D and E, and Sst1 fragment D.

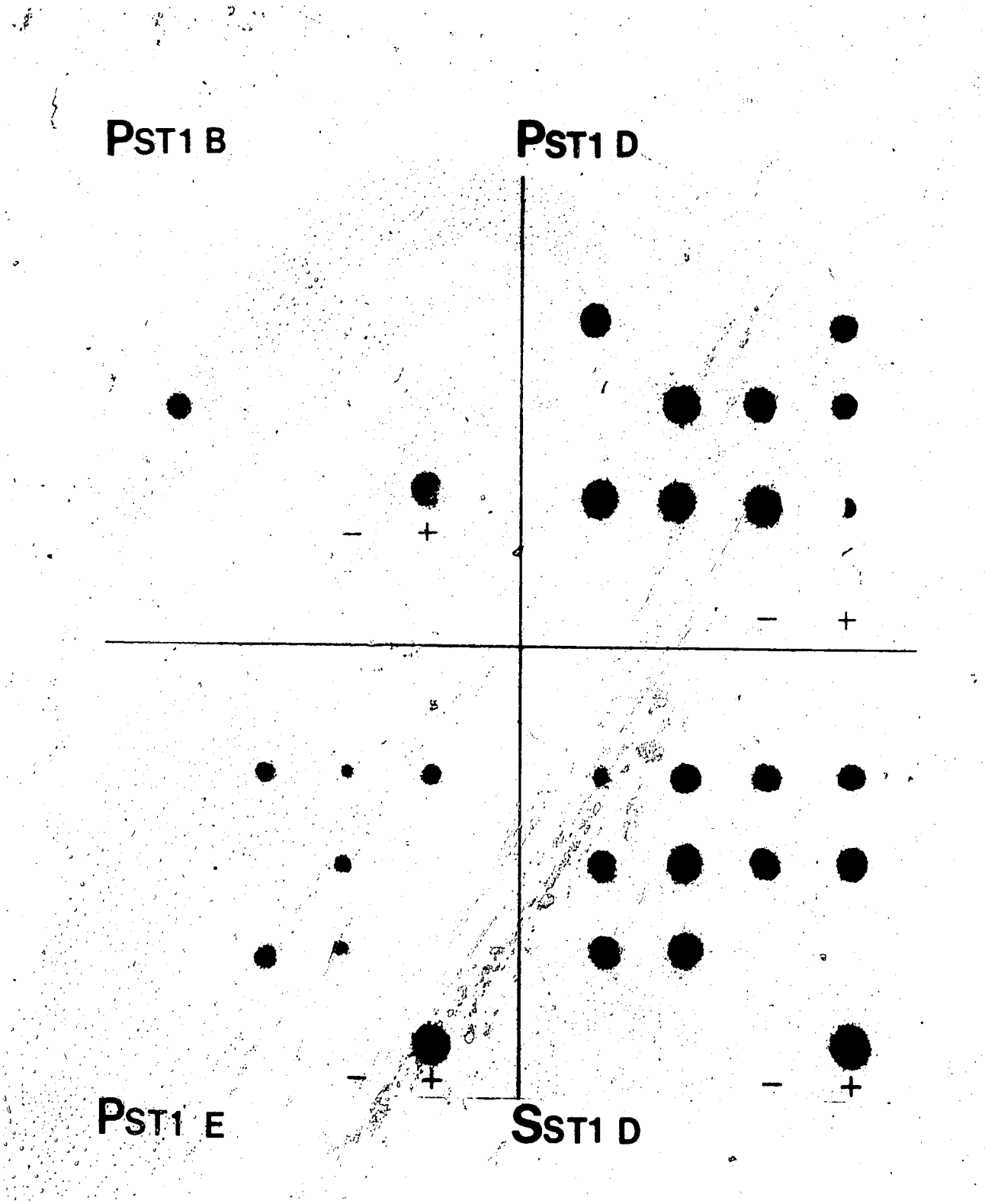
2. Sequencing the gB2 gene.

Both strands of the HSV-2 DNA region between map units

Fig.18.Strategy used for determining the HSV-2 DNA sequence between 0.345 and 0.370 map units. A) The size of the region sequenced (in base pairs). B) The complete restriction map of this region for the enzymes PstI(P), SstI(S) and SalI(Sa) and a partial map for the enzymes Sau3AI(Su), HaeIII(H) and HpaII(Hp). The protein encoding region and gB2 orientation determined by DNA sequence analysis is indicated by the large arrow. C) Map of the sequencing overlaps. The vertical dash at the beginning of each arrow indicates the position of the first nucleotide of a sequenced clone. The thicker arrows (numbered 1-5) represent sequences primed by specific synthetic DNA primers


A.
345
350
355
360
365
370
375
3715
3000
2000
1000
0
B.
B
S
P P
Sa
Sa
S
P
P S
S
Su
Su
Su Su
Su
Hp H
Hp H
C.
3
1
5
4
2

ig.19. Dot blot analysis of M13 clones. DNA derived from M13 phages carrying DNA fragments Pst1 B, D and E, and Sst1 D of Clone 75 was blotted onto nitrocellulose paper. The DNA fragments which were used as inserts to construct the M13 clones were used as hybridization probes to detect positive clones. The positive (+) and negative (-) controls are Clone 75 plasmid DNA and M13mp9(RF)DNA, respectively.


PST1 B
PST1 D
PST1 E
SST1 D
− +
− +
− +
− +

chain-termination method. In most cases M13 universal primers were used to prime the sequencing reaction from the termini of the cloned fragment. In some regions where an M13 clone was not available or sequence confirmation was required, synthetic DNA primers were synthesized to prime sequencing reactions at specific locations (see Fig.18). Table II lists the sequences of the synthetic primers utilized. Examples of nucleotide sequencing gels for sequences primed by synthetic and M13 universal primers are shown in Fig. 20B and 20A, respectively. Sequencing of both strands resolved several ambiguities in the DNA sequence due to band compressions in the sequencing gel. However, determination of the sequence immediately 3' to the PstI site at nucleotide position 495 and between the Sau3A sites (2382-2625) required the use of 7-deaza-2'-deoxyguanosine-5'-triphosphate (c7 dGTP) in place of dGTP in the sequencing reaction. The N-7 position of the guanine ring in this nucleotide analogue is replaced by a methine moiety. Therefore Hoogsteen bond formation is avoided and hairpin structures, which give rise to abnormal band migration especially in G-C rich areas, are avoided (Barr et al.,1986). Fig. 20C shows the sequencing gel of the DNA sequence, 3' to the Sau3A site at position 2382, using c^7dGTP.

B Sequence analysis and antigenic site prediction.

1. DNA sequence analysis.

The nucleotide sequence of the region between 0.345-0.370 is shown in Fig.21. Open reading frame analysis of the

Fig.20. Examples of nucleotide sequencing gels. A) Nucleotide sequence 3' to Sau3A1 recognition site (position 2625) using M13 universal primer. B) Nucleotide sequence 5' to HaeIII recognition site (position 1020) using synthetic primer #4 (see Table II). C) Nucleotide sequence 3' to Sau3A (position 2382) using M13 universal primer and c^7dGTP.

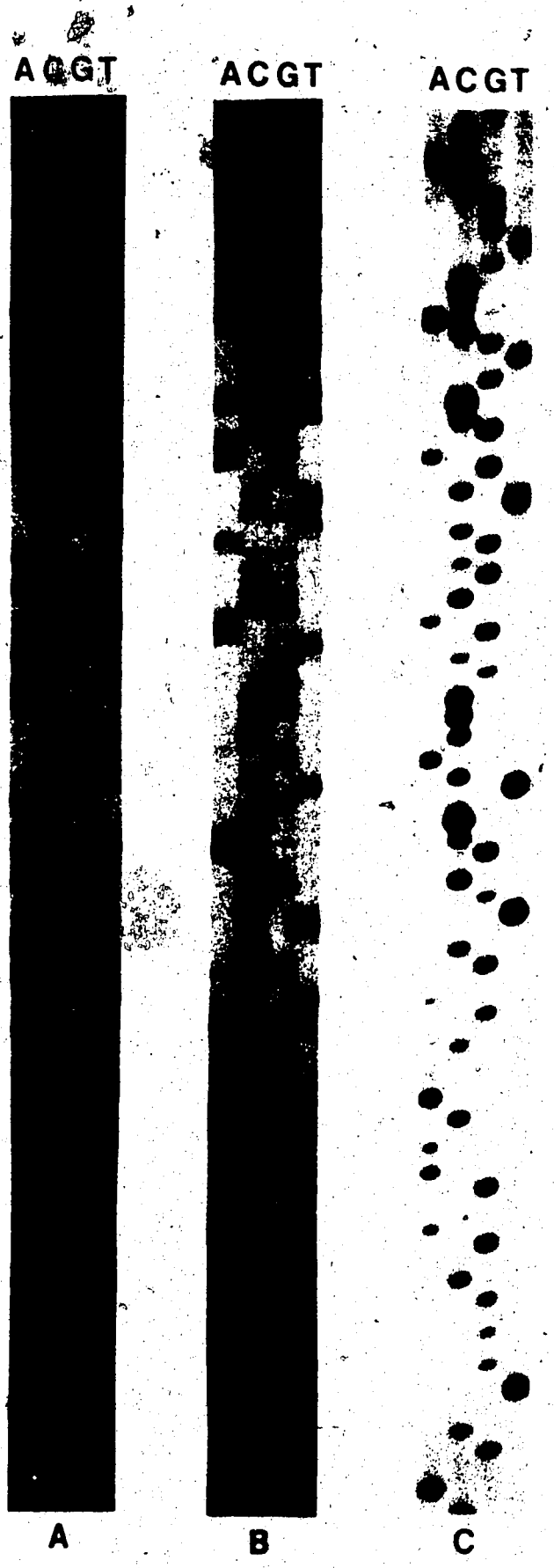

ACGT
ACGT
ACGT
A
B
C

Fig.21. Nucleotide sequence and deduced amino acid sequence of HSV-2 strain 333 gB2. Nucleotide number 2 is the first nucleotide of the Sst1 site at approximately 0.370 map units and nucleotide 3721 is the last nucleotide of the BamHI site located immediately to the right of HSV-2 map coordinate 0.345 (see Fig.17). The nucleotide sequence of the coding strand is presented. Glycoprotein B is transcribed from the right to the left in the P arrangement of HSV-2 DNA. Transcriptional and translational regulatory signals are indicated by boxes and described in the text. Potential N-linked glycosylation sites are underlined.

```
                                                                  27                                                                  54
AGA GCT CGT CAA CGG GCC CCT GTT CGA CCA CTC CAC CCA CAG CTT CGC CCA GCC
                                                                  81                                                                 108
CCC CAA CAC CGC GCT GTA CTA CAG CGT CGA GAA CGT GGG GCT CCT GCC GCA CCT
                                                                 135                                                                 162
CAA GGA GGA ACT CGC CCG CTT CAT CAT GGG CGC GGG GGG CTT CGG GCG CTG ATT
                                                                 189                                                                 216
GGG CCG TCA GCG AGT TTC AAA AGT TCT ACT GTT TTG ACG GTG TTT CCG GAA TCA
                                                                 243                                                                 270
CGC CCA CCC AGC GCG CCG CCT GGC GCT ATA TTC GCG AGC TCA TTA TCG CCA CCA
                                                                 297                                                                 324
CAC TCT TTG CGT CGG TCT ACC GGT GCG GGG AGC TTG AGT TGC GCC GCC CCG ACT
                                                                 351                                                                 378
GCA GCC GCC CGA CCT CCG CAA GTC TGT ACC GCT ACC CGC CGG CGT GTG ACC TCA
                                                                 405                                                                 432
CGT ACA ACT CCG ACT GTC CGC TGG TGG CCA TCG TCG AGA GCG GCC CCG ACG GCT
                                                                 459                                                                 486
GCA TCG GAC CCC GCT CGG TCG TGG TTT ACG ACC GAG ACG TTT TTT CCA TCC TCT
                                                                 513                                                                 540
ACC CGG TCC TGC AGC ACC TCG CCC CCA GAC TAG CGG GCG GCG GGG ACG GAC GGC
                                                                 567                                                                 594
CCC CGT AGG CCC GCC ATG CGC GGG GGG GGC TTG ATT TGC GCG CTG GTC GTG GGG
                        MET Arg Gly Gly Gly Leu Ile Cys Ala Leu Val Val Gly
                                                                 621                                                                 648
GCG CTG GTG GCC GCG GTG GCG TCG GCG GCC CCG GCG GCC CCG GCG GCC CCC CGC
Ala Leu Val Ala Ala Val Ala Ser Ala Ala Pro Ala Ala Pro Ala Ala Pro Arg
                                                                 675                                                                 702
GCC TCG GGC GGC GTC GCC GCG ACC GTC GCG GCG AAC GGG GGT CCC GCC TCC CGG
Ala Ser Gly Gly Val Ala Ala Thr Val Ala Ala Asn Gly Gly Pro Ala Ser Arg
                                                                 729                                                                 756
CCG CCC CCC GTC CCG AGC CCC GCG ACC ACC AAG GCC CGG AAG AAA ACC AAA
Pro Pro Pro Val Pro Ser Pro Ala Thr Thr Lys Ala Arg Lys Lys Thr Lys
                                                                 783                                                                 810
AAG CCG CCC AAG CGG CCC GAG GCG ACC CCG CCC CCC GAC GCC AAC GCG ACC GTC
Lys Pro Pro Lys Arg Pro Glu Ala Thr Pro Pro Pro Asp Ala Asn Ala Thr Val
                                                                 837                                                                 864
GCC GCC GGC CAC GCC ACG CTG CGC GCG CAC CTG CGG GAA ATC AAG GTC GAG AAC
Ala Ala Gly His Ala Thr Leu Arg Ala His Leu Arg Glu Ile Lys Val Glu Asn
                                                                 891                                                                 918
GCC GAT GCC CAG TTT TAC GTG TGC CCG CCC CCG ACG GGC GCC ACG GTG GTG CAG
Ala Asp Ala Gln Phe Tyr Val Cys Pro Pro Pro Thr Gly Ala Thr Val Val Gln
                                                                 945                                                                 972
TTT GAG CAG CCG CGC CGC TGC CCG ACG CGC CCG GAG GGG CAG AAC TAC ACG GAG
Phe Glu Gln Pro Arg Arg Cys Pro Thr Arg Pro Glu Gly Gln Asn Tyr Thr Glu
                                                                 999                                                                1026
GGC ATC GCG GTG GTC TTC AAG GAG AAC ATC GCC CCG TAC AAA TTC AAG GCC ACC
Gly Ile Ala Val Val Phe Lys Glu Asn Ile Ala Pro Tyr Lys Phe Lys Ala Thr
                                                                1053                                                                1080
ATG TAC TAC AAA GAC GTG ACC GTG TCG CAG GTG TGG TTC GGC CAC CGC TAC TCC
MET Tyr Tyr Lys Asp Val Thr Val Ser Gln Val Trp Phe Gly His Arg Tyr Ser
                                                                1107                                                                1134
CAG TTT ATG GGG ATA TTC GAG GAC CGC GCC CCC GTT CCC TTC GAG GAG GTG ATC
Gln Phe MET Gly Ile Phe Glu Asp Arg Ala Pro Val Pro Phe Glu Glu Val Ile
                                                                1161                                                                1188
GAC AAG ATT AAC GCC AAG GGG GTC TGC CGC TCC ACG GCC AAG TAC GTG CGC AAC
Asp Lys Ile Asn Ala Lys Gly Val Cys Arg Ser Thr Ala Lys Tyr Val Arg Asn
                                                                1215                                                                1242
AAC ATG GAG ACC ACC GCG TTT CAC CGG GAC GAC CAC GAG ACC GAC ATG GAG CTC
Asn MET Glu Thr Thr Ala Phe His Arg Asp Asp His Glu Thr Asp MET Glu Leu
                                                                1269                                                                1296
AAG CCG GCG AAG GTC GCC ACG CGC ACG AGC CGG GGG TGG CAC ACC ACC GAC CTC
Lys Pro Ala Lys Val Ala Thr Arg Thr Ser Arg Gly Trp His Thr Thr Asp Leu
                                                                1323                                                                1350
AAG TAC AAC CCC TCG CGG GTG GAG GCG TTC CAT CGG TAC GGC ACG ACG GTC AAC
Lys Tyr Asn Pro Ser Arg Val Glu Ala Phe His Arg Tyr Gly Thr Thr Val Asn
                                                                1377                                                                1404
TGC ATC GTC GAG GAG GTG GAC GCG CGG TCG GTG TAC CCG TAC GAT GAG TTT GTG
Cys Ile Val Glu Glu Val Asp Ala Arg Ser Val Tyr Pro Tyr Asp Glu Phe Val
                                                                1431                                                                1458
TTG GCG ACG GGC GAC TTT GTG TAC ATG TCC CCG TTT TAC GGC TAC CGG GAG GGG
Leu Ala Thr Gly Asp Phe Val Tyr MET Ser Pro Phe Tyr Gly Tyr Arg Glu Gly
                                                                1485                                                                1512
TCG CAC ACC GAG CAC ACC ACG TAC GCC GCC GAC CGC TTC AAG CAG GTC GAC GGC
Ser His Thr Glu His Thr Thr Tyr Ala Ala Asp Arg Phe Lys Gln Val Asp Gly
                                                                1539                                                                1566
TTC TAC GCG CGC GAC CTC ACC ACG AAG GCC CGG GCC ACG TCG CCG ACG ACC CGC
Phe Tyr Ala Arg Asp Leu Thr Thr Lys Ala Arg Ala Thr Ser Pro Thr Thr Arg
                                                                1593                                                                1620
AAC TTG CTG ACG ACC CCC AAG TTT ACC GTG GCC TGG GAC TGG GTG CCG AAG CGA
Asn Leu Leu Thr Thr Pro Lys Phe Thr Val Ala Trp Asp Trp Val Pro Lys Arg
                                                                1647                                                                1674
CCG GCG GTC TGC ACC ATG ACC AAG TGG CAG GAG GTG GAC GAG ATG CTC CGC GCC
Pro Ala Val Cys Thr MET Thr Lys Trp Gln Glu Val Asp Glu MET Leu Arg Ala
                                                                1701                                                                1728
GAG TAC GGC GGC TCC TTC CGC TTC TCC TCC GAC GCC ATC TCG ACC ACC TTC ACC
Glu Tyr Gly Gly Ser Phe Arg Phe Ser Ser Asp Ala Ile Ser Thr Thr Phe Thr
                                                                1755                                                                1782
ACC AAC CTG ACC CAG TAC TCG CTC TCG CGC GTC GAC CTG GGC GAC TGC ATT GGC
Thr Asn Leu Thr Gln Tyr Ser Leu Ser Arg Val Asp Leu Gly Asp Cys Ile Gly
                                                                1809                                                                1838
CGG GAT GCC CGC GAG GCC ATC GAC CGC ATG TTT GCG CGC AAG TAC AAC GCC ACG
Arg Asp Ala Arg Glu Ala Ile Asp Arg MET Phe Ala Arg Lys Tyr Asn Ala Thr
                                                                1863                                                                1890
CAC ATC AAG GTG GGC CAG CCG CAG TAC TAC CTG GCC ACG GGG GGC TTC CTC ATC
His Ile Lys Val Gly Gln Pro Gln Tyr Tyr Leu Ala Thr Gly Gly Phe Leu Ile
```

```
                                                                1917
GCG TAC CAG CCC CTC CTC AGC AAC ACG CTC GCC GAG CTG TAC GTG CGG [illegible] TAC
Ala Tyr Gln Pro Leu Leu Ser Asn Thr Leu Ala Glu Leu Tyr Val Arg Glu Tyr
                                                                1971                                                            1998
ATG CGG GAG CAG GAC CGC AAG CCC CGG AAT GGC ACG CCC GCG CCA CTG CGG GAG
MET Arg Glu Gln Asp Arg Lys Pro Arg Asn Ala Thr Pro Ala Pro Leu Arg Glu
                                                                2025                                                            2052
CGG CCC AGC GCC AAC GCG TCC GTG GAG CGC ATC AAG ACC ACC TCC TCG ATC GAG
Arg Pro Ser Ala Asn Ala Ser Val Glu Arg Ile Lys Thr Thr Ser Ser Ile Glu
                                                                2079                                                            2106
TTC GCC CGG CTG CAG TTT ACG TAT AAC CAC ATA CAG CGC CAC GTG AAC GAC ATG
Phe Ala Arg Leu Gln Phe Thr Tyr Asn His Ile Gln Arg His Val Asn Asp MET
                                                                2133                                                            2160
CTG GGG CGC ATC GCC GTC GCG TGG TGC GAG CTG CAG AAC CAC GAG CTG ACT CTC
Leu Gly Arg Ile Ala Val Ala Trp Cys Glu Leu Gln Asn His Glu Leu Thr Leu
                                                                2187                                                            2214
TGG AAC GAG GCC CGC AAG CTC AAC CCC AAC GCC ATC GCC TCC GCC ACC GTC GGC
Trp Asn Glu Ala Arg Lys Leu Asn Pro Asn Ala Ile Ala Ser Ala Thr Val Gly
                                                                2241                                                            2268
CGG CGG GTG AGC GCG CGC ATG CTC GGA GAC GTC ATG GCC GTC TCC ACG TGC GTG
Arg Arg Val Ser Ala Arg MET Leu Gly Asp Val MET Ala Val Ser Thr Cys Val
                                                                2295                                                            2322
CCC GTC GCC CCG GAC AAC GTG ATC GTG CAG AAC TCG ATG CGC GTC AGC TCG CGG
Pro Val Ala Pro Asp Asn Val Ile Val Gln Asn Ser MET Arg Val Ser Ser Arg
                                                                2349                                                            2376
CCG GGG ACG TGC TAC AGC CGC CCC CTG GTC AGC TTT CGG TAC GAA GAC CAG GGC
Pro Gly Thr Cys Tyr Ser Arg Pro Leu Val Ser Phe Arg Tyr Glu Asp Gln Gly
                                                                2403                                                            2430
CCG CTG ATC GAG GGG CAG CTG GGC GAG AAC AAC GAG CTG CGC CTC ACC CGC GAC
Pro Leu Ile Glu Gly Gln Leu Gly Glu Asn Asn Glu Leu Arg Leu Thr Arg Asp
                                                                2457                                                            2484
GCG CTC GAG CCG TGC ACC GTG GGC CAC CGG CGC TAC TTC ATC TTC GGC GGG GGC
Ala Leu Glu Pro Cys Thr Val Gly His Arg Arg Tyr Phe Ile Phe Gly Gly Gly
                                                                2511                                                            2538
TAC GTG TAC TTC GAG GAG TAC GCG TAC TCT CAC CAG CTG AGT CGC GCC GAC GTC
Tyr Val Tyr Phe Glu Glu Tyr Ala Tyr Ser His Gln Leu Ser Arg Ala Asp Val
                                                                2565                                                            2592
ACC ACC GTC AGG ACC TTC ATC GAC CTG AAC ATC ACC ATG CTG GAG GAC CAC GAG
Thr Thr Val Arg Thr Phe Ile Asp Leu Asn Ile Thr MET Leu Glu Asp His Glu
                                                                2619                                                            2646
TTT GTG CCC CTG GAG GTC TAC ACG CGC CAC GAG ATC AAG GAC AGC GGC CTG CTG
Phe Val Pro Leu Glu Val Tyr Thr Arg His Glu Ile Lys Asp Ser Gly Leu Leu
                                                                2673                                                            2700
GAC TAC ACG GAG GTC CAG CGC CGC AAC CAG CTG CAC GAC CTG CGC TTT GCC GAC
Asp Tyr Thr Glu Val Gln Arg Arg Asn Gln Leu His Asp Leu Arg Phe Ala Asp
                                                                2727                                                            2754
ATC GAC ACG GTC ATC CGC GCC GAC GCC AAC GCC GCC ATG TTC GCG GGG CTG TGC
Ile Asp Thr Val Ile Arg Ala Asp Ala Asn Ala Ala MET Phe Ala Gly Leu Cys
                                                                2781                                                            2808
GCG TTC TTC GAG GGG ATG GGG GAC TTG GGG CGC GCC GTC GGC AAG GTA GTC ATG
Ala Phe Phe Glu Gly MET Gly Asp Leu Gly Arg Ala Val Gly Lys Val Val MET
                                                                2835                                                            2862
GGA GTA GTG GGG GGC GTG GTG TCG GCC GTC TCG GGC GTG TCC TCC TTT ATG TCC
Gly Val Val Gly Gly Val Val Ser Ala Val Ser Gly Val Ser Ser Phe MET Ser
                                                                2889                                                            2916
AAC CCC TTC GGG GCG CTT GCC GTG GGG CTG CTG GTC CTG GCG GGC CTG GTC GCG
Asn Pro Phe Gly Ala Leu Ala Val Gly Leu Leu Val Leu Ala Gly Leu Val Ala
                                                                2943                                                            2970
GCC TTC TTC GCC TTC CGC TAC GTC CTG CAA CTG CAA CGC AAT CCC ATG AAG GCC
Ala Phe Phe Ala Phe Arg Tyr Val Leu Gln Leu Gln Arg Asn Pro MET Lys Ala
                                                                2997                                                            3024
CTG TAT CCG CTC ACC ACC AAG GAA CTC AAG ACT TCC GAC CCC GGG GGC GTG GGC
Leu Tyr Pro Leu Thr Thr Lys Glu Leu Lys Thr Ser Asp Pro Gly Gly Val Gly
                                                                3051                                                            3078
GGG GAG GGG GAG GAA GGC GCG GAG GGG GGC GGG TTT GAC GAG GCC AAG TTG GCC
Gly Glu Gly Glu Glu Gly Ala Glu Gly Gly Gly Phe Asp Glu Ala Lys Leu Ala
                                                                3105                                                            3132
GAG GCC CGA GAA ATG ATC CGA TAT ATG GCT TTG GTG TCG GCC ATG GAG CGC ACG
Glu Ala Arg Glu MET Ile Arg Tyr MET Ala Leu Val Ser Ala MET Glu Arg Thr
                                                                3159                                                            3186
GAA CAC AAG GCC AGA AAG AAG GGC ACG AGC GCC CTG CTC AGC TCC AAG GTC ACC
Glu His Lys Ala Arg Lys Lys Gly Thr Ser Ala Leu Leu Ser Ser Lys Val Thr
                                                                3213                                                            3240
AAC ATG GTT CTG CGC AAG CGC AAC AAA GCC AGG TAC TCT CCG CTC CAC AAC GAG
Asn MET Val Leu Arg Lys Arg Asn Lys Ala Arg Tyr Ser Pro Leu His Asn Glu
                                                                3267                                                            3294
GAC GAG GCC GGA GAC GAA GAC GAG CTC TAA GGG AGG GGA GGG GAG CTG GGC TTG
Asp Glu Ala Gly Asp Glu Asp Glu Leu
                                                                3321                                                            3348
TGT ATA AAT AAA AAG ACA CCG ATG TTC AAA AAT ACA CAT GAC TTC TGG TAT TGT
                                                                3375                                                            3402
TTT GCC TTG GTT TTT ATT TGG GGG GGG GGC GTG TGA CTA GAA AAA CAA ATG CAG
                                                                3429                                                            3456
ACA CTG TGC TAA CGG GAA AAC CAA CCC CAA ACC AAC CCC AAA CCA ACC CCG TCT
                                                                3483                                                            3510
CCC CTG CGA CCG GTC GCT TTC CAC ATC CCC TCC CCG TGG TAG TCC TTC CGG GCC
                                                                3537                                                            3564
TTC GTC GCG TGT GGG GGC CAT CGG TTC GGC TCC TAG CCC CCC CCC CCC TCA CCC
                                                                3591                                                            3618
CTC CGA CCT AAT TTT TGT GTC ATT CGG CCC ACT TTC CCC CCC ACT CCA CCC CCC
                                                                3645                                                            3672
CCC TCT CAA ACA AAA ACA CAA GCA CAC GAA GTG GTA TAC TTT TGT CCG GTT GTT
                                                                3699
TGT TTA TTT AAA ATA TAT GAA AAC ACA CAC CCC CCC CAA GTC CGG ATC C
```

DNA sequence between 0.345 and 0.370 map units revealed one reading frame extending from nucleotide positions 556 to 3270. A larger open reading frame on the opposite strand could not code for gB2 since this strand of DNA did not hybridize to the gB1 mRNA (Holland et al.,1984) and because the predicted protein sequence was not homologous to gB1. An additional open reading frame, which ends at termination codon TAG at nucleotide position 548 and extends beyond the first nucleotide of the sequence, translated into a polypeptide which was homologous to the C-terminal end of HSV-1 polypeptide ICP18.5 (Pellett et al. 1986).

The sequence context around the proposed initiating codon was GCCATGCG (nucleotides 553 to 559) which qualified as a functional sequence context for translation initiation in other eucaryotic mRNAs (Kozak et al.,1983). Putative transcriptional regulatory signals were found in the region 5' to the AUG codon at position 556. Transcription of eucaryotic mRNAs typically begins at an 'A' residue surrounded by pyrimidine residues (Breathnach and Chambon 1981), therefore transcription of the gB2 mRNA probably initiates at one of the 'A' residues in the sequence GCCACCACAC (nucleotides 264 - 273). A 'TATA' box having the sequence ATATATT (nucleotides 242 - 248) and a potential 'CAT' box CGGAAT (nucleotides 209 - 214) are located at 16 and 50 nucleotides, respectively, upstream from the putative transcription initiation site. Nine out of thirteen nucleotides in the sequence CGCCCGCTTCATC (nucleotides 121-

133) are homologous with the ICP4 DNA binding consensus site found in several promoter regions of HSV immediate-early proteins and glycoprotein gD (Faber and Wilcox, 1986; Muller, 1987).

A polyadenylation signal 'AATAAA' (nucleotides 3301 - 3306) was found 30 nucleotides downstream from the termination codon TAA (nucleotides 3268 - 3630). A cleavage site CACATG (3329 - 3334), similar to the consensus RNA cleavage site 'CACTG' (Berget, 1984), occurs 22 nucleotides downstream from the polyadenylation signal. Eight out of ten nucleotides in the sequence 5'-TCCTGCAGCA-3' (nucleotides 547 to 556) are complementary to the 18S ribosomal RNA sequence 3'-AGGAAGGCGT-5' (Hagenbuchle et al. 1978). This sequence probably acts as the ribosomal binding site thought to be required for efficient initiation of translation. All the above regulatory sequences are identical to those found for gB2 strain HG52 (Bzik et al. 1986). The overall DNA sequence of gB2 (nucleotides 333) is 99% homologous to that of HSV-2 (HG52) and 89% homologous to HSV-1 (KOS).

2. Protein sequence analysis.

The predicted polypeptide consists of 904 amino acids and has a molecular weight of 100.4. Analysis of the amino acid sequence reveals a number of features common to membrane bound glycoproteins: (1) a cleavable signal sequence, (2) a hydrophilic surface region containing N-glycosylation sites and several potential antigenic sites, (3) a hydrophobic membrane spanning region and (4) a hydrophilic cytoplasmic

region. These features have also been reported for gB of other HSV strains (Pellett et al. 1985c; Bzik et al. 1986).

The first 22 amino acids of gB2 (333) are characteristic of a signal sequence region. Fifteen out of seventeen residues from positions 6 to 22 are nonpolar. Hydrophilicity analysis, using the algorithm of Kyte and Doolittle (1982), indicated the extensive hydrophobic character of this region (Fig.22). The amino acid sequence ala-ser-ala (residues 20 - 22) is a common signal cleavage-recognition site (Perlman and Halvorson 1983). Alignment of the predicted primary sequences of gB from HSV-2, strains 333 and HG52, and HSV-1, strains KOS and F, shows that the amino acid sequence val-ala-ser-ala-ala-pro (residues 19 to 24) is conserved (Fig.23). Direct amino acid sequencing of the mature gB1 polypeptide revealed that the first N-terminal amino acid residues are: ala-pro-ser (Claesson-Welsh and Spear 1987). Taken together, these observations suggest that both gB1 and gB2 signal peptides are cleaved after the sequence ala-ser-ala.

Hydropathic analysis of gB2 (333) indicated that the domain between residues 723 to 792 contains three major hydrophobic peaks which range from 15 to 22 amino acids in length (Fig.22). This region is postulated to span the cellular membrane and to contain three anti-parallel segments that traverse the membrane three times (Pellett et al. 1985c; Claesson-Welsh and Spear 1987). This region is completely conserved between the two HSV-2 strains and differs from the

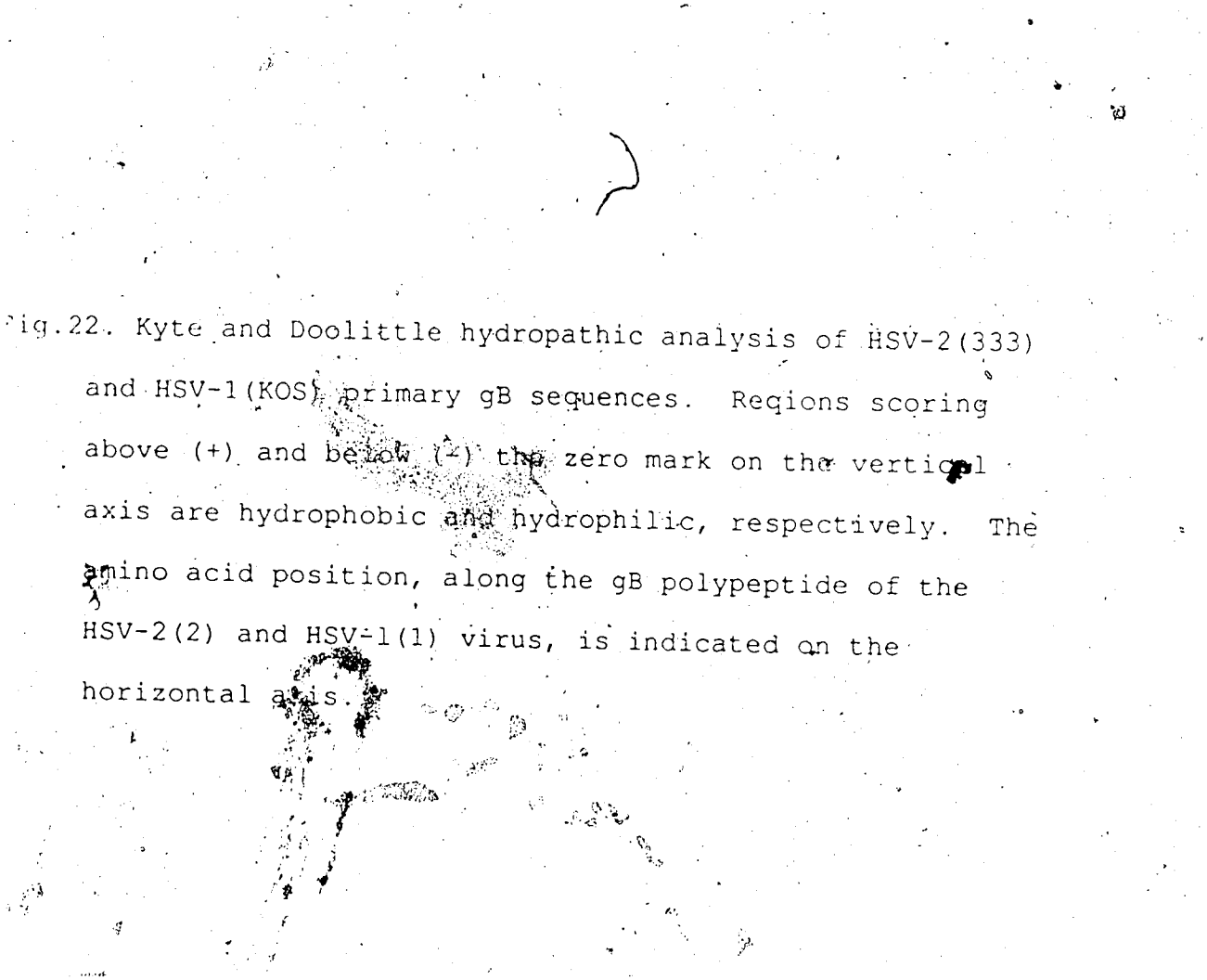


Fig.22. Kyte and Doolittle hydropathic analysis of HSV-2(333) and HSV-1(KOS) primary gB sequences. Regions scoring above (+) and below (-) the zero mark on the vertical axis are hydrophobic and hydrophilic, respectively. The amino acid position, along the gB polypeptide of the HSV-2(2) and HSV-1(1) virus, is indicated on the horizontal axis.

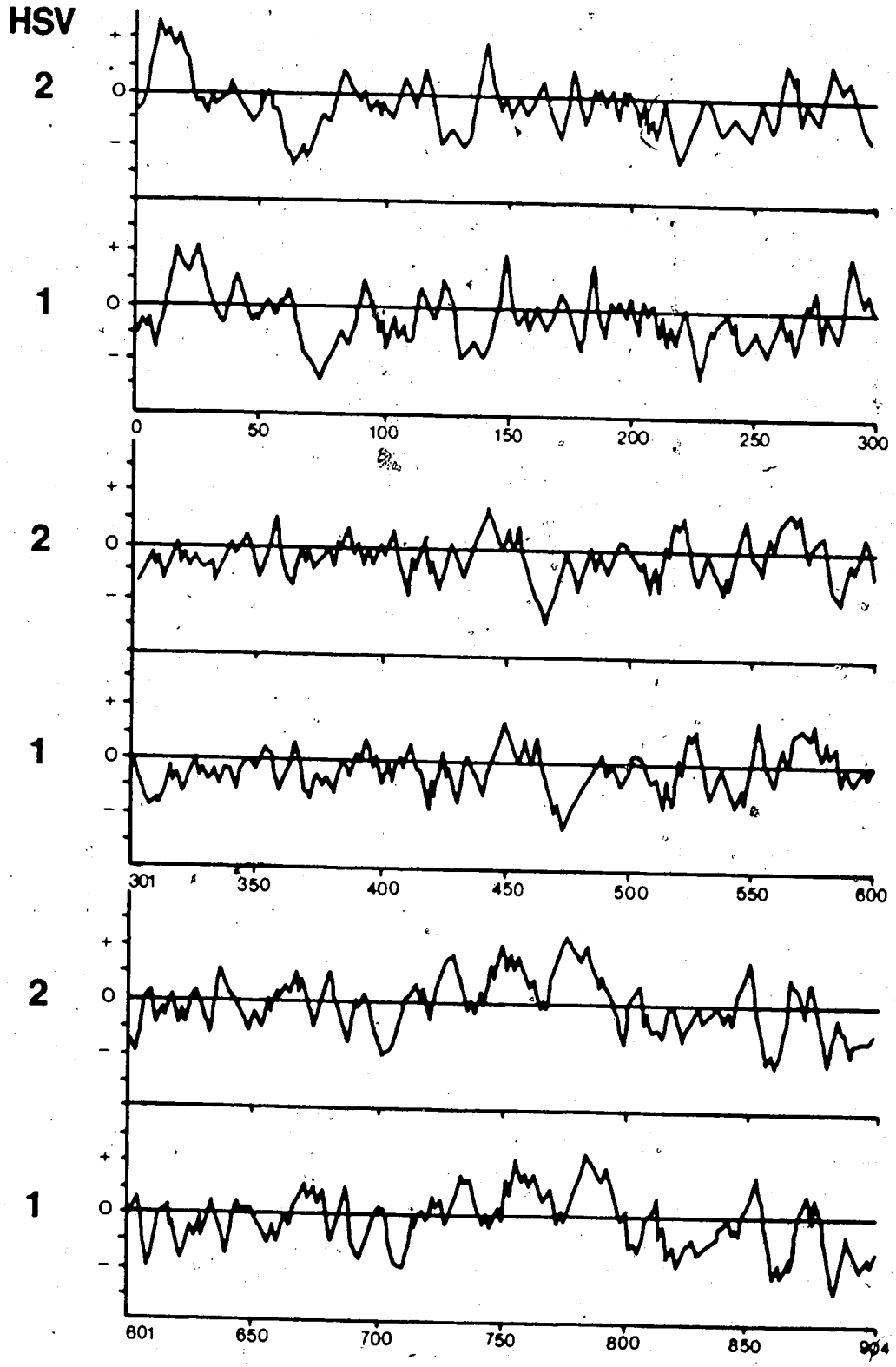
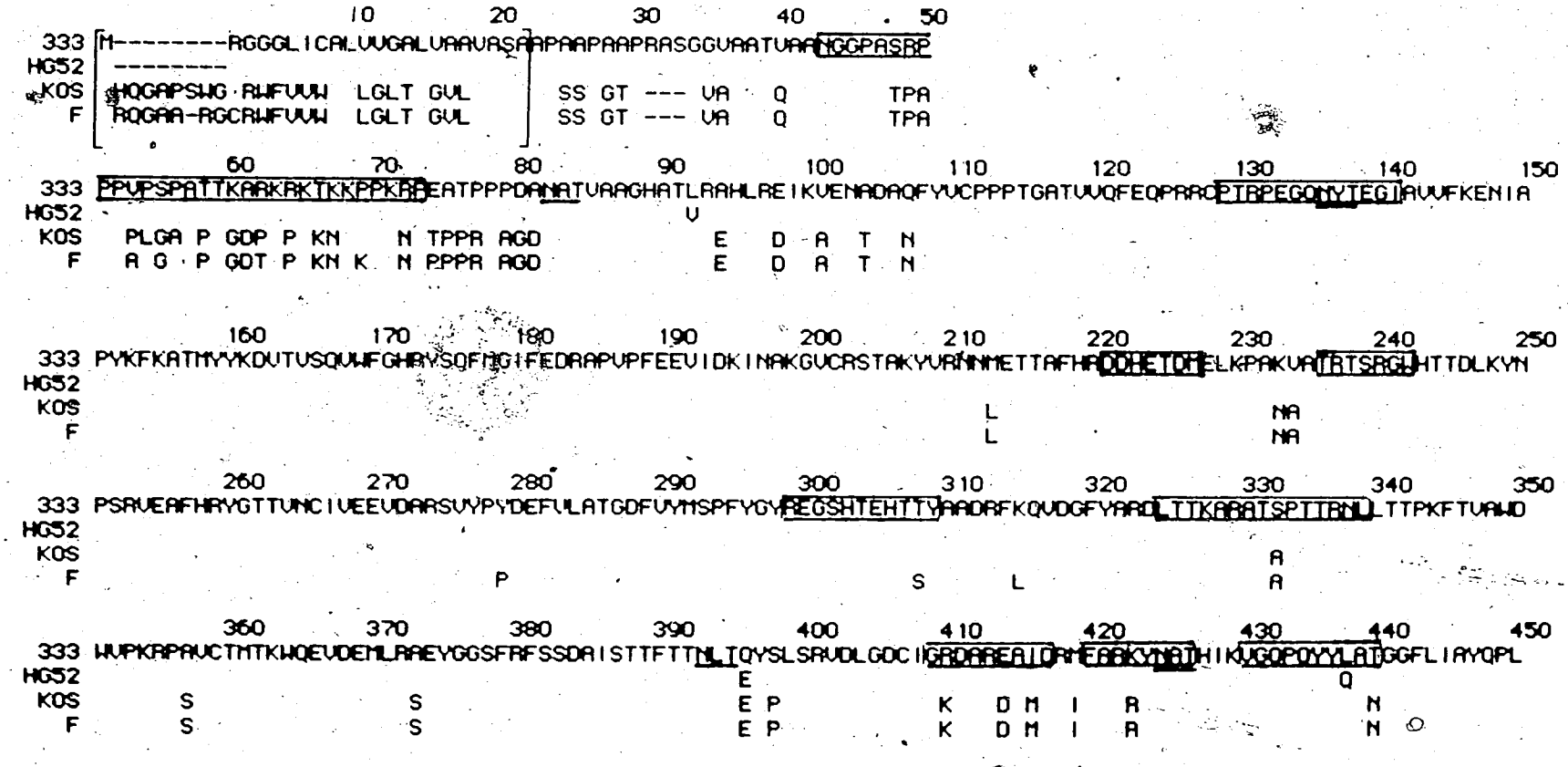
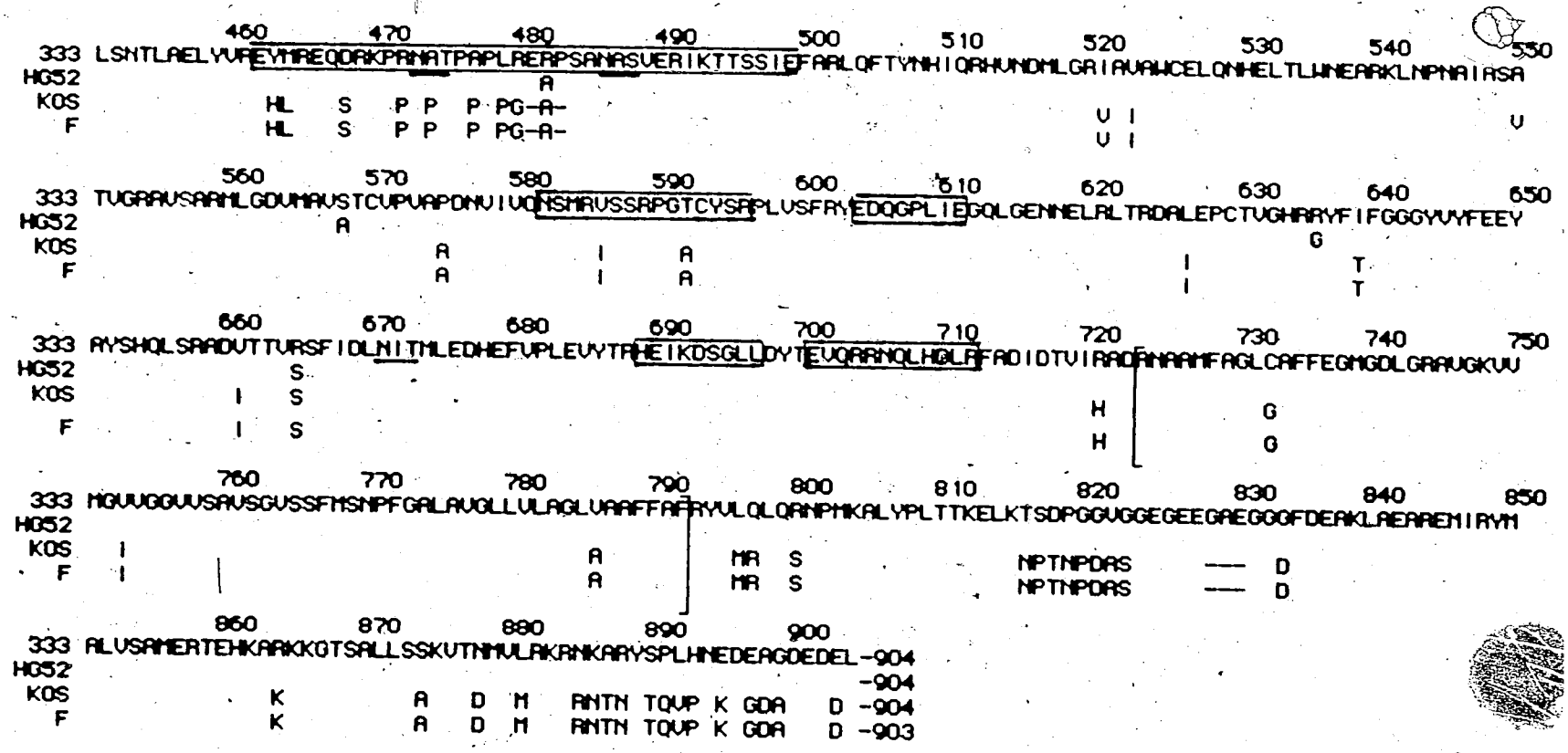

HSV
2
1
+
0
−
0
50
100
150
200
250
300
2
1
301
350
400
450
500
550
600
2
1
601
650
700
750
800
850
904

Fig.23.Alignment of gB primary sequences. The primary sequence of gB2 (strain 333: this paper) is compared with gB2 (strain HG52:Bzik et al.,,1986), gB1 (strain KOS:Bzik et al.,1984b) and gB1 (strain F:Pellett et al.,1985c). For convenience, the sites are numbered according to the amino acid positions of gB2 (333). Residues numbered 1 and 90[illegible] are the N and C-terminal amino acids, respectively. Only amino acids that differ from the gB2 (333) sequence are indicated. Deletion of a residue relative to the same position in another polypeptide is indicated by a dash. The total number of amino acids in each polypeptide is indicated at the C-terminal end. The signal peptide and membrane-spanning regions are indicated by brackets. Major antigenic regions are indicated by boxes, potential N-glycosylation sites are underlined.





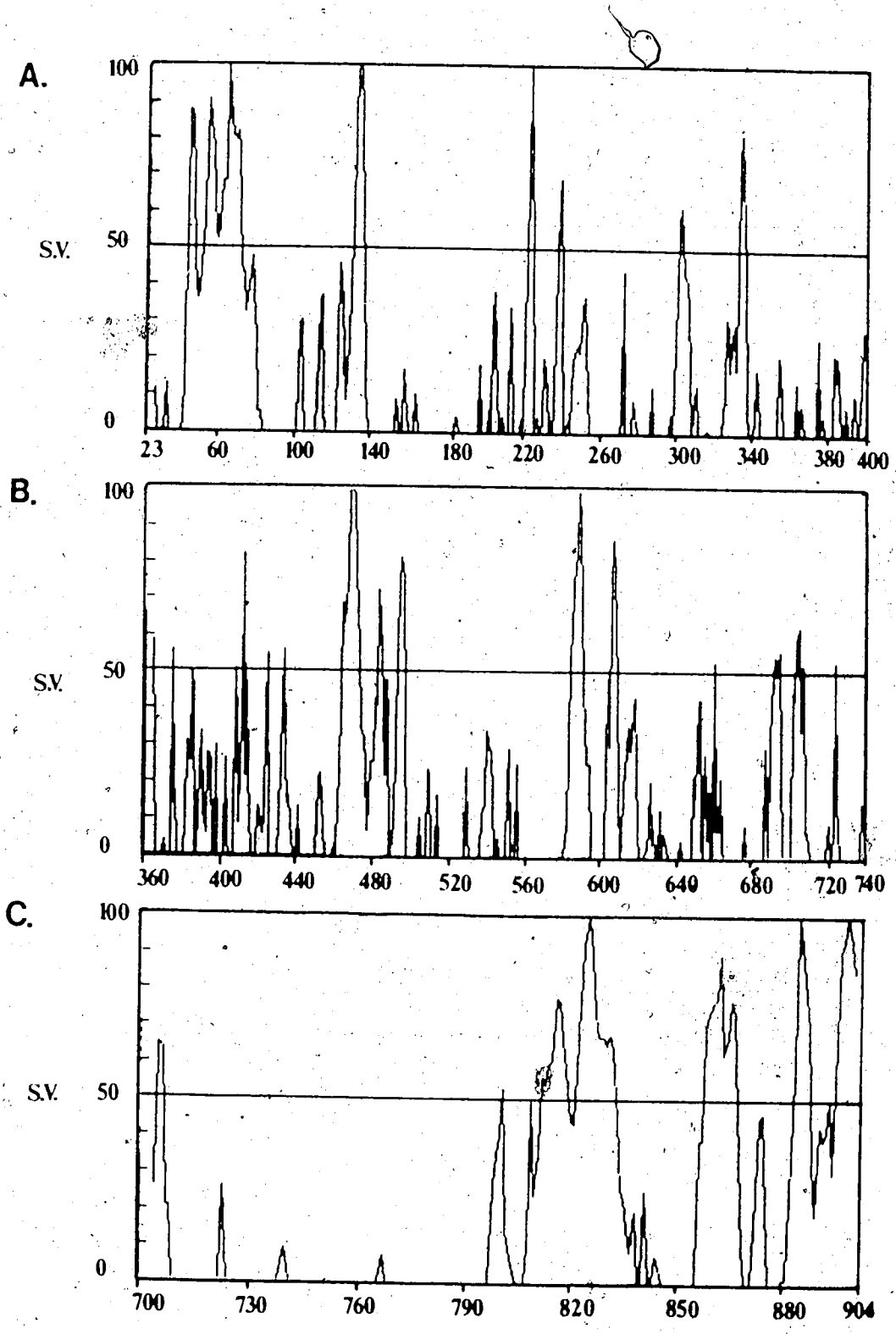
HSV-1 strains at only three positions. The high degree of amino acid conservation in the gB membrane spanning domain suggests that it may be an essential region with respect to gB orientation and function.

The region between amino acids 23 to 723 corresponds to the extracellular surface domain of the gB (333) polypeptide. It contains seven potential N-linked glycosylation sites and many hydrophilic residues. Evidence for this domain is given by analysis of gB translated in vitro in the presence or absence of pancreatic membranes. A large portion of the gB polypeptide which contained N-linked carbohydrate was protected from proteolysis by the membranes (Claesson-Welsh and Spear 1987). Amino acid diversity between gB1 and gB2 is prevalent in this domain. The remainder of gB2, residues 793 to the carboxyl terminal amino acid at position 904, corresponds to the cytoplasmic domain.

3. Primary sequence comparison, secondary structure analysis and antigenic site prediction.

Fig.24 shows the composite surface profile of the mature gB (333) polypeptide as analysed by the program of Parker et al. (1986) (described more fully in Materials and Methods). Regions of the polypeptide that showed surface values which exceed the 50% cut-off valve were defined as major antigenic regions. The hydrophilicity profiles of gB from HSV-1 and HSV-2 were similar (Fig.22). Therefore the positions of the major antigenic sites are likely to be in the same relative location along the length of the gB polypeptide. Amino acid

Fig.24. Predicted composite surface profile for the mature gB2 (333) polypeptide. The analysis was carried out for three separate segments of the polypeptide: A) residues 23 - 400, B) residues 360 - 740 and C) residues 700 - 904. The horizontal axis represents the amino acid position along the length of the polypeptide. The vertical axis represent the relative surface value (S.V.). The solid line represents a 50% cut-off value. Peaks that have surface values greater than 50% were defined as major linear antigenic sites. Peaks in the cytoplasmic domain (residues 792 - 904) were not scored as antigenic sites.

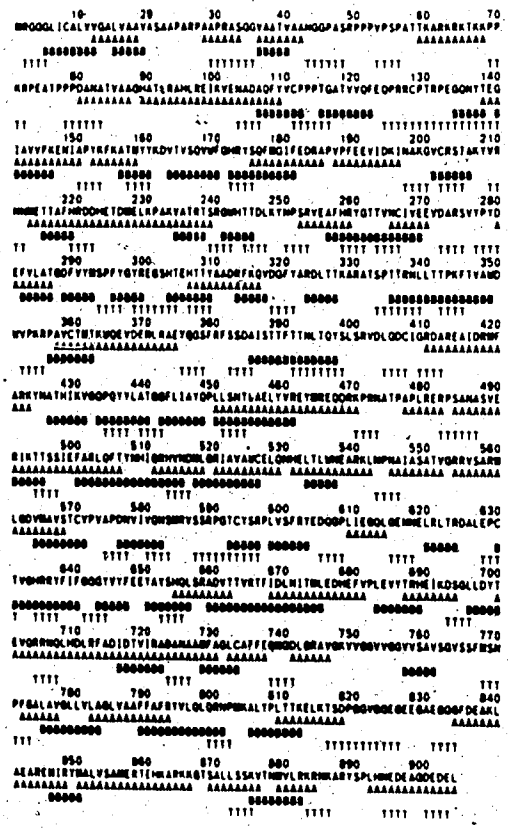

A.
100
S.V.
50
0
23
60
100
140
180
220
260
300
340
380
400
B.
100
S.V.
50
0
360
400
440
480
520
560
600
640
680
720
740
C.
100
S.V.
50
0
700
730
760
790
820
850
880
904

diversity between gB1 and gB2 in a predicted antigenic region could therefore be interpreted as defining a site-specific antigenic determinant.

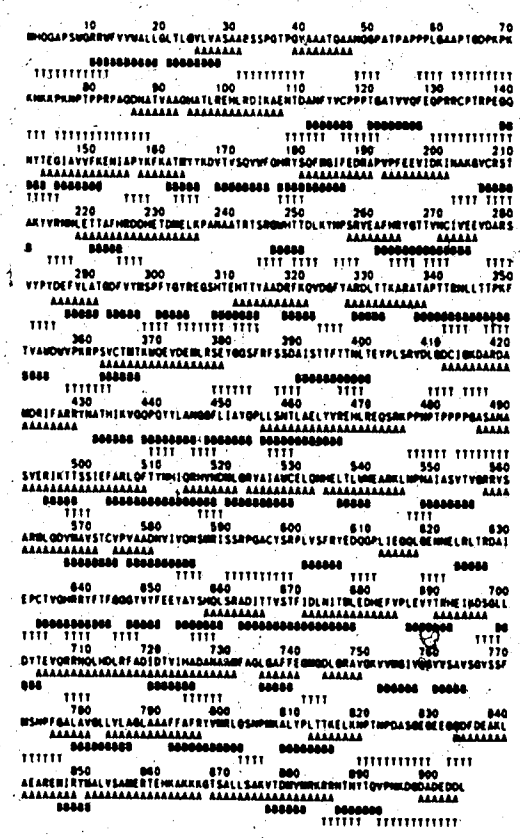
From Figures 23 and 24, five of the major antigenic regions; residues 43 - 82, 409 - 417, 420 - 427, 462 - 499 and 582 - 596, exhibit amino acid diversity between HSV-1 and HSV-2 gB. In some cases, the amino acid changes appear to be clustered. This observation may further delineate the location of a type-specific epitope. For example, all the amino acid substitutions in the region 462 to 499 occur to the left between residues 463 to 483. The surface profile plot indicates three peaks ranging from positions 462 - 475, 475 - 490, and 491 - 499. Taken together, these data suggest at least two type-specific epitopes at positions 463 - 474 and 475 - 483 and a type-common epitope between 491 - 499. Fig. 25 shows a comparison between the secondary structural features for the gB2 (333) and gB1 (KOS) polypeptides predicted by Chou and Fasman analysis. Between amino acids 462 and 499 the polypeptide is predominantly alpha-helical with a few minor turns. Structural predictions of the corresponding region in gB1 showed that the large number of proline residues occurring between positions 472 to 482 induced a major turn. Therefore, it is likely that this region in particular contains a type-specific epitope. Similar analysis of the other antigenic sites showing amino acid diversity indicated the following: the region between position 43 - 83 contains four possible epitopes. Major

Fig.25. Secondary structural features of the gB2 (333) and gB1(KOS) polypeptides predicted by the Chou and Fasman algorithm. AAAA = alpha helix, BBBB = beta sheet, TTTT = turn.

gB2 (333)



gB1 (KOS)



changes in secondary structure are predicted between residues 59 - 68. This segment also coincides with the highest peak suggesting at least one other site-specific epitope in the gB polypeptide.

Few or no amino acid substitutions and changes in secondary structure between gB1 and gB2 occur for the other major antigenic regions shown in Figure 23. An exception to this occurs for antigenic region 325 - 339 where a serine substitution in the gB2 polypeptide disrupts the alpha-helix seen in gB1 between residues 324 to 335. In contrast to this finding, in the regions 409 - 417, 420 - 427 and 582 - 596, the amino acid substitutions do not correlate with any major structural changes. Therefore, these particular sites may be type-common.

Comparison of the gB1 and gB2 regions which were outside the major antigenic sites, by secondary structure analysis, indicated that a few amino acid substitutions induced minor structural perturbations in the polypeptide. Most substitutions and deletions, however, did not produce a significant conformational change.

Intratypic variation in gB between strains of the same virus type is also seen from Figure 23. In the case of gB1, there are amino acid substitutions at position 279, 308, 315, and 550 between strain KOS and F. In addition, there is a single amino acid deletion in the gB signal peptide of strain F. Amino acid substitutions occur at positions 92, 396, 438, 568, 636 and 665 in gB2 between strains 333 and HG52. None

of these intratypic changes had a major predictable effect on the gB polypeptide in terms of antigenicity and second structure.

The position of the proposed hydrophobic membrane spanning region is obvious from the low scoring surface value of the residues between position 723 to 792 (Fig.22 and 23). As mentioned previously, there are few amino acid substitutions in this region. A number of high scoring surface regions occur in the gB domain between amino acids 793 to the C-terminus. These were not considered to be antigenic sites due to the probable internal location of this domain in the cytoplasm of the HSV infected cell and inside the viral envelope.

III Discussion.

Nucleotide sequencing of the gB2 gene revealed sequences in the promoter region which could function as regulatory sequences. One of these regions, CGCCCGCTTCATC (nucleotides 121-133), exhibited striking homology with the ICP4 DNA binding site reported by Faber and Wilcox (1986). A similar sequence is present in the promoter region of gD approximately 100 bp upstream from the transcription initiation site. ICP4 bound specifically to this sequence (Faber and Wilcox, 1986). These data suggest that ICP4 may interact directly with the promoter region of these genes in order to stimulate their transcription. Pizer et al., (1986) showed that the interaction between ICP4 and DNA can inhibit transcription of immedi-

itself, and stimulate early or late genes. This interaction involved the ICP4 consensus sequence (Muller, 1987). It is possible that the ability of ICP4 to both inhibit and stimulate gene expression may be unrelated to the actual DNA sequence in the binding site and perhaps more related to the influence of secondary proteins acting in concert with ICP4.

The gB2 sequence AGGAGGAA (nucleotides 112-119) differs only by one nucleotide from the sequence AGGAGGAG which was found 60 and 150 nucleotides upstream from the mRNA initiation site of gD and TK, respectively (McKnight, 1980; Watson et al.,1982). No function has been assigned to this sequence, however, its presence in the promoter regions of these genes implies that it may have some regulatory role.

Information gathered from comparison of the deduced primary sequence from four different HSV strains, secondary structure and surface profile analysis, was used to predict the location of the major antigenic sites in glycoprotein B. A number of theoretical considerations and empirical observations made this a valid approach.

The antigenicity of a protein is determined by restricted parts of the polypeptide. These discrete regions, known as antigenic determinants or epitopes, correspond to areas on the native protein that are specifically recognized by an appropriate antibody. These sites should therefore be accessible patches on the surface of the protein and are more mobile than the interior regions (Van Regenmortel 1986;

algorithm that combines the parameters of hydrophilicity, accessibility and mobility would most accurately predict the location of the antigenic regions on the gB polypeptide. Indeed, the program utilized in this study accurately predicted antigenic sites for several proteins whose antigenic site locations had already been determined by X-ray crystallography and immunological studies (Parker et al. 1986).

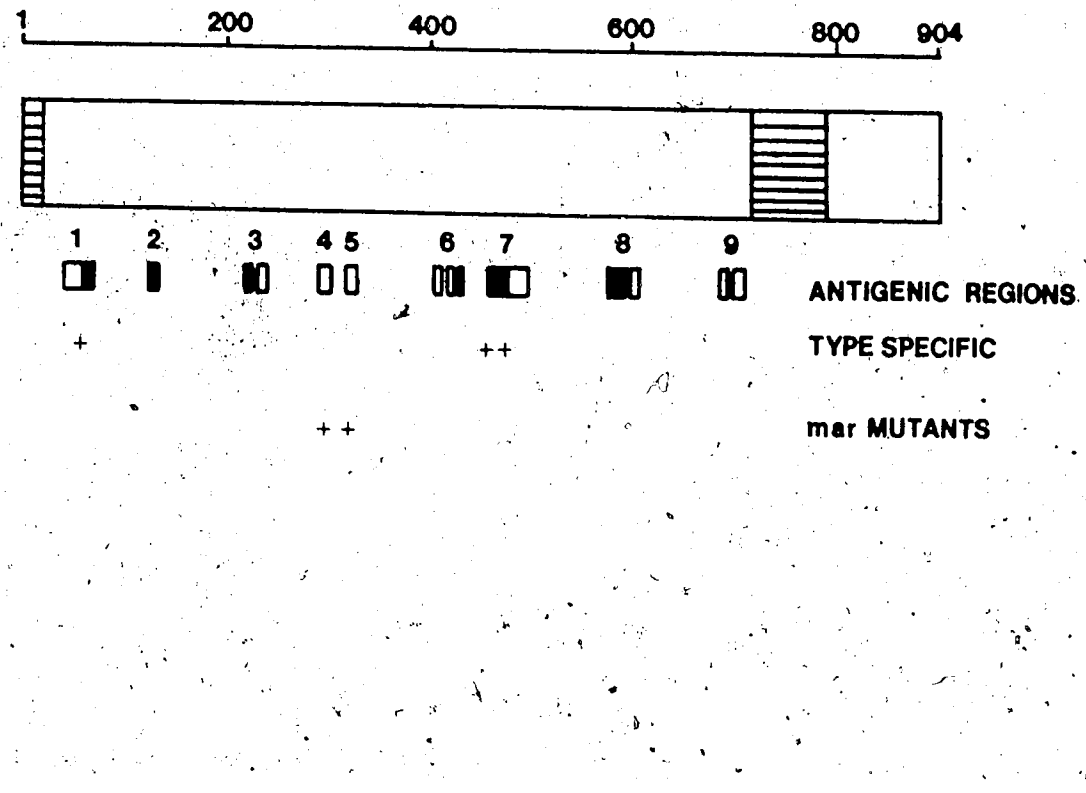
Viral glycoproteins are often expressed on the infected cell surface. Therefore, it could be expected that antigenic regions are especially subject to selective pressures provided by the host immune system. Continual propagation of the virus in the presence of antibody and components of the cell-mediated immune system result in amino acid substitutions and deletions which occur most frequently in epitopes that are highly antigenic. This phenomenon has been well documented for the antigenic determinants of neuraminidase and hemagglutinin for different Influenza virus strains (Gerhard et al. 1981; Webster et al. 1982). *In vitro* cultivation of HSV infected cells in the presence of monoclonal antibody has also demonstrated this process in vitro (Eisenberg et al. 1985a; Marlin et al. 1985). Certain regions of gB, predicted by high surface scores to be major antigenic sites, did in fact correlate with amino acid changes (substitutions and deletions) between gB1 and gB2. A similar observation was made for the predicted antigenic

immunodeficiency virus (HIV) isolates (Modrow et al., 1987). The sites on gB that exhibit amino acid diversity may also represent epitopes that are specific to HSV type 1 and 2. When secondary structural changes were taken into account the data suggested three possible site-specific epitopes between amino acids 59 - 68, 463 - 474 and 475 - 483.

The composite surface profile plot shown in Fig. 24 predicted at least nine major antigenic regions in the gB (333) polypeptide between positions 61 - 76, 129 - 141, 221 - 242, 299 - 309, 325 - 339, 409 - 430, 462 - 499, 582 - 611 and 689 - 712. Fig.26 shows a schematic diagram of the gB2 polypeptide and a summary of the antigenic regions. Hydrophilicity profiles for gB of both HSV-1 and HSV-2 are similar and therefore the relative antigenic site positions should correspond. Five maximal peaks were observed between amino acid positions 61 - 76, 129 - 141, 221 - 227, 462 - 478 and 582 - 596. It is interesting that Marlin et al. (1986), using a panel of 16 virus-neutralizing monoclonal antibodies to react with a series of gB1 mar (monoclonal antibody resistant) mutants, operationally defined at least five non-overlapping epitopes on this antigen.

Pellett et al. (1985c) performed DNA sequence analysis on three HSV-1 (F) mar mutants. Resistance of these strains to neutralization by monoclonal antibodies H126-5 and H233 correlated with amino acid substitutions at positions 273, 283, 285, and 305 in the mature gB1 (F) polypeptide. These

26. The location of the antigenic regions on the gB2(333) polypeptide. The antigenic regions, which were predicted from the composite surface profile, are indicated by the boxes (numbered 1 - 9) under a schematic diagram of the gB2 polypeptide. The shaded boxes correspond to the highest surface value peaks in the composite graph. The scale at the top of the figure indicates the amino acid position. The locations of the antigenic regions which correspond to the monoclonal antibody resistant (mar)-mutations determined by Pellett et al. (1985c), and possible site-specific epitopes, are indicated by a +. The signal and membrane spanning sequences are represented by the hatched regions on the polypeptide.


1
200
400
600
800
904
1
2
3
4 5
6
7
8
9
ANTIGENIC REGIONS
+
++
TYPE SPECIFIC
+ +
mar MUTANTS

of gB (333) shown in Fig. 23. All these residues lie within or immediately adjacent to two major antigenic regions predicted by the surface value plot.

The proposed antigenic site locations will need to be verified by further experimental work. Synthetic peptides, which correspond to the polypeptide regions exhibiting high surface value scores, could be used to immunize animal hosts. The anti-sera raised would then be tested for its ability to bind native gB antigen or protect animals against HSV infection. Such an approach has been successful in delineating epitopes on glycoprotein D (Dietzschold et al. 1984; Eisenberg et al. 1985b). The prediction of antigenic regions by the present method is limited to linear antigenic sites. Minor antigenic regions (less than 50% of the maximum on the surface value scale) were not considered here but may be significant in vivo.

The overall amino acid conservation in gB for the HSV strains examined is probably pertinent to the maintenance of a specific tertiary conformation and function. Ten cysteine residues are conserved between all four strains. At least some of these are likely to be involved in disulfide linkages which stabilize the protein structure. There are seven potential glycosylation sites on the surface domain of gB2 (333). Six of these sites are conserved in gB1. Also of interest is the conservation of alanine at position 550 for all strains except for KOS which has a valine residue. Secondary structure analysis of this region indicated that

valine at this position disrupts an alpha-helix and introduces a beta turn. The virions of strain KOS are known to enter cells more slowly than other HSV virus strains. Bzik et al. (1984a) showed, by DNA sequence analysis, that mutant strain tsB5 had a reversion of valine back to alanine. This reversion correlated with a more rapid entry phenotype of tsB5 virions into the host cell. One notable difference between gB1 and gB2 is the deletions of 8 amino acids in the signal peptide of gB2.

Comparison of the cytoplasmic domains of gB reveals extensive amino acid diversity between gB1 and gB2. However, only the changes between amino acids 874 to the C-terminus cause major conformational changes. Two completely conserved regions exist between position 801 - 815 and 835 - 863. Substitution of histidine in place of arginine at position 858 in gB of the tsB5 virus correlates with the formation of syncytial plaques in HEL and Vero cells infected by this mutant virus (Bzik et al. 1984a). The latter conserved region in the cytoplasmic tail of gB may interact with other membrane or intra-cellular proteins of the HSV-infected cell. A mutation in this region of gB could conceivably perturb this interaction in some way resulting in the syn phenotype.

Time did not permit a follow-up study, using synthetic peptides, to verify the predicted antigenic regions on gB2. The investigation reported in the following chapter was undertaken in order to study another glycoprotein, gD1, for

which several antigenic regions have already been defined (Eisenberg et al., 1985b).

Chapter 6

BIOCHEMICAL CHARACTERIZATION OF GLYCOPROTEIN gD1 SYNTHESIZED IN HSV-1 INFECTED VERO CELLS

I. Introduction

Although considerable information has been obtained from the DNA sequences and deduced amino acid sequences of HSV glycoproteins, the analysis remains indirect and largely predictive. Precise biochemical characterization of a polypeptide requires the development of purification protocol and direct chemical analysis of the purified protein.

This chapter describes the biochemical characterization of gD1 from HSV-1. The reasons for choosing gD were three-fold: 1) gD was the most potent inducer of HSV neutralizing antibodies (Norrild and Vestergaard, 1977), and provided strong protection in animals against lethal virus challenge (Long et al., 1984). Therefore, gD was a prime candidate for HSV subunit vaccine, 2) Only three sites on the gD polypeptide are modified by the attachment of N-linked oligosaccharide (Cohen et al., 1983), analysis of the carbohydrate groups on gD was therefore less complex relative to the other HSV glycoproteins. 3) A monoclonal antibody was available which could immunoprecipitate gD-specific polypeptides.

The pH stability and isoelectric point of native gD1 were determined. The molecular size and number of gD1-

specific polypeptides synthesized in HSV-infected Vero cells were determined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. This study also evaluated methods such as ion-exchange chromatography, ammonium sulfate precipitation, lectin affinity columns and gel filtration as a means to purify gD1 from HSV-1 infected Vero cell extracts. This led to the purification of precursor and mature forms of gD1 by exploiting the unique binding properties of their carbohydrate groups to specific lectin affinity columns.

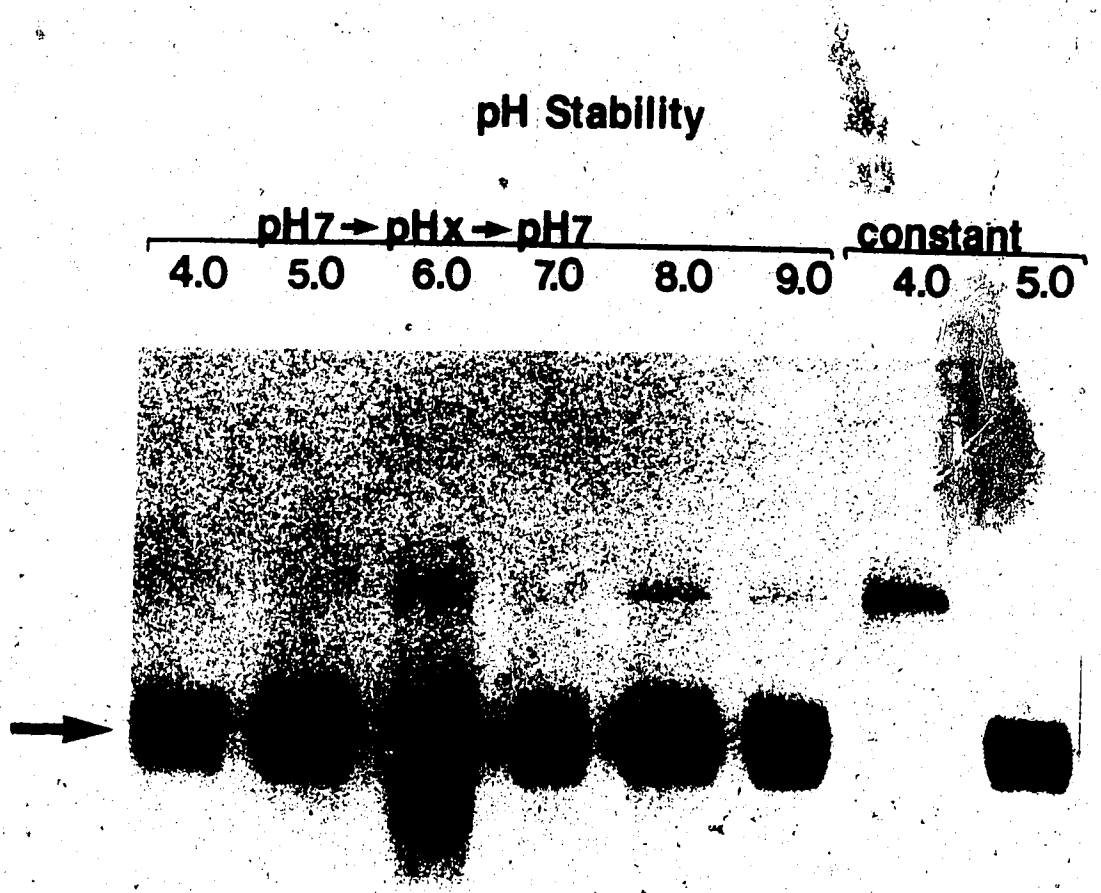
II. Results

A. pH Stability and isoelectric point of gD1

Determination of pH stability and isoelectric point were pre-requisites to the developement of a purification scheme for the gD polypeptide. Figure 27 shows that gD maintained its reactivity with gD specific monoclonal antibody 18βB3 at pH values ranging from 5.0 to 9.0, indicating that the conformation of the epitope responsible for binding to the monoclonal antibody was not markedly altered within this pH range. The binding affinity of gD1 was reduced by short exposure to pH 4.0 and gD1 appeared to disintegrate at prolonged exposure at this pH value.

The iso-electric point of gD1 was determined as a pre-requisite to the development of a purification protocol based on charge. In order to preserve the native polypeptide conformation of gD1, the isoelectric point was determined by flat-bed isoelectric focusing in the absence of urea. Figure 28 shows that gD1 migrated to a single position in the

Fig.27.pH stability of gD1. The pH of ^{35}S-labeled HSV-1 infected-cell extract was adjusted to acidic or alkaline conditions (pH 4.0-9.0) for 30 min. and then titrated back to pH7.0. The long term effect of pH 4.0 and 5.0 on gD1 was determined by maintaining the extract at these pH values throughout the experiment (constant). Glycoprotein gD1 was immunopre-cipitated by monoclonal antibody 18ßB3 and subjected to electrophoresis in 7.5-15% gradient acrylamide gel.


pH Stability
pH7 → pHx → pH7
constant
4.0
5.0
6.0
7.0
8.0
9.0
4.0
5.0

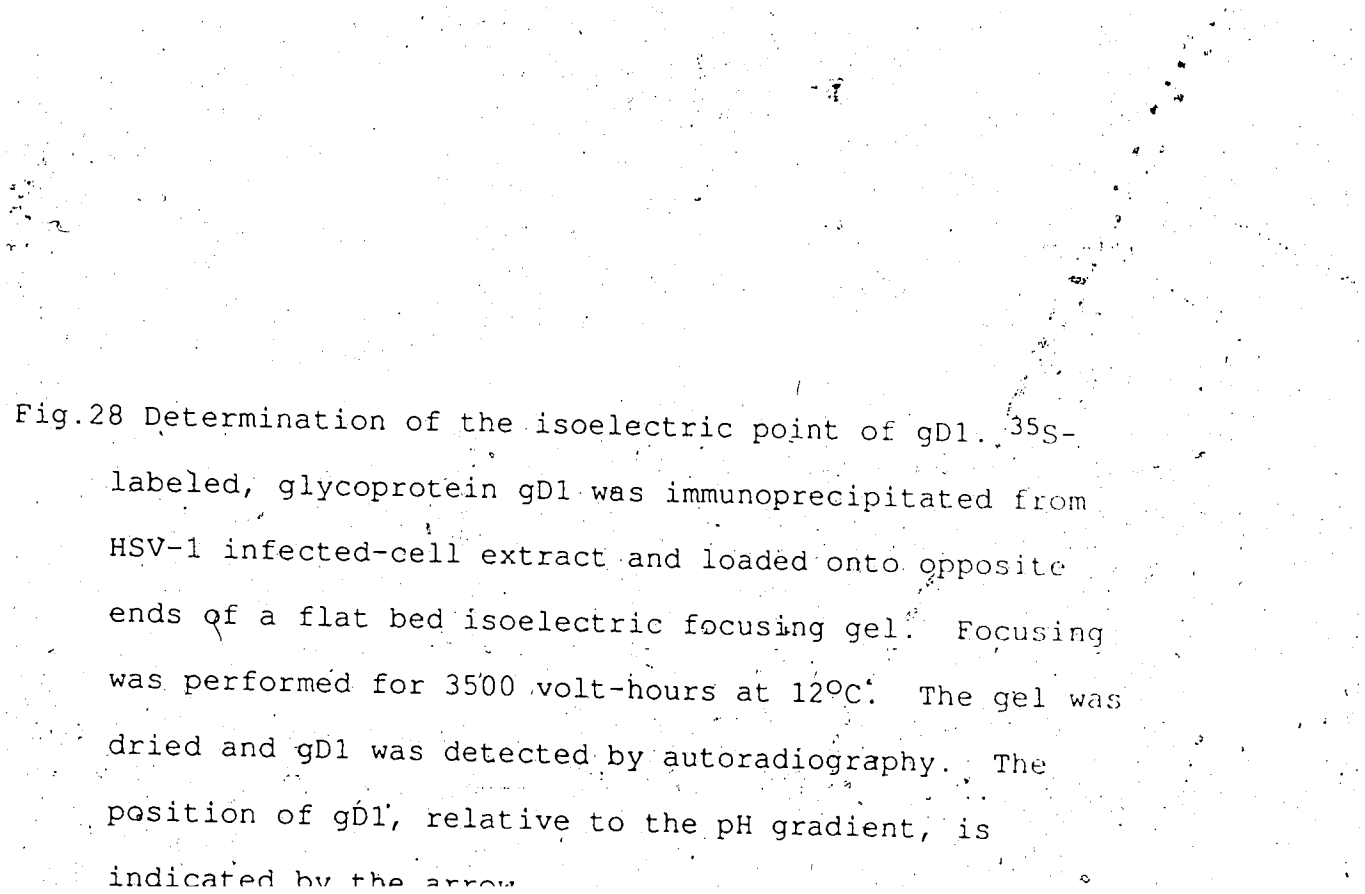
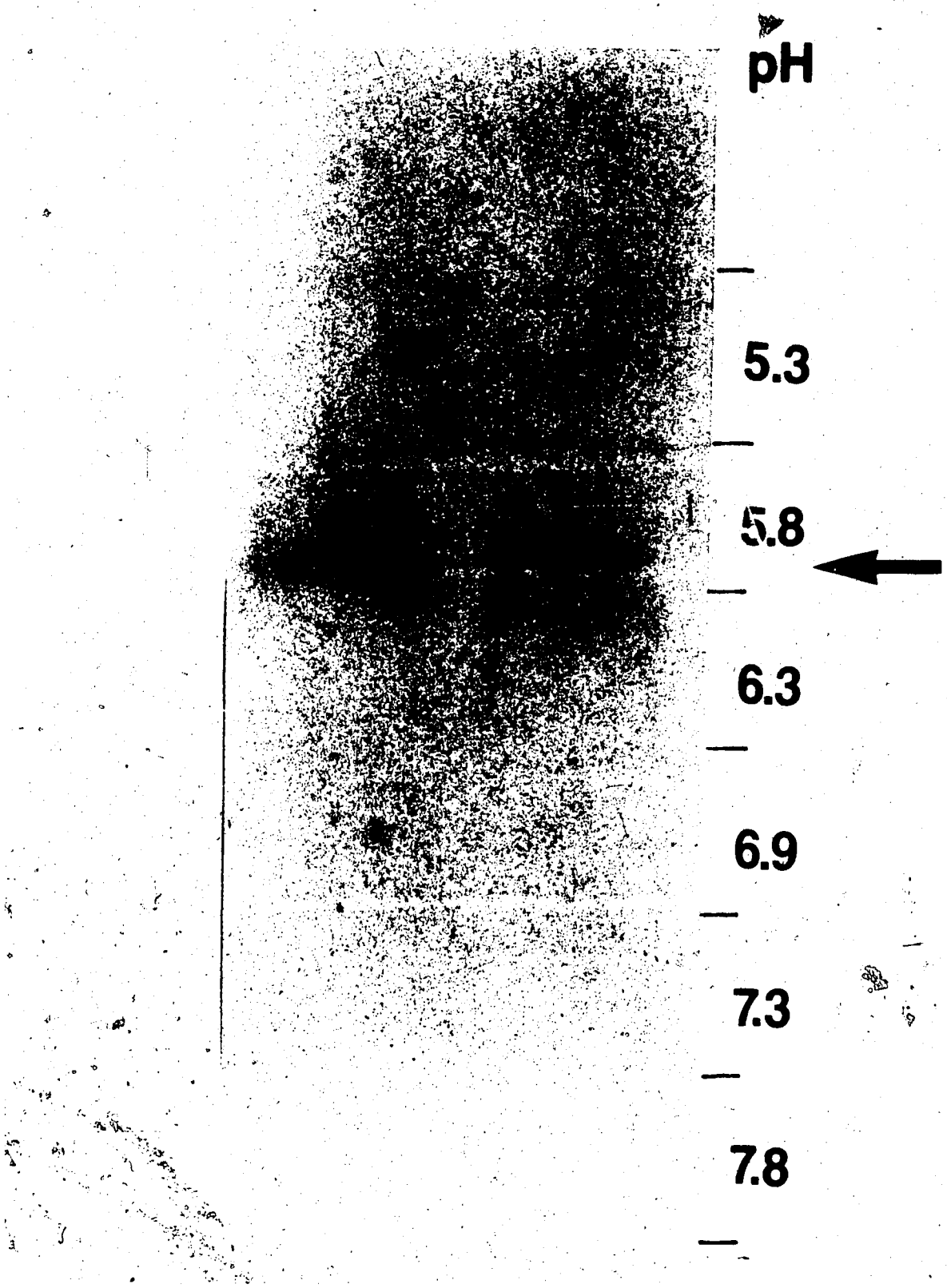


Fig.28 Determination of the isoelectric point of gD1. ^{35}S-labeled, glycoprotein gD1 was immunoprecipitated from HSV-1 infected-cell extract and loaded onto opposite ends of a flat bed isoelectric focusing gel. Focusing was performed for 3500 volt-hours at 12°C. The gel was dried and gD1 was detected by autoradiography. The position of gD1, relative to the pH gradient, is indicated by the arrow.


pH
5.3
5.8
6.3
6.9
7.3
7.8

focusing gel which corresponded to pH 5.9 - 6.0, irrespective of the sample loading location.

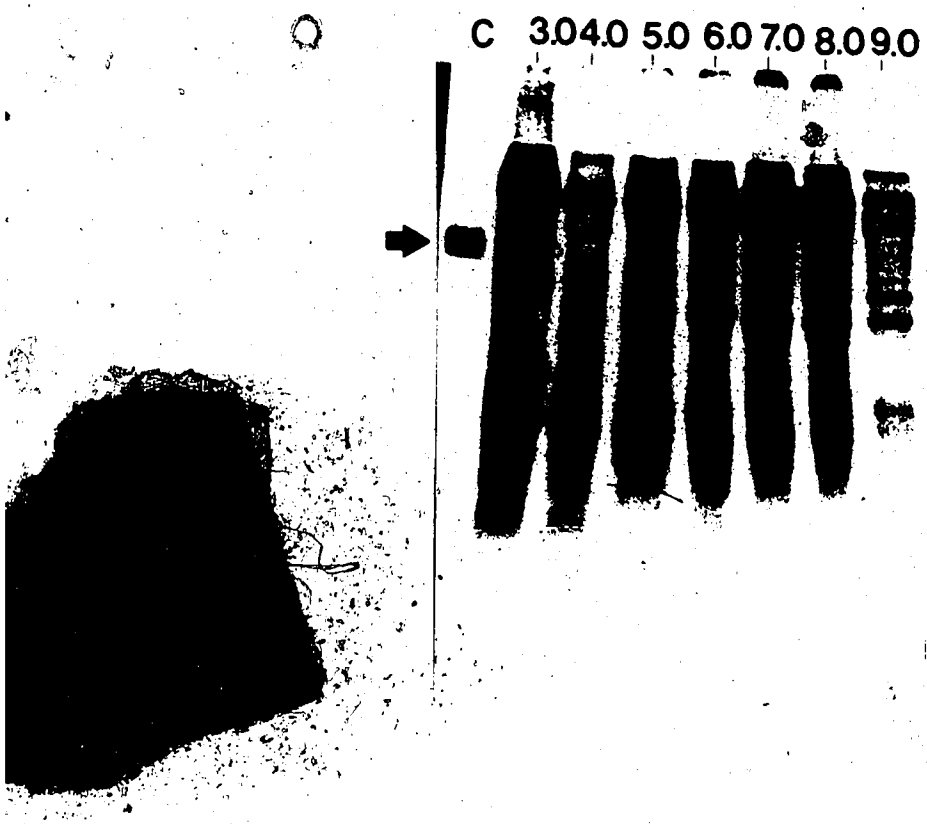
B. Ion exchange chromatography

On the basis of its isoelectric point, gD1 was predicted to be negatively charged above pH 6.0 and therefore should bind to positively charged matrices. HSV-1 infected cell extract was passed through DEAE-Sephadex columns at various pH values ranging from 3.0 to 9.0. Proteins bound to the columns were eluted by 1M sodium chloride. At pH values greater than 6.0, a polypeptide having a molecular size characteristic of gD1 (59K) was present in the eluted fraction (Fig. 29A). Below pH 6.0 this polypeptide could not be detected. To confirm that gD1 did in fact bind to DEAE-Sephadex, the fractions eluted at pH 5.0, 6.0 and 7.0 were immunoprecipitated with 18βB3 monoclonal (Figure 29B). The amount of gD1 bound to DEAE-Sephadex was greatest at pH 7.0, whereas only a minimal amount bound at pH 5.0. These results were consistent with the isoelectric focusing data and suggested that gD1 became negatively charged as the pH increased from 5.0 to 7.0.

A pH value of 6.5 was chosen for purification of gD1 by DEAE- Sepharose chromatography since the polypeptide would be negatively charged at this pH value. HSV-1 infected cell extract was passed through a 10 ml DEAE-Sepharose column and the polypeptides bound to the column were eluted by a continuous sodium chloride gradient (Figure 30A). Most of the bound polypeptides were eluted between 0.2 - 0.47 M

Fig.29. Affinity of gD1 for DEAE-Sephadex at pH values ranging from 3.0-9.0. ^{35}S-labeled HSV-1 infected-cell extract was passed through a DEAE-Sephadex column and the bound proteins were eluted by 1M NaCl. The flow through and eluted protein fractions were subjected to electrophoresis on 7.5% - 15% gradient gel. Panel A shows the proteins which had affinity for DEAE-Sephadex at pH values ranging from 3.0 to 9.0. Lane C = gD1 immunoprecipitated from HSV-1 infected cell extract. The position of gD1 is indicated by the arrow. Panel B shows the polypeptides present in the flow through (F) and elution (E) fractions at pH 5.0, 6.0 and 7.0. The presence of gD1 in the elution fraction was confirmed by immunoprecipitation (I).

A.



B.

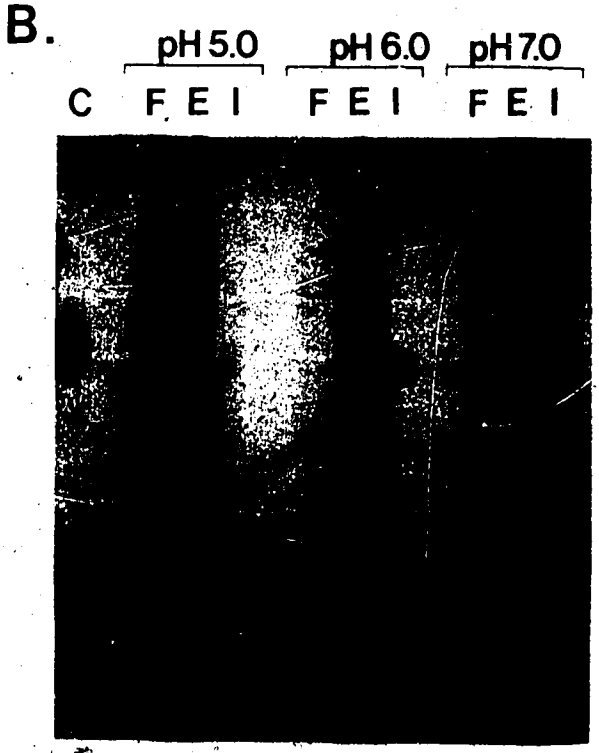
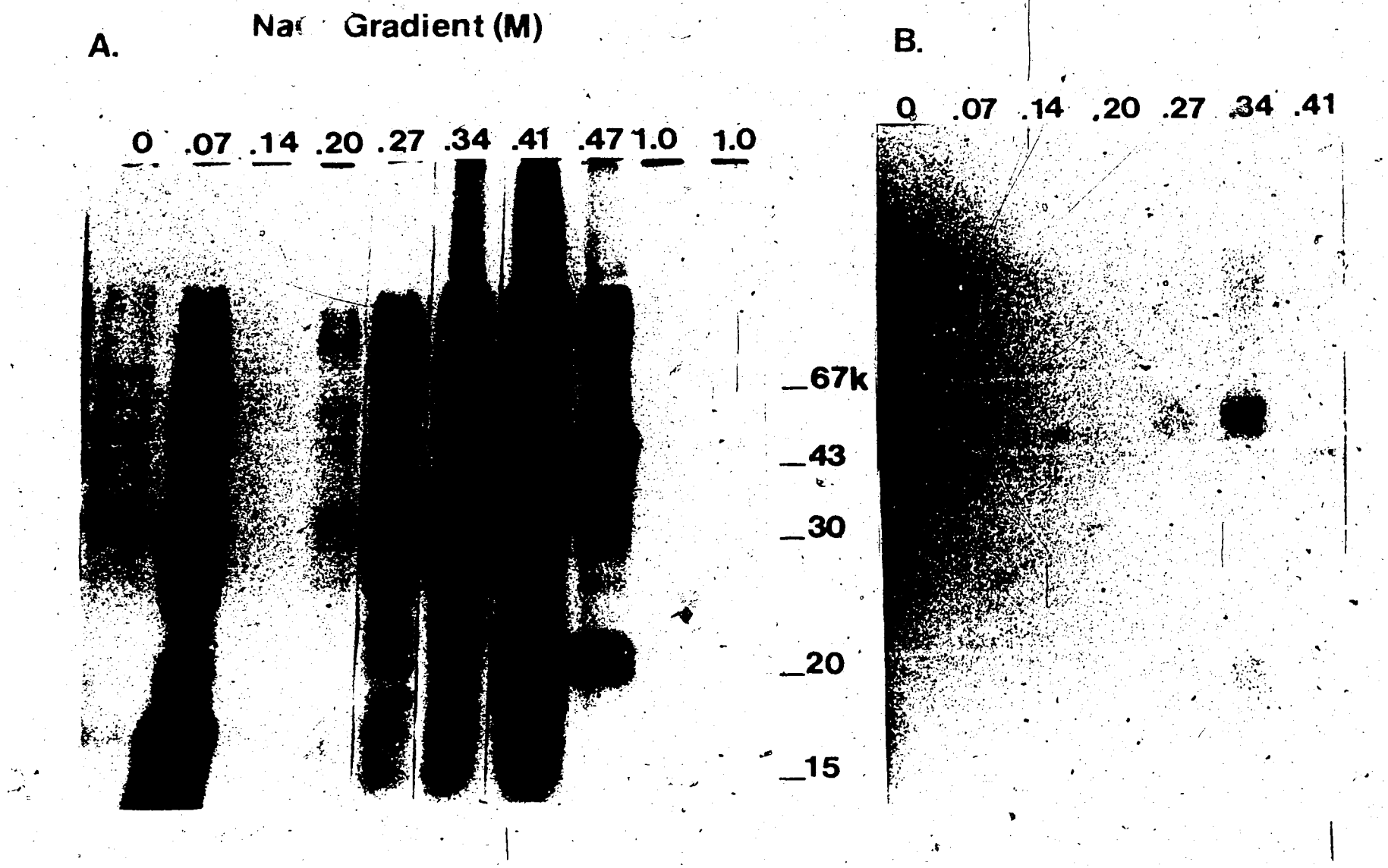


Fig. 30. DEAE-Sepharose chromatography of ^{35}S-labeled HSV-1 infected-cell extract proteins at pH 6.5 The extract was loaded onto the DEAE-Sepharose column. Proteins which bound to the column were eluted by a linear NaCl gradient. A) The proteins present in the eluted fractions. The numbers above the gel indicate the eluting molarity of NaCl for each fraction. The proteins were detected by autoradiography. B) Immunopre- cipitation of gD1 from the fractions using monoclonal antibody 18ßB3.


A.
Na Gradient (M)
0
.07
.14
.20
.27
.34
.41
.47
1.0
1.0
67k
43
30
20
15
B.
0
.07
.14
.20
.27
.34
.41

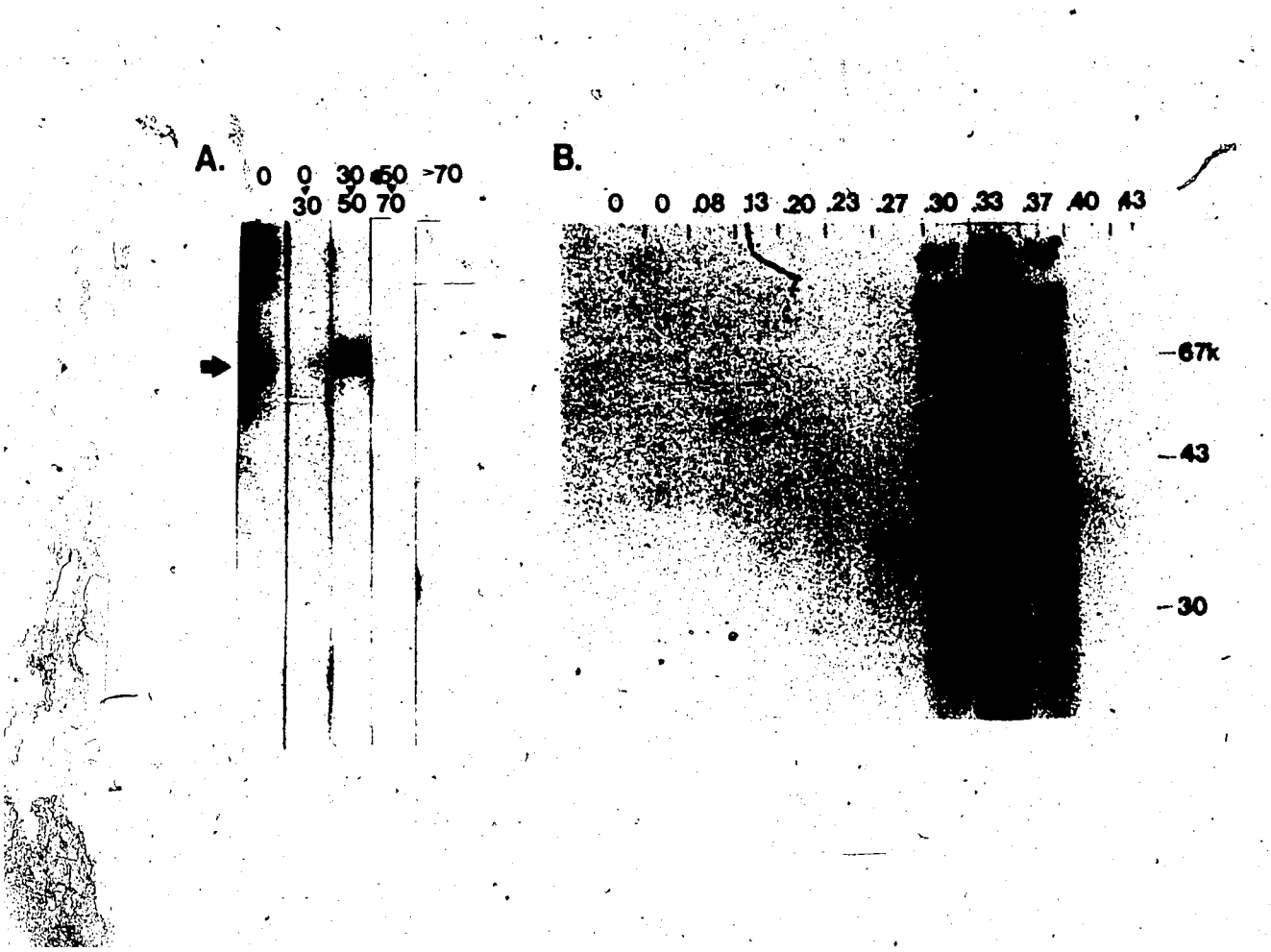
sodium chloride. Immunoprecipitation of the fractions showed that the majority of gD1 was eluted between 0.34 - 0.41 M sodium chloride (Figure 30B). Two gD-specific polypeptide bands were resolved by SDS-polyacrylamide gel electrophoresis indicating that precursor and mature forms of gD were eluted in these fractions.

C. Ammonium sulfate precipitation

The proteins in HSV-1 infected-cell extract were precipitated by varying concentrations of ammonium sulfate at pH 6.0. Figure 31A shows that the majority of gD1 was precipitated between 30-50% ammonium sulfate. Centrifugation of the extract at pH 6.0 precipitated some gD1 in the absence of ammonium sulfate, suggesting that some form of gD may be associated with membrane or particulate matter.

The extent of gD1 purification that could be achieved by a combination of ammonium sulfate precipitation and DEAE-ion exchange chromatography was examined. The fraction of proteins, precipitated by 30-50% ammonium sulfate, was loaded onto a DEAE-Sepharose column and the bound proteins were eluted by a linear NaCl gradient (Figure 31B). SDS-polyacrylamide gel electrophoresis of the collected fractions indicated that a polypeptide having the same molecular size as gD1 (59K) was eluted between 0.33 - 0.37 M sodium chloride. Fig. 30A shows that, in addition to gD1, a large number of other proteins were eluted from the DEAE Sepharose column at 0.34 - 0.41 M NaCl. Comparison between Fig. 30A and Fig. 31B shows that partial purification of the gD1

Fig.31. Ammonium sulfate precipitation and DEAE-Sepharose chromatography of HSV-1 infected-cell extract proteins. A) Immunoprecipitation of gD1 from the proteins which were precipitated in the presence of 0%, 0-30%, 30-50%, 50-70% ammonium sulphate and from the residual proteins (>70%). The proteins were precipitated by adding increasing amounts of ammonium sulphate to the extract at pH6.0. After immunoprecipitation, using 18ßB3 monoclonal antibody, the samples were analyzed by electrophoresis in 7.5-15% gradient polyacrylamide gel and autoradiography. B) DEAE-Sepharose chromatography of the proteins precipitated by 30 - 50% ammonium sulfate. The precipitated proteins were loaded onto a DEAE-Sepharose column and eluted by a gradient of NaCl. The molarity of NaCl is indicated above the figure. Each fraction was analysed by electrophoresis in 7.5-15% polyacrylamide gel.


A.
0 0 30 50 >70
30 50 70
B.
0 0 .08 .13 .20 .23 .27 .30 .33 .37 .40 .43
67k
43
30

polypeptide was achieved by combining ammonium sulfate precipitation with ion exchange chromatography.

D. Lectin affinity columns

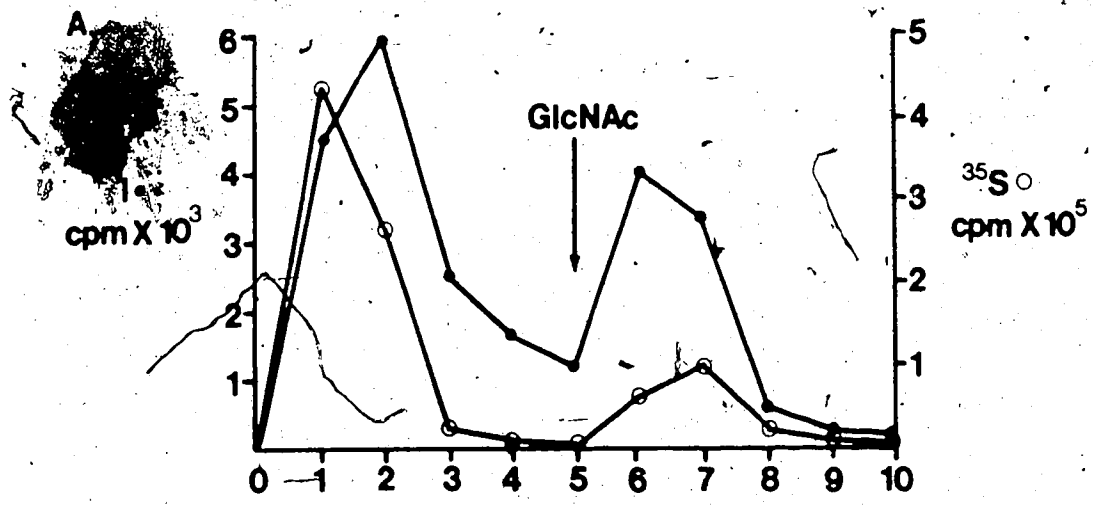
Lectin affinity columns have proven useful for partial purification of HSV glycoproteins gB (Pachl et al., 1987), gC1 (Olofsson et al., 1981b) and gG2 (Olofsson et al., 1986). Therefore several lectins were studied for their ability to bind selectively to gD1.

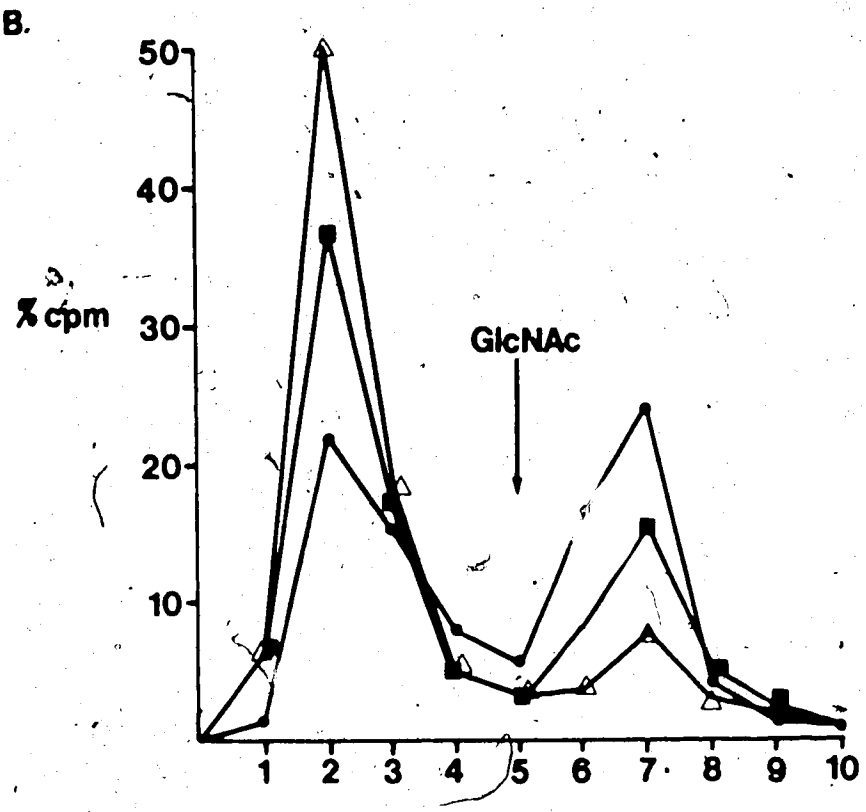
Mild acid conditions or treatment with neuraminidase reduced the molecular size of mature gD1, suggesting that sialic acid was present in the oligosaccharides attached to the gD polypeptide (Cohen et al., 1980; Smiley and Friedman, 1985). Therefore, Wheat germ agglutinin (WGA), a lectin with affinity for terminal sialic acid (NeuAc) and N-acetyl-glucosamin (GlcNAc) residues, was first tested for its ability to bind gD1. Uninfected and HSV-1 infected cell extracts were passed through WGA columns and the bound glycoproteins were eluted by 2.5% GlcNAc. Figure 32 shows that glycoprotein gD1 did not bind to WGA. However, another glycoprotein (approximately 100-120K), present in HSV-1 infected but not in uninfected cell extract, was able to bind. One possible reason for the non-binding of gD1 to WGA could be that the lectin had lost its specificity for sialic acid. To test for this possibility, ^{125}I-labeled fetuin, a glycoprotein which is known to bind to WGA, was included in the HSV-1 infected cell extract, prior to loading onto the column. Figure 33A shows that approximately 45% of labeled

Fig 32. Binding of HSV-1 infected- and uninfected-cell extract proteins to Wheat germ agglutinin (WGA). Infected (panel A) and uninfected (panel B) cell extracts were passed through a WGA column. The proteins bound to WGA were eluted with 0.2M N-acetyl-D-glucosamine (GlcNAc). The pooled flowthrough (FT) fractions (1-3, 4-6, 7-10) and eluted (E) fractions (11-15) were subjected to electrophoresis in 7.5% - 15% polyacrylamide gel[2].


A.
FT
E
1-3
4-6
7-10
11-15
67K
43
30
20
15
B.
FT
E
1-3
4-6
7-10
11-15

Fig.33. Behaviour of fetuin and asialofetuin on Wheat germ agglutinin agarose (WGA) affinity columns. A) Affinity of ^{125}I-fetuin and ^{35}S-protein affinity (from HSV-1 infected cells) for WGA. ^{125}I-fetuin was added to ^{35}S-labeled HSV-1 infected-cell extract and the mixture was passed through a WGA column. The bound proteins were eluted by 0.2M GlcNAc. The number of counts per min (cpm) of ^{125}I-fetuin (●) and ^{35}S-protein (o) was determined for each fraction. The first fraction following the addition of GlcNAc is indicated by the arrow. B) ^{125}I-asialofetuin affinity for WGA after treatment with sialyltransferase. ^{125}I-fetuin or, ^{125}I-asialofetuin was passed through a WGA column. The protein which bound to the column was eluted by 0.2M GlcNAc. The percentage of the total cpm loaded on the column in each fraction was determined. ^{125}I-fetuin (●), ^{125}I-asialofetuin after incubation with α2-6 sialyltransferase and CMP-NeuAc(■), ^{125}I-asialofetuin after incubation in the absence of α2-6 sialyltransferase (Δ).

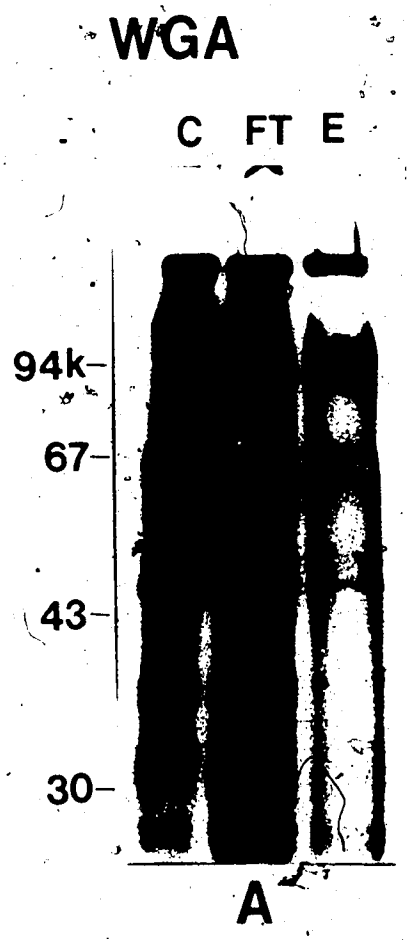

A.
cpm X 10³
GlcNAc
³⁵S ○
cpm X 10⁵
0 1 2 3 4 5 6 7 8 9 10


B.
% cpm
GlcNAc
1 2 3 4 5 6 7 8 9 10

fetuin bound to WGA and could be eluted by GlcNAc. Additional evidence for the specificity of the WGA column for sialic acid groups on fetuin was shown by the fact that treatment of ^{125}I-asialofetuin with α2-6 sialyltransferase in the presence of donor CMP-NeuAc restored at least some of its binding activity (Figure 33B). Approximately 20% of the ^{35}S-labeled polypeptides in the infected cell extract had affinity for WGA (Figure 33A) and was due to the binding of the 100-120K protein species (Figure 34A). Immunoprecipitation with 18βB3 monoclonal confirmed that gD1 was absent from the elution fractions and was only present in the flow-through fractions (Figure 34B). These results suggested that the carbohydrate moieties on gD1 did not contain sialic and/or GlcNAc residues which were available for binding to WGA.

Glycoprotein gD1, and other glycoproteins in the higher molecular weight range, were able to bind to lentil lectin (a lectin with affinity for mannosyl residues) and could be eluted by 0.2 M Methyl α-D-mannoside (Meα-D-Man) (Figure 35). Immunoprecipitation of the proteins present in the pooled flow-through and elution fractions revealed that only the precursor form(s) of gD1 (ie. the form(s) that had a higher migration rate than the mature form) was able to bind to lentil lectin (Figure 36). Interestingly, an additional high molecular weight species was detected in the elution fraction. This species had a molecular size which was

Fig.34. Immunoprecipitation of gD1 from the flow-through and elution fractions from wheat germ agglutinin agarose. A) The ^{35}S-proteins in the flow through (FT) and elution (E) fractions of the experiment shown in fig.33A were subjected to electrophoresis in 10% polyacrylamide gel and detected by autoradiography. Lane C = proteins present in the unfractionated infected-cell extract. B) Immunoprecipitation of the unfractionated cell extract (CI), flow through (FT) and elution (E) fractions with 18ßB3 monoclonal antibody.


WGA
C FT E
94k
67
43
30
A

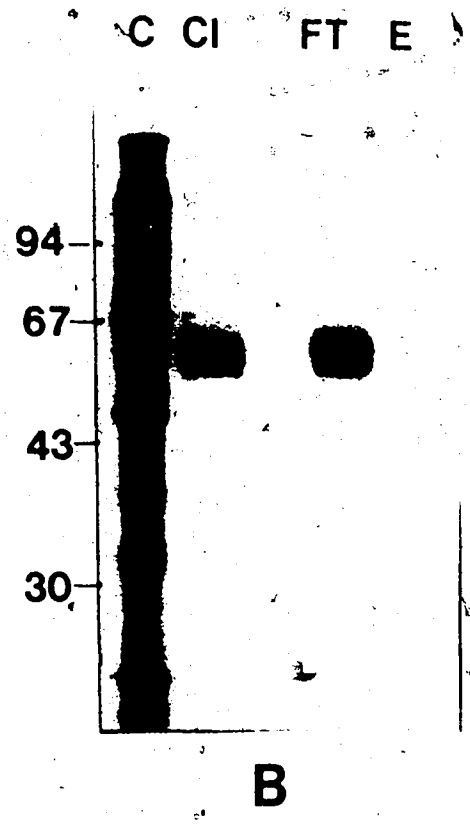

C CI FT E
94
67
43
30
B

Fig.35. Binding of HSV-1 infected-cell extract proteins to lentil lectin. The extract was passed through a lentil lectin column and the bound proteins were eluted by 0.2M methyl α-D-mannoside (Me α-D-Man). The flow-through (FT) fractions (1+2, and 6) and elution (E) fractions (7, 8 and 9) were subjected to electrophoresis in a 10% polyacrylamide gel. Lane CL= unfractionated HSV-1 infected-cell extract.

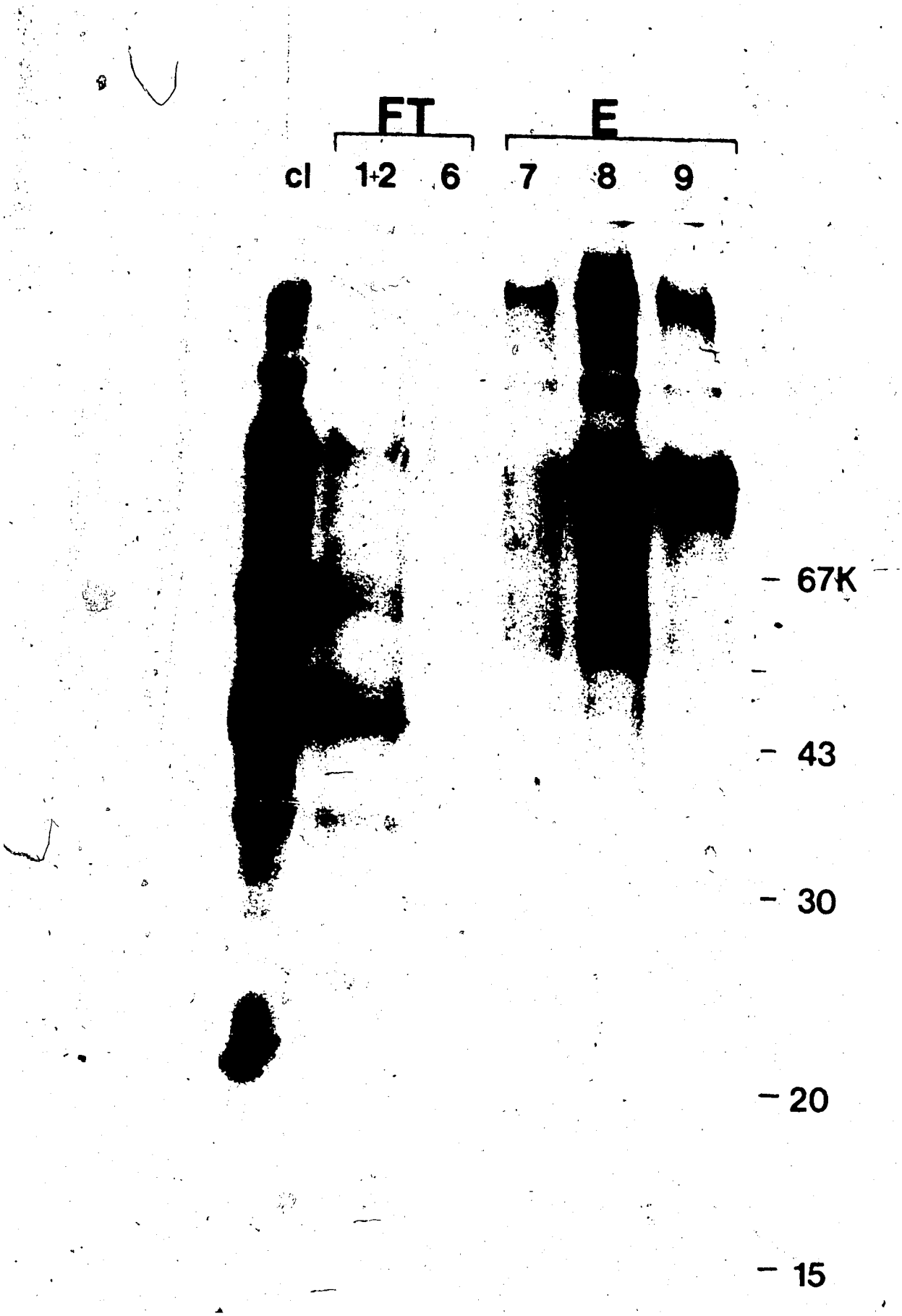
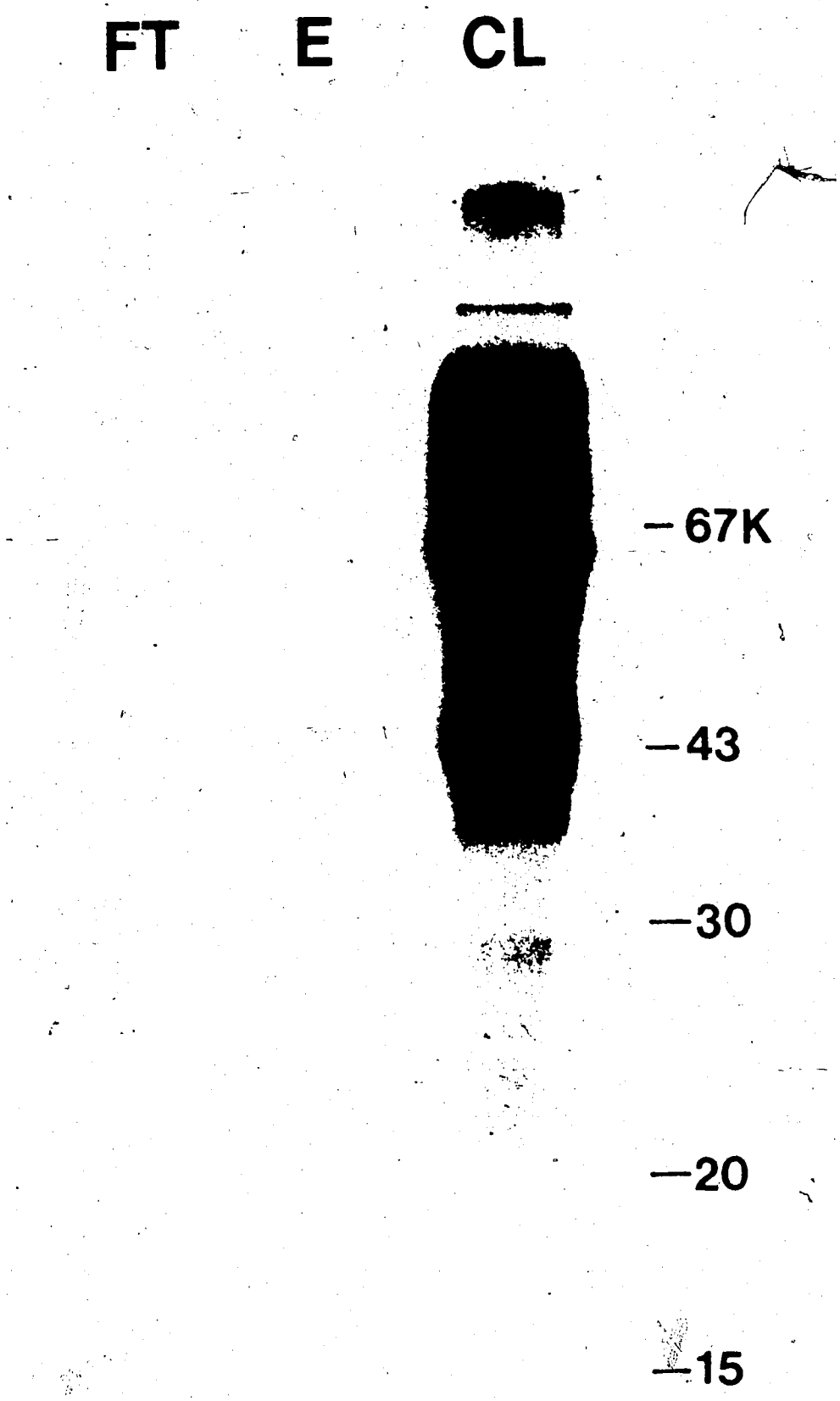

FT
E
cl
1+2
6
7
8
9
67K
43
30
20
15

Fig.36. Immunoprecipitation of gD1 from the flow-through and elution fractions from lentil lectin. The flow through (FT) and elution ( ) fractions were immunoprecipitated with 18ßB3 monoclonal antibody and subjected to electrophoresis in a 10% polyacrylamide gel. Lane CL=unfractionated HSV-1 infected cell extract.


FT
E
CL
67K
43
30
20
15

approximately twice that of gD1 and may represent an oligomeric form of this glycoprotein.

Glycoprotein gD1 and other infected cell glycoproteins were able to bind to Castor bean-120 (CB-120), a lectin with high affinity for D-galactosyl residues and a lower affinity for N-acetyl galactosamine residues. The bound glycoproteins could be eluted by 2.5% D-galactose but not by N-acetyl galactosamine (Figure 37), indicating that the binding specificity was for oligosaccharide chains containing galactose. The flow-through and eluted proteins from CB-120 lectin column were immunoprecipitated with 18βB3 and subjected to electrophoresis in 10% polyacrylamide gels for 8 hours to improve the resolution of gD1 specific polypeptides (Figure 38). At least 3 gD1-specific polypeptides were resolved. The two faster migrating gD1 polypeptides, detected in the flow-through fraction (FT), likely represent immature forms of gD1 which lack terminal galactose. The largest polypeptide (probably mature gD1) was the only form detected in the elution fraction (E). A similar electrophoretic analysis was applied to the gD1 polypeptide species present in the flow-through and elution fractions from lentil lectin column (Figure 38). The majority of the gD1 precursors were bound to the column whereas the mature form(s) was present in both the flow-through and elution fractions. The mature gD1 polypeptide in the flow-through fraction exhibited a slightly slower migration rate (relative to the eluted species, suggesting that it may be more

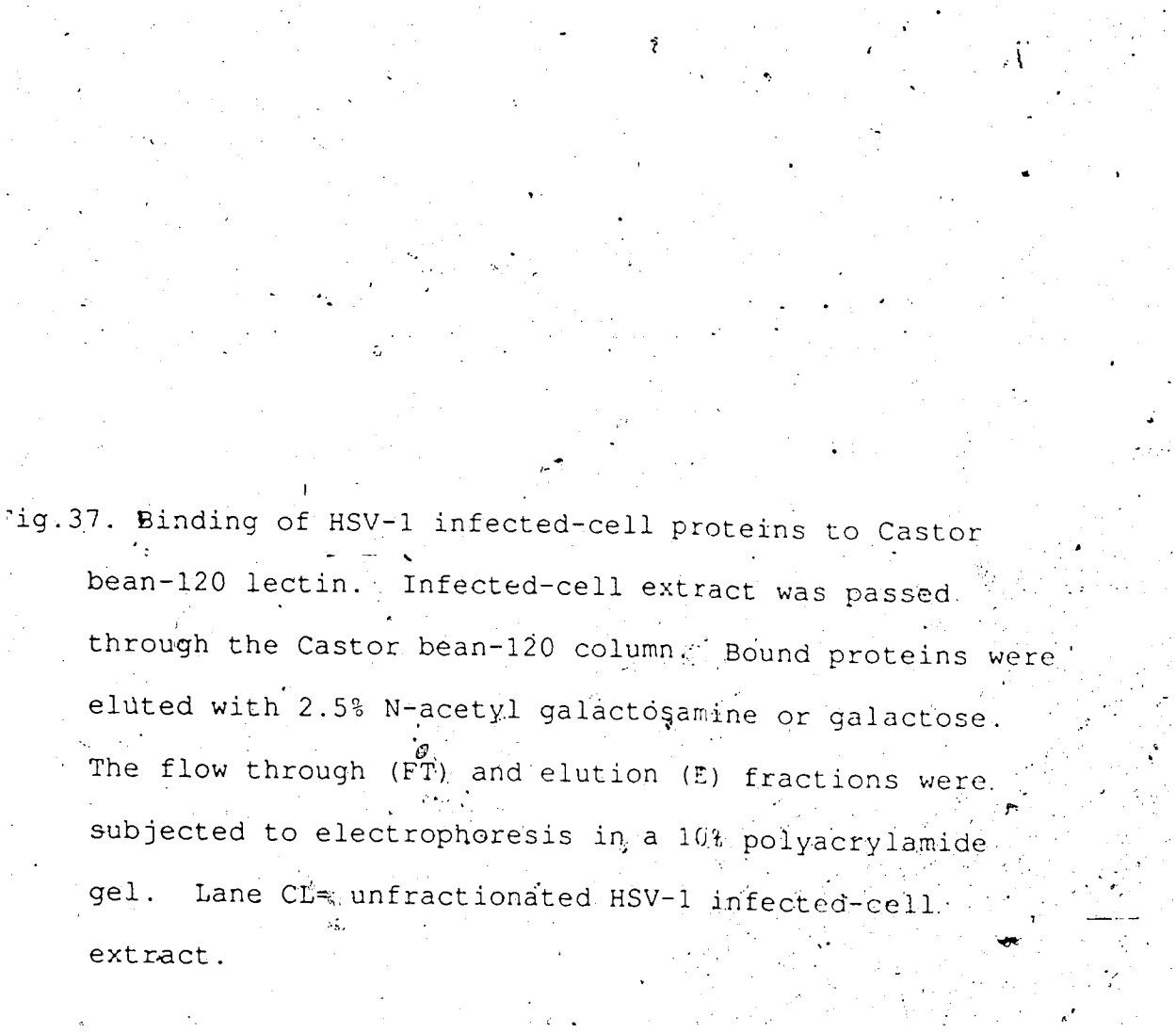


Fig.37. Binding of HSV-1 infected-cell proteins to Castor bean-120 lectin. Infected-cell extract was passed through the Castor bean-120 column. Bound proteins were eluted with 2.5% N-acetyl galactosamine or galactose. The flow through (FT) and elution (E) fractions were subjected to electrophoresis in a 10% polyacrylamide gel. Lane CL= unfractionated HSV-1 infected-cell extract.

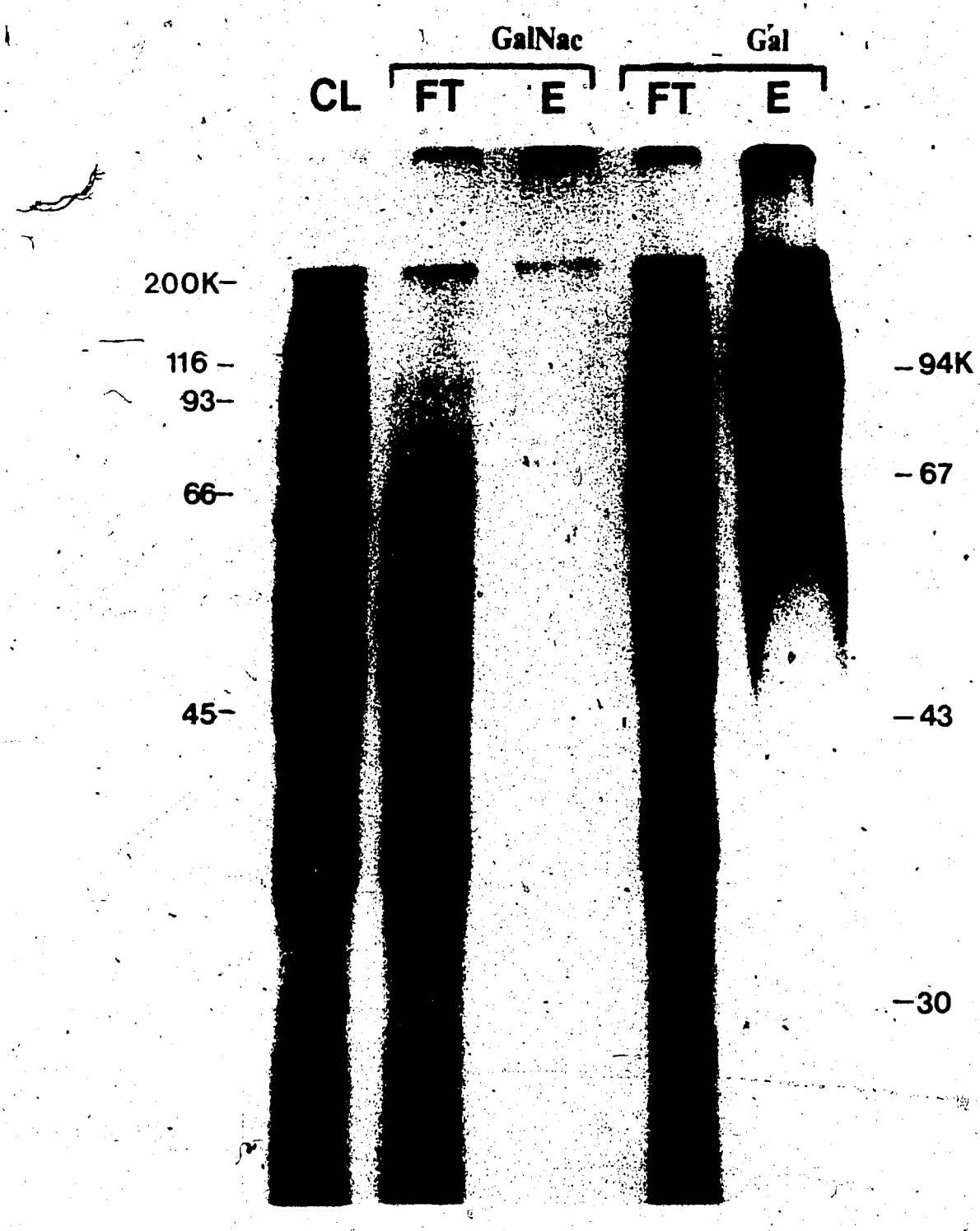
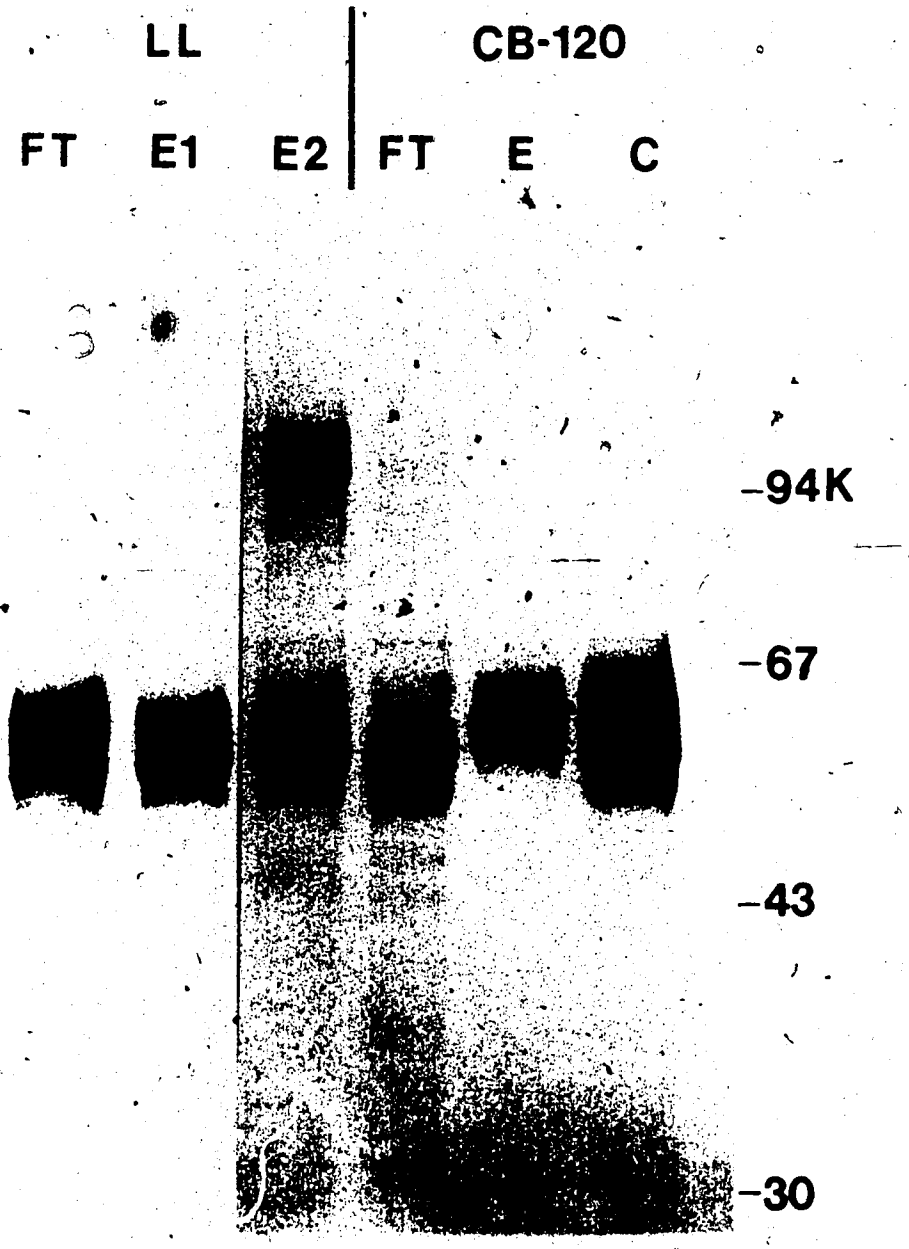

GalNac
Gal
CL
FT
E
FT
E
200K–
116 –
93–
66–
45–
–94K
–67
–43
–30

Fig.38. Immunoprecipitation of gD-1 from the flow-through and elution fractions from Lentil and Castor bean-120 lectins. Infected- cell extracts were passed through lentil lectin (LL) or Castor bean-120 (CB-120) columns and bound proteins were eluted by 0.2M Meα-D-Man and 2.5% galactose, respectively. The flow- through (FT), and elution (E) fractions were immunoprecipitated with 18ßB3 monoclonal and electrophoresed in 10% polyacrylamide gel for 8 hrs at 150 Volts. Lanes E1 and E2 show the gD specific polypeptides present in the lentil lectin elution fractions from two different experiments. Lane C = immunoprecipitation of unfractionated HSV-I infected-cell extract with 18ßB3 monoclonal antibody.

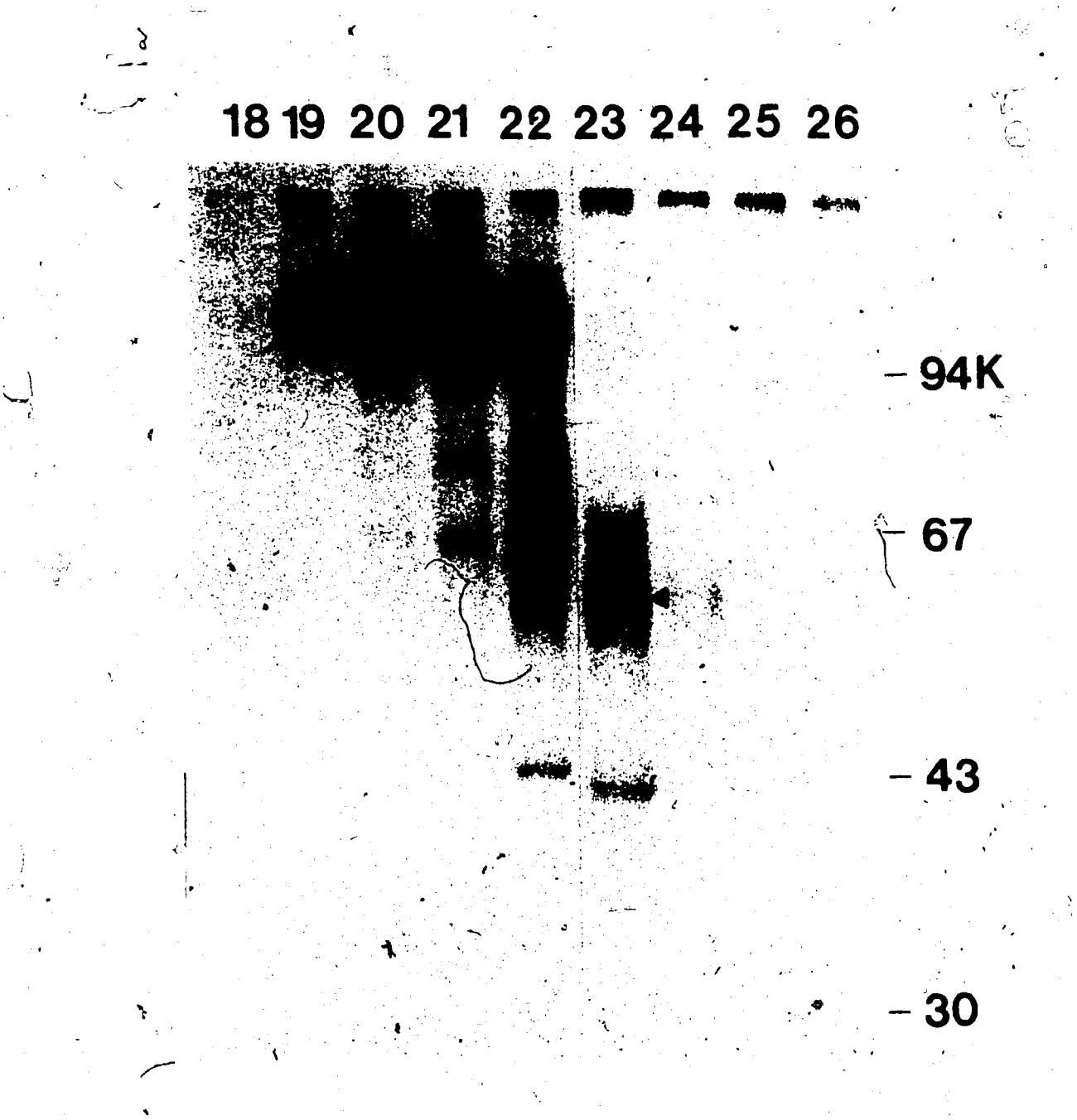

LL
CB-120
FT
E1
E2
FT
E
C
-94K
-67
-43
-30

extensively glycosylated. A high molecular weight protein species was also detected in the elution fraction (E2). This species reacted with gD-specific monoclonal antibody, had a molecular size of approximately 120-125K, and could be resolved into 3 to 4 distinct bands. This larger form of gD1 was repeatedly found in the elution fractions from lentil lectin columns (see also Figure 36) but was not always present in the same relative amounts (compare experiments E1 and E2 in figure 38).

E. Gel Filtration

Glycoprotein gD1 was further purified from the proteins which bound to CB-120 lectin by gel filtration (Figure 39). HSV-1 infected-cell extract was passed through a CB-120 lectin column and the bound proteins were eluted by 2.5% GalNAc. The elution fraction was passed through a Superose 12 gel filtration column. The majority of gD1 was present in fraction 23 and was separated from the higher molecular weight proteins (>67K) present in fractions 19 to 21. Two minor protein bands, approximately 65-67K and 42-43K, were also detected in fractions 22 and 23 by autoradiography. These proteins may be cellular glycoproteins or proteins which bound non-specifically to CB-120 lectin. Their estimated molecular sizes do not correlate with any of the known glycoproteins specified by HSV. The migration rates of these two proteins were distorted slightly between fractions 22 and 23 due to the presence of unlabeled molecular weight standards [BSA(67K) in fraction 22 and ovalbumin (43K) in

Fig.39. Gel filtration of the HSV-1 infected-cell proteins which bound to Castor bean-120 lectin. ^{35}S-labeled HSV-1 infected-cell lysate was passed through a CB-120 lectin column and the bound proteins were eluted with 2.5% GalNAc. The eluted protein sample was loaded onto a Superose 12 gel filtration column which had been equilibriated with 0.05M sodium phosphate (pH7.0), 0.15M NaCl, 0.2% SDS. The proteins were eluted at a flow rate of 0.2 ml/min. and 0.5 ml fractions were collected. The fractions were anlysed by 10% polyacrylamide gel electrophoresis and autoradiography. The fraction numbers are shown above the autoradiogram. The position of gD1 is indicated by the arrow head.


18 19 20 21 22 23 24 25 26
94K
67
43
30

fraction 23] which were added to the sample prior to loading on the column.

III. Discussion

SDS-polyacrylamide gel electrophoresis showed that mature glycoprotein gD1, synthesized in HSV-1 infected Vero cells, had a molecular size of approximately 58-60K. At least 3 to 4 distinct gD1-specific polypeptides, ranging in size from 52 to 60K, could be resolved by polyacrylamide gel electrophoresis. These polypeptides likely represent precursor-and mature-glycosylated forms of the gD1 molecule. Glycoprotein gD1 was shown to be a relatively stable polypeptide which maintained a constant tertiary conformation in the pH range between 5.0-9.0. The isoelectric point (pI) of gD1 was determined by flat-bed isoelectric focusing (IEF) to be 5.9 - 6.0. This agreed with the approximate pI determined for mature gD1 by 2-dimensional gel electrophoresis (Cohen et al., 1980; Eisenberg et al., 1979). Surprisingly, IEF did not resolve the precursors from the mature gD1 polypeptides. Eisenberg et al. (1979), using 2-dimensional IEF, found that the gD1 precursor, synthesized in HSV-1 (HF) infected KB cells, had a pI of 8.0. Urea was not included in the IEF gel utilized in the present study in order to preserve the native conformation of gD1. In the absence of urea, the gD polypeptide may fold in such a way that the charge contribution from the carbohydrate moieties is at least partially masked and hence the overall net charge difference between the precursor and mature forms would be

negligible. The fact that both of these forms of gD1 bound to DEAE-Sepharose at pH 6.5 and could be eluted by 0.27 - 0.34 M sodium chloride (see Figure 30) lends additional support to the notion that they exhibit similar net charges in the absence of denaturing agent. A gD1 precursor having a pI of 8.0 would be positively charged at this pH and could not bind to a positively charged matrix such as DEAE-Sepharose. Alternatively, the single gD1 species observed on the IEF gel may be due to association between precursor and mature molecules.

Partial purification of gD1 was achieved by DEAE-Sepharose chromatography and the extent of purification was improved by combining this technique with ammonium sulfate precipitation. Due to the large number of polypeptides present in infected cell extracts, complete purification of gD1 was not possible. However, ammonium sulfate precipitation was useful as a technique for concentrating the polypeptides from the extract, and, in combination with ion exchange chromatography, as an initial purification step.

Lectin affinity columns provided a means to specifically purify HSV glycoproteins and yielded information regarding the identity of various sugar residues contained in the oligosaccharides of gD1. The inability of gD1 to bind to WGA suggested that it did not contain terminal sialic acid or GlcNAc residues since the presence of either of these residues is a requirement for high affinity binding to WGA (Wright, 1984). WGA has a low affinity for α2-6 linked

sialic acid (Kronis and Carver, 1985). Therefore, if any terminal sialic acid residues are present on the gD oligosaccharides, they may be joined by an α2-6 rather than an α2-3 linkage. The fact that fetuin, a glycoprotein that contains two terminal α2-3 linked sialic acid residues (Takasaki and Kobata, 1986), was able to bind to WGA negated the possibility that the WGA column had lost its specificity for this residue. One HSV-specific glycopeptide (120-130K) did bind to WGA (Figure 32). On the basis of its molecular size this glycoprotein may be gC. Olofsson et al. (1983) showed that a portion of the gC population could bind to WGA.

A fraction of both the precursor and mature gD1 had affinity for lentil lectin. Comparison of the intensity of the bands between the elution and flow-through fractions suggested that a larger percentage of the precursor gD1 population bound to lentil lectin (Figure 38). This result was consistent with the expectation that the glycans on precursor gD1 would be largely composed of high-mannose core carbohydrates. Lentil lectin has specificity for glycoproteins having oligosaccharide molecules with at least one of the α-linked mannose residues substituted at positions C-2 and C-6 and which also have a fucose residue linked to the GlcNAc proximal to the polypeptide (Kornfeld et al., 1981; Cummings and Kornfeld, 1982). The portion of the precursors and mature forms of gD1 which did not bind to the column may lack either or both of these features.

Respess et al. (1984) found that none of the mature gD1 polypeptide synthesized in HEp-2 cells was able to bind to lentil lectin. This result was in contrast to the present study which found that a portion of mature gD1 did bind. This discrepancy may reflect differential glycan processing between HEp-2 and Vero cells. Several other glycoproteins, within the range 80-130K, had affinity for lentil lectin (see Figure 35). Respess et al. (1984) showed that gB (120K), gC (128K), and gE (84K) bound to lentil lectin.

CB-120 lectin has a high affinity for carbohydrates containing terminal galactose. The mature form of gD1 bound specifically to CB-120 whereas the precursor forms did not bind, suggesting that terminal galactose was present on the mature gD molecule and that addition of this residue was a late step in processing of gD1 in Vero cells. At least two additional glycoproteins in the molecular size range of 118-130K also bound to CB-120. Eberle and Courtney (1980), using gels cross-linked with methylenebisacrylamide, determined that gB and gC polypeptides (synthesized in Vero and HEp-2 cells) migrated to positions that corresponded to the molecular ~~size~~ range of 118-128K. Kumarosamy and Blough (1985) showed that the number of cell surface galactose residues increased dramatically following HSV infection and that this increase was largely due to the incorporation of galactose onto the N-linked carbohydrates of gB and gC but not gD and gE. Therefore, the high molecular weight CB-120 binding glycoproteins likely correspond to mature, and

possibly some immature, forms of glycoproteins gB and gC. The results of Kumarosamy and Blough (1985) suggested that terminal galactose is absent from gD1 synthesized in BHK-21 and HEp-2 cells. However, in this study, 100% of mature gD1 bound to CB-120 (see Figure 38) indicating that terminal galactose addition to gD1 may be a unique feature of Vero cells.

A gD1-related species (approximately 120K) was detected in the elution fraction from lentil lectin. On the basis of its size, it may represent a dimeric form of gD1. Multimeric forms of gD were not detected in the CB-120 elution or flow-through fraction. Taken together, these results suggested that enrichment of the lentil lectin binding gD1 species may have promoted the formation of gD1 oligomers. Another possibility is that lentil lectin binds specifically to gD1 oligomers which are already present in HSV-1 infected cell extract. However, this explanation is less likely since equal molar amounts of gD1 oligomers were not consistently detected in every experiment using the same cell extract. This species could not be dissociated by boiling in the presence of SDS and β-mercaptoethanol and was composed of at least 3 bands which were resolvable by SDS polyacrylamide gel electrophoresis. These results suggested that the putative gD1 dimer may consist of tightly bound, multiple forms of glycosylated gD1. Other investigators have detected oligomeric forms of gD1 (Eisenberg et al., 1982; Snowden and Haliburton, 1985) but this is the first report to suggest

that the presence of certain carbohydrate structures, recognized by lentil lectin, may be important for their formation. Specific endoglycosidase cleavage and chemical analysis of the oligosaccharides attached to the lentil lectin-binding gD1 species will determine the nature of this interaction more precisely.

Further purification of gD from the lectin-bound glycoprotein was achieved by gel filtration. It was necessary to fractionate the glycoproteins in the presence of SDS. In the absence of SDS all the glycoproteins which bound to CB-120 lectin were eluted as one fraction. This was perhaps due to interaction between hydrophobic regions in these proteins causing them to aggregate together. Inclusion of zwitterions in the eluent buffer and running the column at lower temperatures should reduce this problem and further improve the extent of protein separation so that gD could be purified from the contaminating proteins at 43K and 67K. The structural integrity of the proteins would be better maintained under these conditions rather than in the presence of SDS or 2-mercaptoethanol. Silver staining of the proteins present in the CB-120 elution fraction and the gel filtration fractions indicated the presence of two additional proteins in the 30-35K range which were not detected by autoradiography (data not shown). These proteins were eluted in fractions 25 and 26 from the gel filtration column and therefore were resolved from gD-1 present in fractions 22 and 23.

Lectin affinity chromotography was useful as a method to isolate gD and fractionate individual gD species on the basis of their carbohydrate moieties. This method therefore has an advantage over immunoaffinity chromotography, a technique which is not necessarily specific for precursor and mature forms of the glycoprotein. Further characterization, using specific monoclonal antibodies, will be required in order to ascertain the usefulness of CB-120 and lentil lectins for the purification of other HSV glycoproteins. Scale-up of the lectin procedure in conjunction with immunoaffinity chromatography or gel filtration should provide sufficient quantities of gD for detailed biochemical analysis of the protein and carbohydrate structures. The lectins will also be used as probes which detect variations in the carbohydrate residues added to HSV glycoproteins in different cell lines.

The results of this study suggest the following purification protocol for the purpose of isolating gD1 from Vero cells infected by HSV-1 or cells expressing gD1 after transformation by an expression vector containing this gene: 1) Concentration of proteins from the cell culture by 30-50% ammonium sulfate followed by suspension and dialysis in the appropriate buffer for lectin affinity chromatography. 2) Affinity chromatography of this fraction first on a CB-120 lectin column and then pass the flow-through fraction through a lentil lectin column. The elution, fractions, from these two columns can be combined or processed separately depending

on whether separation of the different forms of gDl is desired or not. 3) Dialysis against gel filtration buffer and performance of gel filtration in order to purify gDl to homogeneity.

CHAPTER 7

SUMMARY

Two glycoproteins, gB and gD, of HSV were characterized in this thesis project. The following is a list of the major findings:

1) Glycoproteins gB1 and gB2 were localized, by northern blot analysis, to within 0.345 and 0.386 map units on the HSV genome.

2) The DNA sequence between 0.345 and 0.370 map units on the HSV-2 genome was determined. Open reading frame analysis of the sequence indentified the gB2 gene and showed that it was transcribed from right to left (on the prototype HSV genome) and coded for a polypeptide consisting of 904 amino acid residues.

3) Analysis of the amino acid (a.a.) sequence suggested four structural regions; a signal peptide (22 a.a.), an external region (701 a.a.), a cytoplasmic membrane-spanning sequence (68 a.a.) and a cytoplasmic region (111 a.a.).

4) Surface profile analysis of the external sequence predicted 9 major antigenic regions. Several of these regions corresponded to segments on the gB1 and gB2 polypeptides which exhibited amino acid diversity.

5) Glycoprotein gD1 was resolved into 3 to 4 forms (52-60K) by electrophoresis in SDS-10% polyacrylamide gels. These forms could be fractionated by lectin affinity chromatography using Lentil and CB-120 lectins.

6) The binding of the high molecular weight form(s) of gD1 to CB-120 lectin indicated that mature gD1, synthesized in Vero cells, contains terminal galactose residues on its carbohydrate moieties. This form(s) was purified by a two step procedure utilizing CB-120 lectin affinity chromatography followed by gel filtration.

Additional work will be required to verify the antigenic regions on the gB polypeptide. Synthetic peptides, which include the predicted epitopes, could be synthesized and analysed for their ability to raise HSV-neutralizing antibody or their ability to react with gB-specific monoclonal antibody. The fractionation of the different glycosylated forms of gD1 could be further refined by combining lectin affinity chromatography with immunoaffinity chromatography. This would enable the purification of the gD1 forms which were bound and not bound to the lectin columns.

REFERENCES

Ackerman, M., Longnecker, R., Roizman, B. and L. Pereira, 1986. Identification, properties, and gene location of a novel glycoprotein specified by herpes simplex virus 1. Virology 150:207-220.

Arsenakis, M., Hubenthal-Voss, J., Campadelli-Fiume, G., Pereira, L. and B. Roizman, 1986a. Construction and properties of a cell line constitutively expressing the herpes simplex virus glycoprotein B dependent on functional α4 protein synthesis. J. Virol. 60:674-682.

Arsenakis, M., Tomasi, L.F., Speziali, V., Roizman, B. and G. Campadelli-Fiume, 1986b. Expression and regulation of glycoprotein C gene of herpes simplex virus 1 resident in a cloned L-Cell line. J. Virol. 58:367-376.

Averett, D.R., Furman, P.A. and T. Spector. 1984. Ribonucleotide reductase of herpes simplex virus type 2 resembles that of herpes simplex virus type 1. J. Virol. 52: 981-983.

Balachandran, N.D., Harnish, D., Killington, R.A., Bacchetti, S., and W.F. Rawls. 1981. Monoclonal antibodies to two glycoproteins of herpes simplex virus type 2. J. Virol. 39:438-446.

Balachandran, N.D., Harnish, D., Rawls, W.E., and S.Bacchetti, 1982a. Glycoproteins of herpes simplex virus type 2 as defined by monoclonal antibodies. J. Virol. 44:344-355.

Balachandran, N., Bacchetti, S. and W.E. Rawls, 1982b. Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. Infect. Immun., 37:1132-1137.

Banks, L.M., Halliburton, I.W., Purifoy, D.J.M., Killington, R.A. and K.L. Powell, 1985. Studies on the herpes simplex virus alkaline nuclease: detection of type-common and type-specific epitopes on the enzyme. J. Gen. Virol. 66:1-14.

Barr, P.J., Thayer, R.M., Laybourn, P., Najarian, R.C., Seela, F.and D.R. Tolan. 1986. 7-deaza-2'-deoxyguanosine-5'-triphosphate: enhanced resolution in M13 dideoxy sequencing. Bio Techniques 4:428-431.

Batterson, W. and B. Roizman, 1983. Characterization of the herpes simplex virion-associated factor responsible for induction of α genes. J. Virol. 46:371-377.

Baucke, R.B. and P.G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc binding glycoprotein. J. Virol. 32:779-789.

Berget, S.M. 1984. Are U4 small nuclear ribonucleoproteins involved in polyadenylation? Nature, 309:179-182.

Bibor-Hardy, V., Dagenais, A. and R. Simard. 1985. *In situ* hybridization of the major capsid protein during lytic infection by herpes simplex virus. J. Gen. Virol. 66:897-901.

Bishop, G.A., Glorioso, J., and S.A. Schwartz, 1983. Relationship between expression of herpes simplex virus glycoproteins and susceptibility of target cells to human natural killer activity. J. Exp. Med. 157:1544-1561.

Bishop, G.A., Marlin, S.D., Schwartz, S.A. and J.C. Glorioso, 1984. Human natural killer cell recognition of herpes simplex virus type 1 glycoproteins: specificity anlysis with the use of monoclonal antibodies and antigenic variants. J. Immunol., 133:2208-2214.

Bishop, G.A., Kumel, G., Schwartz, S.A. and J. Glorioso, 1986. Specificity of human natural killer cells in limiting dilution culture for determinants of herpes simplex virus type 1 glycoproteins. J. Virol. 57:294-300.

Bonner, W.M. and R.A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83.

Braun, D.K., B. Roizman, and L. Pereira, 1984. Characterization of posttranslational products of herpes simplex virus gene 35 proteins binding to the surface of full but not empty capsids. J. Virol. 49:142-153.

Breathnach, R. and P. Chambon. 1981. Organization and expression of eukaryotic split genes coding for proteins. Annu. Rev. Biochem. 50:349-383.

Buckmaster, E.A., Gompels, U. and A. Minson, 1984. Characterization and physical mapping of an HSV-1 glycoprotein of approximately 115 x 10^3 molecular weight. Virology 139:408-413.

Bzik, D.J., Fox, B.A., Deluca, N.A. and S. Person. 1984a. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology, 137:185-190.

Bzik, D.J., Fox, B.A., Deluca, N.A. and S. Person. 1984b. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology, 133:301-314.

Bzik, D.J., Debroy, C., Fox, B.A., Pederson, N.E. and S. Person, 1986. The nucleotide sequence of the gB glycoprotein gene of HSV-2 and comparison with the corresponding gene of HSV-1. Virology, 155:322-333.

Cabrera, C.V., Wohlenberg, C. and H. Openshaw, 1980. Herpes simplex virus DNA sequences in the CNS of latently infected mice. Nature 288:288-290.

Camacho, A. and P. Spear, 1978. Transformation of hamster embryo fibroblasts by a specific fragment of herpes simplex virus genome. Cell 15:993-1002.

Campadelli-Fiume, G., Sinibaldi-Vallebora, P., Cavrini, V. and A. Mannini-Palenzona, 1980. Selective inhibition of herpes simplex virus glycoprotein synthesis by a benzamidinohydrazone derivative. Arch. Virol. 66:179-191.

Campadelli-Fiume, G., Poletti, L., Dall'Olio, F. and F. Serafini-Cessi, 1982. Infectivity and glycoprotein processing of herpes simplex virus type 1 grown in a ricin-resistant cell line deficient in N-acetylglucosaminyl transferase I. J. Virol. 43:1061-1071.

Campadelli-Fiume, G. and Serafini-Cessi, F., 1985. Processing of the oligosaccharide chains of herpes simplex virus type 1 glycoproteins. In The Viruses, Vol. 3, The Herpesviruses. (B. Roizman ed.) pp. 357-382. Plenum Press, New York.

Campbell, M.E.M., Palfreyman, and C.M. Preston, 1984. Identification of herpes simplex virus DNA sequence which encode a transacting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. 180:1-19.

Carter, V.C., P.A. Schaffer, and S.S. Tevethia, 1981. The involvement of herpes simplex type 1 glycoproteins in cell mediated immunity. J. Immunol. 126:1655-1660.

Chatterjee, S., Hunter, E. and R. Whitley, 1985. Effect of cloned human interferons on protein synthesis and morphogenesis of herpes simplex virus. J. Virol. 56:419-425.

Chiou, H.C., Weller, S.K., and D.M. Coen. 1985. Mutations in the herpes simplex virus major DNA-binding protein gene leading to altered sensitivity to DNA polymerase. Virology 145:213-226.

Chou, P.Y., and G.D. Fasman, 1978. Empirical predictions of protein conformation. Ann. Rev. Biochem. 47:251-276.

Claesson-Welsh, L. and P.Spear, 1987. Amino-terminal sequence, synthesis, and membrane insertion of glycoprotein B of herpes simplex virus type 1. J. Virol. 61:1-7.

Cohen, G.H., Long, D. and R. Eisenberg, 1980. Synthesis and processing of glycoproteins gD and gC of herpes simplex virus type 1. J. Virol. 36:429-439.

Cohen, G.H., Long, D., Matthews, J.T., May, M. and R. Eisenberg, 1983. Glycopeptides of the type-common glycoprotein gD of herpes simplex virus types 1 and 2. J. Virol. 46:679.

Cohen, G.H., Dietzschold, B., Ponce de Leon, M., Long, D., Golub, E., Varrichio, A., Pereira, L., and R.J. Eisenberg, 1984. Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates the production of neutralizing antibody. J. Virol. 49:102-108.

Cohen, G.H., Isola, V.J., Kuhns, J., Berman, P.W. and R.J. Eisenberg, 1986. Localization of discontinuous epitopes of herpes simplex virus glycoprotein D: use of a nondenaturing ("native" gel) system of polyacrylamide gel electrophoresis coupled with western blotting. J. Virol. 60:157-166.

Compton, T. and R.J. Courtney, 1984a. Evidence for post-translational glycosylation of a nonglycosylated precursor protein of herpes simplex virus type 1. J. Virol. 52:630-637.

Compton, T. and R.J. Courtney, 1984b. Virus specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1- infected cells. J. Virol. 49:594-597.

Conley, A.F., Knipe, D.M., Jones, P.C. and B. Roizman, 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by *in vitro* mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. J. Virol. 37:191-206.

Constanzo, F., Campadelli-Fiume, G., Foa-Tomas, L. and E. Cassai, 1977. Evidence that herpes simplex virus DNA is transcribed by cellular RNA polyperase II. J. Virol. 21:996-1001.

Corey, L. and P.G. Spear, 1986. Infections with herpes simplex viruses. New Engl. J. Med. 314:749-757.

Costa, R.H., Draper, K.G., Devi-Rao, G., Thompson, R.L., and E.W. Wagner, 1985a. Virus-induced modification of the host cell is required for expression of the bacterial chloramphenicol acetyltransferase gene controlled by a late herpes simplex virus promoter. J. Virol. 56:19-30.

Costa, R.H., Draper, K.G., Kelly, T.J. and E.K. Wagner. 1985b. An unusual spliced herpes simplex virus type 1 transcript with sequence homology to epstein-barr virus DNA. J. Virol. 54:317-328.

Cremer, K.J., Mackett, M., Wohlenburg, C., Natkins, A.L. and B. Moss, 1985. Vaccinia virus recombinant expressing HSV type 1 glycoprotein D prevents latent herpes in mice. Science 228:737-740.

Crumpacker, C.S., Chartrand, P., Subak-Sharpe, J.H. and N.M. Wilkie, 1980. Resistance of herpes simplex virus to acycloquanisine-genetic and physical analysis. Virology 105:171-184.

Cummings, R.D. and S. Kornfeld, 1982. Fractionation of asparagine-linked oligosaccharides by serial lectin-agarose affinity chromatography. J. Biol. Chem. 257:11235-11240.

Dalyrymple, M.A., McGeoch, D.J., Davison, A.J. and C.M. Preston, 1985. DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate early promoter. Nucl. Acid. Res. 13:7865-7879.

Daksis, J.I. and V.L. Chan, 1987. Physical mapping of two herpes simplex virus type 1 host shutoff loci: Rescue of each ts mutation occurs with two unique cloned regions of the viral genome. J. Virol. 61:143-150.

Davidson, A.J., Edson, C.M., Ellis, R.W., Forghandi, B., Gilden, D., Grose, C., Keller, P.M., Vafai, A., Wroblewska, Z., and K.Yamanishi, 1986. New common nomenclature for the glycoprotein genes of varicella zoster Virus and their glycosylated products. J. Virol. 57:1197-1197.

Delius, H. and J.B. Clements, 1976. A partial denaturation map of herpes simplex virus type 1 DNA: Evidence for inversions of the unique DNA regions. J. Gen. Virol. 33:125-133.

DeLuca, N., Bzik, D., Bond, V.C., Person, S., and W. Snipes, 1982. Nucleotide sequences of herpes simplex type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB ((P7). Virology 122:411.

DeLuca, N., Pearson, S., Bzik, D.J. and W. Snipes, 1984. Genome locations of temperature-sensitive mutants in glycoprotein gB of herpes simplex virus type 1. Virology 137:382-389.

Dietzschold, B., Eisenberg, R.J., Ponce De Leon, M., Golub, E., Hudec, F., Varrichio, A. and G.H. Cohen. 1984. Fine structure analysis of type-specific and type-common antigenic sites of herpes simplex virus glycoprotein D. J. Virol. 52:431-435.

Dowbenko, D.J. and L.A. Lasky, 1984. Extensive homology between the herpes simplex virus type 2 glycoprotein F gene and the herpes simplex virus type 1 glycoprotein C gene. J. Virol. 52:154-163.

Duff, R. and F. Rapp, 1973. Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus 1. J. Virol. 12:209-217.

Eberle, R. and R.J. Courtney, 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. J. Virol. 35:902-917.

Efstathiou, S., Minson, A.C., Field, H.J., Anderson, J.R. and P. Wildy, 1986. Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J. Virol. 57:446-455.

Eisenberg, R., Hydrean-Stern, C., and G.H. Cohen, 1979. Structural analysis of precursor and product forms of type common envelope gD (CP-1 antigen) of herpes simplex virus type 1. J. Virol. 31:608-620.

Eisenberg, R.J., Ponce de Leon, M., Pereira, L., Long, D. and G.H. Cohen, 1982. Purification of glycoprotein gD of herpes simplex virus types 1 and 2 by use of monoclonal antibody. J. Virol. 41:1099-1104.

Eisenberg, R.J., Long, D., Hogue-Angeletti, R. and G. Cohen, 1984. Amino-terminal sequence of glycoprotein D of herpes simplex viruses types 1 and 2. J. Virol. 49:265-268.

Eisenberg, R.J., Long, D., Ponce de Leon, M., Matthews, J.T., Spear, P.G., Gibson, M.G., Lasky, L.A., Berman, P., Golub, E., G.H. Cohen, 1985. Localization of epitopes of herpes simplex virus type 1 glycoprotein D. J. Virol. 53:634-644.

Eisenberg, R.J., Cerini, C.P., Heilman, C.J., Joseph, A.D. Dietzschold, B., Golub, E., Long, D., Ponce De Leon, M. and G. Cohen. 1985b. Synthetic glycoprotein D related peptides protect mice against herpes simplex virus challenge. J. Virol. 56:1014-1017.

Epstein, A.L., Jacquemont, B. and I. Machuca, 1984. Infection of a restrictive cell line (XC Cells) by intertypic recombinants of HSV-1: Relationship between penetration of the virus and relative amounts of glycoprotein C. Virology 132:315-324.

Everett, R.D., 1983. DNA sequence elements required for regulated expression of the HSV-1 glycoprotein D gene lie within 83 bp of the RNA capsites. Nucl. Acids Res. 11:6647-6667.

Faber, S.W. and K.W. Wilcox, 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucl. Acids Res. 14: 6067-6083.

Felser, J.M, Straus, S.E., and J.M. Ostrove, 1987. Varicella-Zoster Virus complements herpes simplex virus type 1 temperature sensitive mutants. J. Virol. 61:225-228. Fenwick, M.L. and M.J. Walker, 1978. Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J. Gen.Virol. 41:37-51.

Frame, M.C., Marsden, H.S. and D.J. McGeoch, 1986. Novel herpes simplex virus type 1 glycoproteins identified by antiserum against synthetic oligopeptide from the predicted product of gene US4. J. Gen. Virol. 67:745-751.

Frankel, M.E., Gerhard, W., Wroblewska, Z, Fraser, N.W., and H. Koprowski, 1985. Possible relationship between antigenic properties and isolation history of HSV-1 strains. Virus. Res. 3:323-377.

Fraser, N.W., Lawrence, W.C., Wroblewska, Z., Gilden, D.H., and H. Koprowski, 1981. Herpes simplex virus type 1 DNA in human brain tissue. Proc. Natl. Acad. Sci. USA 78:6461-6465.

Frenkel, N., Roizman, B., Caussai, E. et al., 1972. A herpes simplex virus type 2 DNA fragment and its transcription in human cervical cancer tissue. Proc. Natl. Acad. Sci. USA 69:3784.

Friedman, H.M., Cohen, G.H., Eisenberg, R.J., Seidel, C.A., and D.B. Cines, 1984. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature (London) 309:633-635.

Fries, L.F., Friedman, H.M., Cohen, G.H., Eisenberg, R.J., Hamer, C.H. and M.M. Frank, 1986. Glycoprotein C of herpes simplex virus 1 is an inhibitor fo the complement cascade. J. Immunol. 137:1636-1641.

Frink, R.J., Eisenberg, R., Cohen, G. and E.K. Wagner, 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. 45:634-647.

Fuller, A.O., and P.G. Spear, 1985. Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. J. Virol. 55:475-482.

Gaffney, D.F., McLauchlan, J., Whitton, J.L., and J.B. Clements, 1985. A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. Nucl.Acids Res. 13:7847-7862.

Galloway, D.A., Fenoglio, C., Shewchuk, M. and J.K. McDougall, 1979. Detection of herpes simplex RNA in human sensory ganglia. Virology 95:265-268.

Galloway, D.A. and J.M. McDougall, 1981. Transformation of rodent cells by a cloned DNA fragment of herpes simplex virus type 2. J. Virol. 38:749-760.

Galloway, D.A. and J.K. McDougall, 1983. The oncogenic potential of herpes simplex viruses: evidence for a "hit and run" mechanism. Nature 302:21-24.

Gerhard, W., Yewdell, J. and M.E. Frankel, 1981. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature, 290:713-717.

Gibson, M.G. and P.G. Spear, 1983. Insertion mutations of herpes simplex virus have a duplication of the gD gene and express two different forms of gD. J. Virol. 48:396.

Glorioso, J., Szczesiul, M.S., Marlin, S.D. and M. Levine, 1983. Inhibition of glycosylation of herpes simplex virus glycoproteins: immunogenicity and antigenicity of partially glycosylated glycoproteins. Virology 126:1-18.

Glorioso, J., Schroder, C.H., Kumel, G., Szczesiul, M., and M. Levine, 1984. Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. J. Virol. 50:805-812.

Godowski, P.J. and D.M. Knipe, 1983. Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 results in increased levels of viral gene expression. J. Virol. 47:478-486.

Godowski, P.J. and D.M. Knipe, 1986. Transcriptional control of herpes gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA 83:256-260.

Gompels, U. and A. Minson, 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology 153:230-247.

Gong, M., Ooka, T., Matsuo, T. and E. Kieff, 1987. Epstein-Barr virus glycoprotein homologous to herpes simplex virus gB. J. Virol. 61:499-508.

Green, M.T., Courtney, R.J. and E.G. Dunkel, 1981. Detection of an immediate early herpes simplex virus type 1 polypeptide in trigeminal ganglia from latently infected animals. Infect.Immun. 34:987.

Haarr, L. and H.S. Marsden, 1981. Two dimensional gel analysis of HSV type 1 induced polypeptides and glycoprotein processing. J. Gen. Virol. 52:77-92.

Hagenbuchle, O., Santer, M., Steitz, J.A. and R.J. Mans, 1978. Conservation of the primary structure of the 3'

end of 18S rRNA from eukaryotic cells. Cell 13:551-563.

Hayashi, Y., Wada, T. and R. Mori, 1983. Protection of newborn mice against herpes simplex virus infection by prenatal and postnatal transmission of antibody. J. Gen. Virol. 64:1007.

Hayward, G.S., Jacob, R.J. Wadsworth, S.C. and B. Roizman, 1975. Anatomy of herpes simplex virus DNA: Evidence for four populations of molecules that differ in the relative orientations of their long and short segments. Proc. Natl. Acad. Sci. USA, 72:4243-4247.

Hill, T.J., 1985. Herpes simplex virus latency. In The Viruses, Vol. 3, The Herpesviruses (B. Roizman, ed.) pp. 175-225. Plenum Press, New York.

Ho, M., 1977. Viral infections after transplantation in man. Arch.Virol. 55:1.

Hodgman, T.C. and A.C. Minson, 1986. The herpes simplex virus type 2 equivalent of the herpes simplex virus type 1 US7 gene and its flanking sequences. Virology 153:1-11.

Holland, T.C., Sandri-Goldin, R.M., Holland, L.E., Marlin, S.D., Levine, M. and J.C. Glorioso, 1983. Physical mapping of the mutation in an antigenic variant of herpes simplex virus type 1 by use of an immunoreactive plaque assay. J. Virol.46:64

Holland, L.E., Sandri-Goldin, R.M., Goldin, A., Glorioso, J.C. and Levine, M. 1984a. Transcriptional and Genetic analyses of the Herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. 49:947.

Holland, T.C., Homa, F.L., Marlin, S.D., Lvine, M. and J. Glorioso, 1984b. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. 52:566-574.

Homa, F.L., Otal, T.M., Glorioso, J.C., and M. Levine, 1986. Transcriptional control signals of herpes simplex virus type 1 late (γ_2) gene lie within bases -34 to +124 relative to the 5' terminus of mRNA. Mol. Cell. Biol. 6:3652-3666.

Honess, R.W., 1984. Herpes simplex and "The Herpes Complex": diverse observations and a unifying hypothesis. J. Gen. Virol. 65:2077-2107.

Honess, R.W. and B. Roizman, 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14:8 19.

Honess, R.W. and B. Roizman, 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA 72:1276-1280.

Hope, R.G., Palfreyman, J., Suh, M., and H.S. Marsden, 1982. Sulphated glycoproteins induced by herpes simplex virus. J. Gen. Virol. 58:399-415.

Horohov, D.W., Moore, R.N., and B.T. Rouse, 1985. Regulation of herpes simplex virus-specific lymphoproliferation by suppressor cells. J. Virol. 56:1-6.

Husar, D. and S. Bachetti, 1981. Partial purification and characterization of the ribonucleotide reductase induced by herpes simplex virus infection of mammalian cells. J. Virol. 37:580.

Jacob, R., Morse, L.S. and B. Roizman, 1979. Anatomy of herpes simplex virus DNA. XII. Accumulation of head to tail concatomers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of DNA. J. Virol. 29:448-457.

Jarivalla, R.J., Aurelian, L., and P.O.P. Ts'0, 1980. Tumorigenic transformation induced by a specific fragment of DNA from herpes simplex virus type 2. Proc. Natl. Acad. Sci. USA 77:2279-2283.

Jennings, S.R., Rice, P.L., Kloszewski, E.D., Anderson, R.W., Thompson, D.L. and S.T. Tevethia, 1985. Effect of herpes simplex virus types 1 and 2 on surface expression of class I major histocompatability complex antigens on infected cells. J. Virol. 56:757-766.

Jennings, S.R., Lippe, P.A., Pauza, K.J., Spear, P.G., Pereria, L. and S. Tevethia, 1987. Kenetics of expression of herpes simplex virus type 1-specific glycoprotein species on the surfaces of infected murine, simian, and human cells: flow cytometric analysis. J. Virol. 61:104-112.

Jing, G., Liu, A. and W.C. Leung, 1986. A method for the preparation of size marker for synthetic oligonucleotides Analytical Biochem. 155:376-378.

Johansson, P.J.H., Hallberg, T., Oxelius, V.A., Grubb, A. and J. Blomberg, 1984. Human immunoglobulin class and

subclass specificity of Fc receptors induced by herpes simplex virus type 1. J. Virol. 50:796-804.

Johnson, D.C. and P.G. Spear, 1982. Monensin inhibits the processing of herpes simplex virus glycoprotein, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. 43:1102-1112.

Johnson, D.C. and P.G. Spear, 1983. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell 32:987-997.

Johnson, D.C. and J.R. Smiley, 1985. Intracellular transport of herpes simplex virus gD occurs more rapidly in uninfected cells than infected cells. J. Virol. 54:682-684.

Jones, K., Fenoglio, C., Shevchuk-Chabon, et al., 1978. Detection of herpes simplex virus type 2 mRNA in human cervical biopsies by in situ cytological hybridization. In Oncogenesis and Herpesviruses III. (G. de-The et al. eds.) pp. 917. IARC, France.

Jones, T.R. and R.W. Hyman, 1983. Species hybridization between herpes simplex virus DNA and human cellular DNA. Virology 131:555-560.

Jones, T.R., Parks, C.L., Spector, D.J. and R.W. Hyman, 1985. Hybridization of herpes simplex virus DNA and human ribosomal DNA and RNA. Virology 144:384-397.

Josephs, S.F., Salahuddin, S.Z., Ablashi, D.V., Schachter, F., Wong-Staal, F. and R. Gallo, 1986. Genomic analysis of the human B-lymphotropic virus (HBLV). Science 234:601-603.

Keller, P.M., Davison, A.J., Lowe, R.S., Bennett, C.D. and R.W. Ellis, 1986. Identification and structure of the gene encoding gpII, a major glycoprotein of varicella-zoster virus. Virology, 152:181-191.

Kikuchi, G.E., Coligan, J.E., Holland, T.C., Levine, M., Glorioso, J.C. and R. Nairn, 1984. Biochemical characterization of peptides from herpes simplex virus glycoprotein gC: loss of CNBr fragments from the carboxy terminus of truncated, secreted gC molecules. J. Virol. 52:806-815.

Kinchington, P.R., Remenick, J., Ostrove, J.M., Straus, S., Ruyechan, W.T. and J. Hay, 1986. Putative glycoprotein gene of varicella-zoster virus with variable copy numbers of a 42 base-pair repeat

sequence has homology to herpes simplex virus glycoprotein C. J. Virol. 59:660-668.

Kino, Y., Eto, T., Ohtomo, N., Hayashi, Y., Yamamoto, M. and R. Mori, 1985. Passive immunization of mice with monoclonal antibodies to glycoprotein gB of herpes simplex virus. Microbiol. Immunol. 29:143-149.

Kino, Y., Eto, T., Nishiyama, K., Ohtomo, N. and R. Mori, 1986. Immunogenicity of purified glycoprotein gB of herpes simplex virus. Archives of Virology, 89:69-80.

Kirchner, H., Peter, H.H., Hirt, H.M., Zawatzky, R., Dalugge, H. and P. Bradstreet, 1979. Studies of the producer of interferon in human lymphocyte cultures. Immunobiology 156:65-75.

Kitamura, K., Manazue, J., Campo-Vera, H., Ogino, T. and K. Yamanishi, 1986. Induction of neutralizing antibody against varicella-zoster virus (VZV) by VZV gp3 and cross-reactivity between VZV gp3 and herpes simplex viruses gB. Virology 149:74-82.

Kit, S., and D.R. Dubbs, 1963. Acquisition of thymidine kinase activity by herpes simplex infected mouse fibroblast cells. Biochem. Biophys. Res. Commun. 11:55-59.

Kornfeld, K., Reitman, M.L. and R. Kornfeld, 1981. The carbohydrate-binding specificty of pea and lentil lectins. J. Biol. Chem. 256:6633-6640.

Kousoulas, K.G., Pellett, P.E., Pereira, L. and B. Roizman, 1984. Mutations affecting conformation or sequence of neutralizing epitopes identified by reactivity of viable plaques segregate from syn to ts domains of HSV-1 (F) gB gene. Virology 135:379.

Kozak, M., 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes and organelles. Microbiol. Rev. 47:1-45.

Kreil, G., 1981. Transfer of proteins across membranes. Annu Rev Biochem. 50:317.

Kronis, K.A. and J.P. Carver, 1985. Wheat Germ Agglutanin dimers bind sialyloligosaccharides at four sites in solution: proton nuclear magnetic resonance temperature studies at 360 MHz. Biochemistry 24:826-833.

Kuman, A. and U. Lindberg. 1972. Characterization of messenger ribonucleoprotein and messenger RNA from KB cells. Proc. Natl. Acad. Sci. USA. 69:681.

Kumarasamy, R. and H.A. Blough, 1985. Galactose-rich glycoproteins are on the cell surface of herpes virus-infected cells. Arch.Biochem.Biophys. 236:593-602.

Kumel, G., Kaerner, H.C., Levine, M., Schroder, C.H., and J.C. Glorioso, 1985. Passive immune protection by herpes simplex virus-specific monoclonal antibodies and monoclonal antibody-resistant mutants altered in pathogenicity. J. Virol. 56:930-937.

Kyte, J. and R.F. Doolittle, 1982. A single method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105-132.

Lasky, L.A., Dowbenko, D., Simonsen, C.C., and P.W.Berman, 1984. Protection of mice from lethal herpes simplex virus infection by vaccination with a secreted form of glycoprotein D. Biotechnology, June:527-532.

Lebbon, P., 1985. Inhibition of herpes simplex virus type-1 induced interferon synthesis by monoclonal antibodies against viral glycoprotein D and by lysosomotropic drugs. J. Gen. Virol. 66:2781-2786.

Lee, G.T.Y., Para, M.F. and P.G. Spear, 1982a. Location of the structural genes for glycoproteins gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA. J. Virol. 43:44.

Lee, G.T.Y., Pogue-Geile, K.L., Pereira, L. and P.G. Spear, 1982b. Expression of herpes simplex virus glycoprotein C from a DNA fragment inserted into the thymidine kinase gene of this virus. Proc. Natl. Acad. Sci. USA. 79:6612.

Lee, C.K. and Knipe, D.M., 1983. Thermolabile *in vivo* DNA binding activity associated with a protein encoded by mutants of herpes simplex virus type 1. J. Virol. 46:909.

Lehner, T., Witton, J.M.A., and E.J. Shillitoe, 1975. Immunological basis for latency, recurrences and putative oncogenicity of herpes simplex viruses. Lancet ii:60-62.

Leinbach, S.S. and J.F. Casto, 1983. Identification and characterization of deoxyribonucleoprotein complexes containing the major DNA-binding protein of herpes simplex virus type 1. Virology 131:274-286.

Little, S.P., Jofre, J.T., Courtney, R.J. and P.A. Schaffer, 1981. A virion-associated glycoprotein essential for

infectivity of herpes simplex virus type 1. Virology 115:149.

Locker, H. and N. Frenkel, 1979. BamH1, Kpn1 and Sall restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurence of heterogeneities in defined regions of the viral DNA. J. Virol. 32:424-441.

Long, D., Madara, T.J., Ponce de Leon, M., Cohen, G.H., Montgomery, P.C. and R.J. Eisenberg, 1984. Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. Infect. Immun. 43:761-764.

Longnecker, R. and B. Roizman, 1986. Generation of an inverting herpes simplex virus type 1 mutant lacking the L-S junction a sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and α47 gene. J. Virol. 58:583-591.

Lycke, E., Kristensson, K., Svennerholm, B., Vahlne, A. and R. Ziegler, 1984. Uptake and transport of herpes simplex virus in neurites of rat dorsal root ganglia cells in culture. J. Gen. Virol. 65:55-64.

Mackem, S. and B. Roizman, 1982. Differentiation between alpha promoter regulator regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha regulator. Proc. Natl. Acad. Sci. USA. 79:4917-4921.

Maniatis, T., Fritsch, E.F. and J. Sambrook, 1982. Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Manservigi, R., Spear, P.G. and A. Buchan, 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA 74:3913-3917.

Marlin, S.D., Holland, T.C., Levine, M. and J.C. Glorioso, 1985. Epitopes of herpes simplex virus type 1 glycoprotein gC are clustered in two distinct antigenic sites. J. Virol. 53:128-136.

Marlin, S.D., Highlander, S.L., Holland, T.C., Levine, M. and J.C. Glorioso, 1986. Antigenic variation (mar mutations) i herpes simplex virus glycoprotein B can induce temperature-dependent alterations in gB processing and virus production. J. Virol. 59:142-153.

Marsden, H.S., Stow, N.D., Preston, V.G., Timbury, M.C., N.M. Wilkie, 1978. Physical mapping of HSV induced polypeptides J. Virol. 28:624-642.

Martin, S., Moss, B., Berman, P.W., Laskey, L.A. and B.T. Rouse, 1987. Mechanisms of antiviral immunity induced by vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D; cytotoxic T cells. J. Virol. 61:726-734.

Matthews, R.E.F., 1982. Classification and nomenclature of viruses. Fourth Report of the International Committee on Taxonomy of Viruses. Intervirology 17:1-199.

Matthews, J.T., Cohen, G.H. and R.J. Eisenberg, 1983. Synthesis and processing of glycoprotein D of herpes simplex virus types 1 and 2 in an *in vitro* system. J. Virol. 48:521.

McGeoch, D.J., 1985. On the predictive recognition of signal peptide sequences. Virus Res. 3:271-286.

McGeoch, D.J., Dolan, A., Donald, S. and F.J. Rixon, 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. J. Mol. Biol. 181:1-13.

McKnight, S.L. 1980. The nucleotide sequences and transcription map of herpes simplex virus thymidine kinase gene. Nucleic Acid Res. 8:5949-5964.

McLaughlan, J. and J.B. Clements, 1982 A 3'co-terminus of two early herpes simplex virus type 1 mRNAs. Nucl. Acid. Res. 10:501-512.

McMaster, G.K. and G.G. Carmichael. 1977. Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyosyl and acridine orange. Proc. Natl. Acad. Sci. USA. 74:4835.

Mocarski, E.S. and B. Roizman, 1981. Site specific inversion sequence of herpes simplex virus genome: Domain and structural features. Proc. Natl. Acad. Sci. USA. 78:7047-7051.

Mocarski, E.S. and B. Roizman, 1982a. Herpesvirus-dependent amplification and inversion of cell associated viral thymidine kinase gene flanked by viral α sequences and linked to an origin of viral DNA replication. Proc. Natl. Acad. Sci. USA. 79:5626-5630.

Mocarski, E.S. and B. Roizman, 1982b. The structure and role of herpes simplex virus DNA termini in inversion,

circularization and generation of virus DNA. Cell 31:89-97.

Modrow, S., Hahn, B.H., Shaw, G.M., Gallo, R.C., Wong-Staal, F. and H. Wolf. 1987. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. J. Virol. 61:570-578.

Montreiul, J., 1980. Primary structure of glycoprotein glycans: basis for the molecular biology of glycoproteins. Adv. Carbohydr. Chem. Biochem. 37:158-213.

Morgan, C., Rose, H.M., Holden, M. and E.P. Jones, 1959. Electron microscope observations on the development of herpes simplex virus. Journal of Exp. Med. 110:643-656.

Morgan, C., Rose, H.M. and B. Mednis, 1968. Electron microscopy of herpes simplex virus I. Entry. J. Virol. 2:507-516.

Muller, M.T., 1987. Binding of the herpes simplex virus immediate early gene product ICP4 to its own transcription start site. J. Virol. 61:858-865.

Nahmias, A.J. and B. Norrild, 1980. Oncogenic potential of herpes simplex viruses and their association with cervical neoplasia. In: Oncogenic Herpesviruses Vol. 2 (F. Rapp, ed.) pp. 25-45., CRC Press, Inc., Boca Raton, FL.

Nahmias, A.J. and B. Roizman, 1973. Infection with herpes simplex viruses 1 and 2. New England J. of Med, 289:781-789.

Nash, A.A., Leung, K.N., and P. Wildy, 1985. The T-Cell-Mediated Immune response of mice to herpes simplex virus. In Th Viruses, Vol. 4. The Herpesviruses (Roizman, B and C. Lopez, eds.) pp. 87-102, Plenum Press, New York.

Norrild, B. 1985. Humoral response to herpes simplex virus infections. In The Viruses, Vol. 4. The Herpesviruses (Roizman, B. and C. Lopez, eds.) pp. 69-86. Plenum Press, New York.

Norrild, B. and B.F. Vestergaard, 1977. Polyacrylamide gel electrophoretic analysis of herpes simplex virus type 1 glycoproteins and their expression on the cell surface. J. Virol. 43:395.

Norrild, B., Shore, S.L., and A.J. Nahmias, 1979. Herpes simplex virus glycoproteins: participation in immune cytolysis and their correlation with previously identified glycopeptides. J. Virol. 2:741-749.

Norrild, B., Shore, S.L., Cromeans, T.L. and A.J. Nahmias, 1980. Participation of three major glycoprotein antigens of herpes simplex virus type 1 early in the infectious cycle as determined by antibody-dependent cell-mediated ctyotoxicity Infect. Immun. 28:38-44.

Norrild, B. and B. Pederson, 1982. Effect of tunicamycin on the synthesis of herpes simplex virus type 1 glcyoproteins and their expression on the cell surface. J. Virol. 43:395-402.

Norrild, B., Lehto, V.P., and I. Virtanen, 1986. Organization of cytoskeleton elements during herpes simplex virus type I infection of human fibroblasts: an immunofluorescence stud . J. Gen. Virol. 67:97-105.

O'Hare, P. and G.S. Hayward, 1985. Three transacting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. 56:723-733.

Olofsson, S., Khanna, B., and E. Lycke, 1980. Altered kinetic properties of sialyl and galactosyl transferases associated with herpes simplex virus infection of GMK and BHK cells. J. Gen. Virol. 47:1-9.

Olofsson, S., Blomberg, J. and E. Lycke, 1981a. O-glycosidic carbohydrate-peptide linkages of herpes simplex virus glycoproteins. Arch. Virol. 70:312-329.

Olofsson, S., Jeansson, S. and E. Lycke, 1981b. Unusual lectin-binding properties of a herpes simplex virus type 1-specific glycoprotein. J. Virol. 38:564-570.

Olofsson, S., Sjoblom, I., Lundstrom, M., Jeansson, S. and E. Lycke, 1983. Glycoprotein C of herpes simplex virus type 1: characterization of O-linked oligosaccharides. J. Gen. Virol. 64:2735-2747.

Olofsson, S., Lundstrom, M., Marsden, H., Jeanson, S. and A. Vahlne, 1986. Characterization of a herpes simplex virus type 2-specified glycoprotein with affinity for N Acetylgalactosamine-specific lectins and its identification as g92K or gG. J. Gen. Virol. 67:737-744.

Pachl, C., Burke, R.L., Stuve, L.L., Sanchez-Pescador, L., Van Nest, G., Masiary, F. and D. Dina, 1987. Expression of cell-associated and secreted forms of herpes simplex virus type 1 glycoprotein gB in mammalian cells. J. Virol. 61:315-325.

Para, M.F., Goldstein, L. and P.G. Spear, 1982a. Similarities and differences in the Fc-binding glycoprotein (gE) of herpes simplex virus types 1 and 2 and tentative mapping of the viral gene for this glycoprotein. J. Virol. 41:137.

Para, M.F., Bauke, R.B. and P.G. Spear, 1982b. Glycoprotein gE of herpes simplex virus type 1: Effects of anti gE on virion infectivity and on virus induced Fc binding receptors. J. Virol. 41:129-136.

Parker, J.M.R, Guo, D., R.S. Hodges, 1986. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: Correlation of predicted surface residues with antigenicity and X-ray derived accessible sites. Biochemistry, 25:5425-5432.

Paul, M., Goldsmith, M.R., Hansley, J.R. and F.C. Kafatos. 1972. Specific protein synthesis in cellular differentiation. J. Cell. Biol. 55:653.

Pellett, P., McKnight, J.L.C., Jenkins, F. and B. Roizman, 1985a. Nucleotide sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing α genes. Proc. Natl. Acad. Sci. USA 82:5870-5874.

Pellett, P.E., Biggin, M.D., Barrell, B., and B. Roizman, 1985. Epstein-Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1. J. Virol. 56:807-813.

Pellett, P.E., Kousoulas, K.G., Pereira, L. and B. Roizman, 1985c. Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: Primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. J. Virol. 53:243-253.

Pellett, P.E., Jenkins, M., Ackerman, M., Sarmiento, M. and B. Roizman, 1986. Transcription initiation sites and nucleotide sequence of a herpes simplex virus 1 gene conserved in Epstein-Barr virus genome and reported to affect the transport of viral glycoproteins. J. Virol. 60:1134-1140.

Pereira, L, Klassen, T. and J.R. Baringer, 1980. Type common and type specific monoclonal antibody to herpes simplex virus type 1. Infect. Immun. 29:724-732.

Pereira, L., Donder, D., Norrild, B. and B. Roizman, 1981. Differential immunologic reactivity and processing of glycoprotein gA and gB of herpes simplex virus types 1 and 2 in vero and HEp-2 cells. Proc. Natl. Acad. Sci. USA 78:5202-5206.

Pereira, L., Dondero, D.V., Gallo, D., Devlin, V., and J.D. Woodie, 1982. Serological analysis of herpes simplex virus types 1 and 2 with monoclonal antibodies. Infect. Immun. 35:363-367.

Perlman, D. and H.O. Halvorson, 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. 167:391-404.

Petrovskis, E.A., Timmins, J.G., Armentrout, M.A., Marchioli, C.C.Yancey, R.J. and L. Post, 1986. DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J. Virol. 59:216-223.

Pizer, L.I., Cohen, G.H. and R.J. Eisenberg, 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. J. Virol. 34:142-153.

Pizer, L.I., Tedder, D.G., Bety, J.L., Wilcox, K.W., and P. Beard, 1986. Regulation of transcription in vitro from herpes simplex virus genes. J. Virol. 60:950-959.

Poliquin, L., Wolfe, G. and G.C. Shore, 1985. Involvement of the Golgi apparatus and a restructured nuclear envelope during biogenesis and transport of herpes simplex virus glycoproteins. J. Histochem. Cytochem. 33:875-883.

Post, L.E. and B. Roizman, 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus type 1 is not essential for growth. Cell 25:227-232

Powell, K.L., Letter, E. and D. J. Purifoy, 1981. Nonstructural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J. Virol. 39:894-902.

Preston, C.M., 1979. Abnormal properties of an immediate early polypeptide in cells infected with herpes simplex virus type 1 mutant tsK. J. Virol. 32:357.

Preston, C.M. and M.G. Cordingley, 1982. mRNA- and DNA-directed synthesis of herpes simplex virus-coded exonuclease in Xenopus laevis oocytes. J. Virol. 43:386-394.

Preston, V.G., Davison, A.J., Marsden, M.S., Timbury, M.C., Subak-Sharpe, J.H. and N.M. Wilkie, 1978. Recombinants between herpes simplex virus types 1 and 2: Analyses of genome structures and expression of immediate early polypeptides. J. Virol. 28:499-517.

Preston, V.G., Coates, J.A.V. and F.J. Rixon, 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. 45:1056-1064.

Preston, V.G. and F.B. Fisher, 1984. Identification of the herpes simplex virus type 1 gene encoding the UTPase. Virology 138:58-68.

Price, R.W. and A. Kahn, 1981. Resistance of peripheral autonomic neurons to *in vivo* productive infection by herpes simplex virus mutants deficient in thymidine kinase activity. Infect. Immun. 43:571.

Purifoy, D.J.M. and K.L. Powell, 1977. Herpes simplex virus DNA polymerase as a site of phosphonoacetate sensitivity: temperature sensitive mutants. J. Virol. 24: 470-477.

Rafield, L.F. and D.M. Knipe, 1984. Characterization of the major mRNAs transcribed from the genes for glycoprotein B and DNA-binding protein ICP8 of Herpes simplex virus type 1. J. Virol. 49:960.

Rapp, F., 1984. The challenge of herpes viruses. Cancer Research 44:1309-1315.

Rawls, W.E. and E. Adam, 1977. Herpes simplex viruses and human malignancies. In Origins of Human Cancer (H.H. Hiatt, J.D. Watson, and J.A. Winsten, eds.) pp. 1133-1155. Cold Spring Harbor Laboratory, New York.

Read, G.S. and N. Frenkel, 1983. Herpes simplex virus mutants defective in the virion associated shut-off of host polypeptide synthesis of α (immediate early) viral polypeptides. J. Virol. 46:498-512.

Respess, R.A., Pancake, B.A., Edson, C.M. and P.A. Schaffer, 1984. A rapid procedure for the enrichmant of undernatured, antigenically active herpes simplex virus glycoproteins. J. Virol. Methods 8:27-45.

Richman, D.D., Buchmaster, A., Bell, S., Hodgman, C. and A.C. Minson, 1986. Identification of a new glycoprotein of herpes simplex virus type 1 and genetic mapping of the gene that codes for it. J. Virol. 57:647-655.

Robinson, J.E. and G. Miller, 1982. Biology of lymphoid cells transformed by Epstein-Barr virus. In The Herpesviruses Vol.1 (B. Roizman, ed.) pp. 151-207, Plenum Press, New York.

Rock, D.L. and N.W. Fraser, 1983. Detection of HSV-1 genome in central nervous system of latently infected mice. Nature 302:523-525.

Rock, D.L. and W. Fraser, 1986. Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. J. Virol. 55:849-852.

Roizman, B., 1965. An inquiry into the mechanism of recurrent herpes infections of man. Perspectives in Virology 4:283-301.

Roizman, B., Norrild, B., Chan, C. and L. Pereira, 1965. Macromolecular synthesis in cells infected with herpes simplex virus. Nature 206:1374-1375.

Roizman, B., 1974. Herpesvirus, latency and cancer: a biochemical approach. J. Reticuloendothel. Soc. 15:312.

Roizman, B., 1979. The organization of the herpes simplex virus genomes. Ann. Rev. Genet. 13:25-57.

Roizman, B., 1982. The family Herpesviridae. General description, taxonomy and classification. In The Viruses, Vol. 1, Herpesviruses (B. Roizman ed.) pp. 1-23. Plenum Press, New York.

Roizman, B., Norrild, B., Chan, C. and L. Pereira, 1984. Identification and preliminary mapping with monoclonal antibodies of a herpes simplex virus 2 glycoprotein lacking a known type 1 counterpart. Virology 133:242-247.

Roizman, B. and W. Batterson, 1985. Herpesviruses and their replication. In Virology (B.N. Fields et al. eds.) pp. 497-526. Raven Press, New York.

Rosenthal, K., Perey, R. and C. Hodnichak, 1985. Inhibition of herpes simplex virus type 1 penetration by cytochalasins B and D. J. Gen. Virol. 66:1601-1605.

Ruyechan, W.T., Morse, L.S., Knipe, D.M. and B. Roizman, 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. 29:677.

Ruyechan, W.T. and A.C. Weir, 1984. Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. J. Virol. 52:727-733.

Sacks, W.R., Greene, C.C., Aschman, D.P. and P.A. Schaffer, 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. 55:796-805.

Salahuddin, S.Z., Ablashi, D.V., Markham, P.D., Joshephs, S.F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfield, P., Wong-Staal, F., Kramarsky, B., and R. Gallo, 1986. Isolation of a new virus, HBLV. in patients with lymphoproliferative disorders. Science 234:596-601.

Sandri-Goldin, R., Goldin, A., Holland, L., Glorioso, J. and M. Levine, 1983. Expression of herpes simplex virus β and γ genes integrated in mammalian cells and their induction by an α gene product. Mol. Cell. Biol. 2028-2044.

Sanger, F., Nicklen, S. and A.R. Coulson, 1977. DNA sequencing with chain terminating inhibitors. Proc. Nat. Acad. Sci. USA. 74:5463-5467.

Sarmiento, M., Haffey, M. and P.G. Spear, 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B2) in virion infectivity, J. Virol. 29:1149.

Schmid, D.S. and T.B. Rouse, 1983. Cellular interactions in the cytotoxic T lymphocyte response to herpes simplex virus antigens: differential antigen activation requirements for the helper T lymphocyte and cytotoxic T lymphocyte precursors. J. Immunol. 131:479-484.

Serafini-Cessi, F., Dall'Olio, F., Scannavini, M., Costanzo, F., and G. Campadelli-Fiume, 1983a. N-acteylgalactosaminyl transferase activity involved in O-glycosylation of herpes simplex virus type 1 glycoproteins. J. Virol. 48:325-329.

Serafini-Cessi, F., Dall'Olio, F., Scannavini, M., G. Campadelli-Fiume, 1983b. Processing of herpes simplex virus glycams in cells defective in glycosyl

transferases of the Golgi system: Relationship to cell fusion and virion egress. Virology 131:59.

Serafini-Cessi, F., Dall'Olio, F., Pereira, L. and G. Campadelli-Fiume, 1984. Processing of N-linked oligosaccharides from precursor-to-mature-form herpes simplex virus type 1 glycoprotein C. J. Virol. 51:838-844.

Sharp, J.A., Wagner, M.J. and W.C. Summers, 1983. Transcription of herpes simplex virus gene *in vivo*: overlap of a late promoter with the 3' end of the early thymidine kinase gene. J. Virol. 45:10-17.

Sheldrick, P., Laithier, M., Larria, D., and M.L. Ryhinder, 1970. Infectious DNA from herpes simplex virus: Infectivity of double- and single-stranded molecules. Proc. Natl. Acad. Sci. USA 70:3621-3625.

Sheridan, J.F., Donneberg, A.D., Aurelian, L. and D.J. Elpern, 1982. Immunity to herpes simplex virus type 2. IV. Impaired lymphokine production during recrudescence correlates with an imbalance in T lymphocyte subsets. J. Immunol. 129:326-331.

Showalter, S.D., Zweig, M., and B. Hampar, 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP-4. Infect. Immun. 34:684- 692.

Silver, S. and B. Roizman, 1985. γ_2-Thymidine Kinase chimeras are identically transcribed but regulated as γ_2 genes in the herpes simplex virus genomes and as β genes in cell genomes. Mol. Cell. Biol. 5:518-528.

Silverstein, S., Millette, R., Jones, P. and B. Roizman, 1976. RNA synthesis in cells infected with herpes simplex virus XII. Sequence complexity and properties of RNA differing in extent of adenylation. J. Virol. 18:977-991.

Smiley, J., Fong, B. and W.C. Leung, 1981. Construction of a double jointed herpes simplex virus DNA molecule: Inverted repeats are required for segment inversion and direct repeat promote deletions. Virology 111:345-362.

Smiley, M.L. and H.M. Friedman, 1985. Binding of complement C3b to glycoprotein C is modulated by sialic acid on herpes simplex virus type 1-infected cells. J. Virol. 55:857-861.

Smith, H.O. and M.L. Birnstiel. 1976. A simple method of DNA restriction site mapping. Nucl. Acids. Res. 3:2387.

Snowden, W. and I.W. Halliburton, 1985. Identification of cross reacting glycoproteins of four herpesviruses by western blotting. J. Gen. Virol. 66:2039-2044.

Snowden, B.W., Kinchington, P.R., Powell, K.L. and I.W. Halliburton, 1985. Antigenic and biochemical analysis of gB herpes simplex virus type 1 and type 2 and of cross-reacting glycoprotein induced by bovine mammillitis virus and equine herpes virus type 1. J. Gen. Virol. 66:231-247.

Spear, P.G., 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. J. Virol. 17:991-1008.

Spear, P.G., 1985. Glycoproteins specified by herpes simplex viruses. In The Herpesviruses, Vol. 3. (B. Roizman, ed.). pp. 315-347, Plenum Press, New York.

Srinivas, R.V., Balachandran, N., Alonso-Caplen, F.V., and R.W. Compans, 1986. Expression of herpes simplex virus glycoproteins in polarized epithelial cells. J. Virol. 58:689-693.

Stevens, J.G., Wagner, E.K., Devi-Rao, G.B., Cook, M.L. and L.T. Feldman. 1987. RNA complementary to a herpesvirus α gene mRNA is prominent in latently infected neurons. Science 235:1056-1059.

Stow, N.D., 1985. Mutagenesis of a herpes simplex virus origin of DNA replication and its effect on viral interference. J. Gen. Virol. 66:31-42.

Stringer, J., Holland, J.L., Swanstrom, R., Pivo, K. and E. Wagner, 1977. Quantitation of herpes simplex virus type 1 RNA in infected HeLa cells. J. Virol. 21:889-901.

Tainer, J.A., Getzoff, E.D., Alexander, H., Houghten, R.A., Olson, A.J. and R. Lerner, 1984. The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in the protein. Nature, 312:127-134.

Takasaki, S. and A. Kobata, 1986. Asparagine-linked sugar chains of fetuin: occurrence of tetrasialyl triauntennary chains containing the Galβ1- 3GlcNAc sequence. Biochemistry 25:5709-5715.

Tenser, R.B. and M.E. Dunstan, 1979. Herpes simplex virus thymidine kinase expression in infection of the trigeminal ganglion. Virology, 99:417-422.

Tenser, R.B., Miller, R.L. and F. Rapp, 1979. Trigeminal ganglion infection by thymidine kinase-negative mutants of herpessimplex virus. Science 205:915.

Thomas, P., 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad.Sci. USA 77:5201-5205.

Tognon, M., Manservigi, R., Cavini, V., and G. Campadelli-Fiume, 1984. Characterization of a herpes simplex virus mutant resistant to benzyhydrazone, a selective inhibitor of herpesvirus glycosylation. Proc. Natl. Acad. Sci., USA 81:2440-2443.

Vahlne, A., Svennerholm, B., Sandberg, M., Hamberger, A., and E. Lycke, 1980. Differences in attachment between herpes simplex type 1 and 2 viruses to neurons and glial cells. Infect. Immun. 28:675-680.

Van Regenmortel, M.H., 1986. Which structural features determine protein antigenicity? T.I.B.S. 11:36-39.

Vestergaard, B.F., 1980. Herpes simplex virus antigens and antibodies. A survey of studies based on quantitative immuno-electrophoresis. Rev. Infect. Dis. 2:899-913.

Vlazny, D.A., Kwong, A. and N. Frenkel, 1982. Site specific cleavage packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full length viral DNA. Proc. Natl. Acad. Sci. USA 79: 1423-1427.

Von Heifne, G., 1984. How signal sequences maintain cleavage specificity. J. Mol. Biol. 173:243-251.

Wadsworth, S., Jacob, R.J. and B. Roizman, 1975. Anatomy of herpes simplex virus DNA. II. Size, composition and arrangement of inverted terminal repetitions. J. Virol. 15:1487-1497.

Wagner, E.K., 1985. Individual HSV transcripts: Characterization of specific genes. In Herpesviruses, Vol. 3 (B. Roizman, ed.). Plenum Press, New York.

Warren, K.G., Brown, S.M., Wroblewska, Z., Gilden, D., Koprowski, H. and J. Sharpe, 1978. Isolation of latent herpes simplex virus from the superior cervical and vagus ganglions of human beings. N. Eng. J. Med. 298:1068-1070.

Watson, R.J. and J.B. Clements, 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature 285:329-330.

Watson, K., Stevens, J.G., Cook, M.L. and J.H. Subak-Sharpe, 1980. Latency competence of thirteen HSV-1 temperature sensitive mutants. J. Gen. Virol., 49:149.

Watson, R.J., Weis, J.H., Salstrom, J.S. and L.W. Enquist. 1982. Herpes simplex virus type-1 glycoprotein D gene. Nucleotide sequence and expression in Escherichia coli. Science 218:381-384.

Webster, R.G., Laver, W.G., Air, G.M. and G.C. Schild, 1982. Molecular mechanisms of variation in influenza viruses. Nature, 296:115-121.

Weinstein, J., de Souza-e-Silva, U and J.C. Paulson. 1982a. Purification of a Galß1->4GlcNAc α2->6 Sialyltransferase and a Galß1->3(4)GlcNAc α2->3 Sialyltransferase to homogeneity from rat liver. J. Biol. Chem. 257:13835-13844.

Weinstein, J., de Souza-e-Silva, and J.C. Paulson, 1982b. Sialylation of glycoprotein oligosaccharides N-linked to asparagine. J. Biol. Chem. 257:13845-13853.

Weller, S.K., Spadaro, A., Schaffer, J.E., Murray, A.W., Maxam, A.M. and P.A. Schaffer, 1985. Cloning, sequencing and functional analysis or ORI_L, a herpes simplex virus type 1 origin of DNA synthesis. Mol. Cell. Biol. 5:930-942.

Weis, J.H., Enquist, L.W., Salstrom, J.S. and R.J. Watson, 1983. An immunologically active chimeric protein containing herpes simplex virus type 1 glycoprotein D. Nature 302:72-74.

Weizhong, C., Person, S., Warner, S.C., Zhou, J., and N.A. DeLuca, 1987. Linker-insertion nonsense and restriction-site deletion mutants of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. 61:714-721.

Wenske, E.A., and R.J. Courtney, 1983. Glycosylation of herpes simplex virus type 1 glycoprotein C in the presence of tunicamycin. J. Virol. 46:297-301.

Wheeler, C.E., 1975. Pathogenesis of recurrent herpes simplex infections. J. Invest. Dermatol. 65:341.

Whitley, R.J., Nahmias, A.J., Visintine, A.M., Fleming, C.L. and C.A. Alford, 1980. The natural history of herpes simplex virus infection in the mother and newborn. Pediatrics 66:489-494.

Whitley, R.J., Levin, M. and N. Barton, 1984. Infections caused by herpes simplex virus in immunocompromised host:natural history and topical acyclovir therapy. J. Infect. Dis.150:323-329.

Whitton, J.L. and J.B. Clements, 1984. Replication origins and a sequence involved in coordiante induction of the immediate-early gene family are conserved in an intergenic region of herpes simplex virus. Nucl. Acids. Res. 12:2061-2079.

Wright, C.S., 1984. Structural comparison of the two distinct sugar binding sites in wheat germ agglutanin Isolectin II. J. Mol.Biol. 178:91-104.

Yasukawa, M. and J.M. Zarling, 1984. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. I. Lysis restricted by HLA class II MB and DR antigens. J. Immunol. 133:422-427.

Zarling, J.M., Moran, P.A., Lasky, L.A. and B. Moss, 1986. Herpes simplex virus (HSV)-specific human T-cell clones recognize HSV glycoprotein D expressed by recombinant vaccinia virus. J. Virol 59:506-509.

Zezulak, K.M. and P.G. Spear, 1983. Characterization of a herpes simplex virus type 2 75,000 molecular-weight glycoprotein antigenically related to herpes simplex virus type 1 glycoprotein C. J. Virol. 47:553

Zezulak, K.M., and P.G. Spear, 1984a. Mapping of the structural gene for the herpes simplex virus type 2 counterpart ofherpes simplex virus type 1 glycoprotein C and identification of a type 2 mutant which does not express this glycoprotein. J. Virol. 49:741.

Zezulak, K.M. and P.G. Spear, 1984b. Limited proteolysis of herpes simplex virus glycoproteins that occurs during their extraction from Vero cells. J. Virol. 50:258-262.

Zweig, M., Showalter, S.D., Bladen, S.V., Heilman, C.J. and B. Hampar, 1983. Herpes simplex virus type 2 glycoprotein gF and type 1 glycoprotein gC have related antigenic determinants. J. Virol. 47:185.

Zweig, M., Showalter, S.D., Simms, D.J. and B. Hampar, 1984. Antibodies to a synthetic oligopeptide that react with

herpes simplex virus type 1 and 2 glycoprotein C. J. Virol. 51:430- 436.