THE PROTEIN PRODUCTS OF HERPES SIMPLEX VIRUS TYPE 1 GENES UL31, UL45, UL46 AND UL47

by

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Gordon William McLean (December 1990).

SUMMARY

Herpes simplex virus type 1 (HSV-1), contains a large (153Kb) double-stranded DNA genome, the complete sequence of which has now been fully determined. Of the predicted open-reading frames (ORFs), a number have still to have a protein product assigned to them and their ability to encode a polypeptide confirmed. The availability of this sequence has allowed the use of short oligopeptides to generate antisera reactive with HSV-1 proteins of which the peptide was predicted to form a part. This technique has been used successfully to identify a number of HSV-1 encoded polypeptides or to assign previously identified viral encoded proteins to their ORF. Unfortunately the antipeptide sera produced using the standard method whereby peptides are coupled to carrier proteins are often of low titre making experimentation difficult.

Work presented here has shown that peptides presented in a branched form attached to a polylysine core were more immunogenic than monomeric peptides coupled to carrier proteins or peptides attached to a resin matrix. Branched peptides elicited both higher titre antipeptide and antiprotein immune reponses in rabbits. In addition, these responses were achieved after a single immunisation of peptide, whereas both other forms of antigen required two or more immunisations to produce a response. Branched peptides were also shown to be successful at producing high titre antipeptide responses in inbred mice when presented in combination with a "foreign" T_H-cell epitope.

Antisera generated in this study were used to identify the protein products of four HSV-1 genes namely; UL31, UL45, UL46 and UL47. The product of gene UL31 has an apparent M_r of 30,000. This polypeptide was first detected in infected cells in minor amounts at 5 hours after infection at 37° C but was later (from 10-18 hours) present in large amounts. UL31 regulation was investigated using phosphonoacetic acid (PAA), an inhibitor of viral DNA synthesis: the regulation of the 30K protein was compared

with 65K$_{DBP}$, an early gene product, and 21/22K, a true-late gene product. This data demonstrated that UL31 is regulated as a true-late gene.

The product of gene UL45 has an apparent M_r of 38,000. This polypeptide is a component of HSV-1 virions; it was first detected in infected cells in minor amounts at 1 hour after infection at 37° C but was later (12-24 hours) present in large amounts. This polypeptide therefore displayed the kinetics of an early protein.

The product of gene UL46 has an apparent M_r of 100,000. This protein was first detected in infected cells at 2 hours after infection at 37° C but was later (8-24 hours) present in large amounts. This polypeptide also displayed the kinetics of an early protein.

An antiserum raised against a peptide from the predicted product of gene UL47 has identified two encoded proteins. They have apparent M_r of 82,000 and 81,000 and have both been demonstrated to be major components of the virion located in the tegument. The 82/81K proteins were first detected in infected cells in minor amounts 6 hours after infection at 37° C but were later (from 10 to 24 hours) present in large amounts. Regulation was investigated using PAA as described for the UL31 gene product and the data showed that UL47 is regulated as a true-late gene.

ABBREVIATIONS

ABTS	2'2 azino-bis(3-ethylbenzthazoline-6-sulfonic acid)
ATP	adenosine triphosphate
BHK	baby hamster kidney
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumin
Ci	Curie(s)
cm	centimeters
CMV	cytomegalovirus
cpe	cytopathic effects
DAB	bis-diazotised benzidine
DADT	N, N' -diallyltartardiamide
DBP	DNA binding protein
DCCI	dicyclohexylcarbodiimide
DCM	dichloromethane
DMF	dimethylformamide
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
Fab	antibody binding fragment (immunoglobulin)
Fc	crystalisable fragment (immunoglobulin)
Fmoc	9-Fluorenyl-methoxycarbonyl
FMDV	foot and mouth disease virus
g	gram(s)
g(B)	glycoprotein (B)
h	hour(s)
HCMV	human cytomegalovirus
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7

HPLC	high pressure liquid chromatography
HPR	horseradish peroxidase
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVS	herpes virus saimiri
HOBT	1-hydroxylbezotriazole-monohydrate
ICP	infected cell polypeptide (HSV-1)
ICSP	infected cell specific polypeptide (HSV-2)
IE	immediate early
Ig	immunoglobulin
IgG	immunoglobulin G
IR	internal repeat
K	kilodalton
kb	kilobase
l	litre(s)
M	molar
mA	milliamp(s)
mCi	millicurie(s)
MDB	major DNA binding protein
2-ME	2-mercaptoethanol
MI	mock infected
min.	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
moi	multiplicity of infection
mol. wt.	molecular weight
M_r	relative molecular mass
mRNA	messenger ribonucleic acid

Mtr	4-Methoxy-2,3,6-trimethylphenyl-sulphonyl
nm	nanometre(s)
NPT	non-permissive temperature
NRS	normal rabbit serum
OD	optical density
ORF	open reading frame
ori	origin of replication
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFP	Pentafluorophenyl ester
pfu	plaque forming units
pi	post infection
PI	pre-immune
R1	large subunit ribonucleotide reductase
R2	small subunit ribonucleotide reductase
rpm	revolutions per minute
RT	room temperature
s	second(s)
SDS	sodium dodecyl sulphate
SWM	sperm whale myoglobin
t-Boc	tert-Butyloxycarbonyl
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethylethylene diamine
TFA	trifluoroacetic acid
TR	terminal repeat
ts	temperature sensitive
Tween-20	polyoxyetheylene sorbitan monolaurate
U	unique
uv	ultra violet

Vmw	virus-specific polypeptide of apparent molecular weight
vol	volume
v/v	volume per volume
VP	viral polypeptide
VZV	varicella-zoster virus
w/v	weight per volume
uCi	microCurie(s)
ug	microgram(s)
ul	microlitre(s)
uM	micromolar

THREE AND ONE LETTER AMINO ACID CODES

AMINO ACID	THREE LETTER CODE	ONE LETTER CODE
alanine	Ala	A
arginine	Arg	R
arparagine	Asn	N
aspartic acid	Asp	D
cysteine	Cys	C
glutamic acid	Glu	E
glutamine	Gln	Q
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

INTRODUCTION.

The work presented in this thesis concerns a qualitative and quantitative evaluation of three different approaches to antigen delivery for the generation of antipeptide antisera. It further describes the use of these antisera to identify and characterise the products of four HSV-1 genes, namely UL31, UL45, UL46 and UL47. The protein products of genes UL31, UL45 and UL46 had not previously been detected while that of UL47 had not been assigned to a gene nor had it been characterised.

1. THE FAMILY HERPESVIRIDAE

Members of the *Herpesviridae* form a large virus family with over eighty identified members and have a range of hosts spanning many species (reviewed by Roizman, 1982). The family is characterised by possessing a linear, double-stranded DNA genome which undergoes an intranuclear replicative cycle. The virion particles are 150-200nm in diameter and conserve a distinct morphological structure comprised of four distinct elements.

The core, which is an electron dense fibrillar spindal surrounded by the genomic DNA (Epstein, 1962; Furlong *et al.*, 1972; Nazerian, 1974). A capsid which is icosahedral in shape and formed from 162 capsomeres of which 150 are hexameric and 12 are pentameric prisms surrounding the core (Wildy *et al.*, 1960; Schrag *et al.*, 1989). Surrounding the capsid is an undefined layer of proteinaceous material known as the tegument (Schwartz and Roizman, 1969; Morgan *et al.*, 1968; Roizman and Furlong, 1974) which comprises about 65% of the virion by volume (Schrag *et al.*, 1989). The capsid and tegument are surrounded by a lipid membrane, containing numerous viral encoded glycoprotein spikes (Morgan *et al.*, 1959;

Wildy *et al.*, 1960; Asher *et al.*, 1969; Spear and Roizman, 1972).

In addition to being morphologically identical and having an intranuclear replicative cycle the herpesviruses share the ability to persist in a latent state in their infected hosts. Despite these similarities, they have however been divided on the basis of their biological properties, into three groups; alpha, beta and gamma, divided on criteria of host range, reproductive cycle, cytopathology and the characteristics of their latent infection (Roizman, 1981, 1982; Mathews, 1982).

The **alphaherpesviruses** frequently establish latency in the ganglia. They have a relatively short replication cycle of less than 24 hours in cell culture. Members of this family include HSV-1, HSV-2, VZV and pseudorabies virus.

The **betaherpesviruses**, have a restricted host range and a longer reproductive cycle, with infection spreading slowly in culture. Infection results in enlarged infected cells and generally establishment of latency in lymphoreticular cells, secretory glands and kidneys. HCMV is an example of a member of this sub-family.

The final sub-family are the **gammaherpesviruses**, these too have a narrow host range which is generally restricted to B or T lymphocytes. They have a variable reproductive cycle, often leading to a persistent rather than lytic infection with establishment of latency in lymphoid tissue. This family includes Epstein-Barr virus.

2. THE HUMAN HERPESVIRUSES.

To date seven different herpesviruses have been identified that are capable of infecting humans: HSV-1, HSV-2, VZV, HCMV, EBV ; HHV-6 (Salahuddin *et al.*, 1986) and HHV-7 (Frenkel *et al.*, 1990). The majority of the

infections caused by these viruses are either mild or asymptomatic, however more serious problems can occur especially in immunocompromised individuals.

2.1 THE PATHOGENICITY OF THE HUMAN HERPESVIRUSES

HSV-1 AND HSV-2: Herpes simplex virus is unusual among viruses in causing a wide variety of clinical syndromes. Diseases that are due to the virus can be divided into two forms; either the primary infection or those resulting from the reactivation of latent virus. A large majority of the population become infected with HSV, and although most primary infections are asymtomatic, there are several exceptions.

The most common primary infection is gingivostomatitis, which results in ulceration of the buccal mucosa. The eye can also be a target for primary infection with the virus able to infect both the conjunctiva and the cornea, producing a vesicular eruption and swelling of the eyelid. More serious manifestations can also occur, acute necrotising encephalitis is a very rare but extremely serious disease, resulting in severe necrosis of the temporal lobe of the brain. This disease can also take a milder form which has a better prognosis and is usually found in children. It is unclear whether encephalitis due to HSV is as a result of a primary infection or a reactivation.

The genital region is also a common site of primary infection, which takes the form of a vesicular eruption. In the majority of cases this infection is caused by HSV-2, however a growing number of cases of genital herpes have been shown to be as a result of HSV-1 infection. Also more commonly associated with HSV-2 is a severe generalised infection in neonates due to a genital infection in the mother. This can prove extremely serious with a high case mortality rate.

Reactivation of latent HSV can be triggered by

nerve damage or trauma and also by stimulation of the neuron and can occur sporadically throughout life (Stevens, 1975; Wildy *et al.*, 1982; Hill, 1985). The most common clinical forms of reactivation are "cold sores", which occur around the mucocutaneous junctions of the nose and mouth. Reactivation causing genital lesions can also occur and is generally associated with HSV-2. Less common, is reactivation affecting the eye, recurring dendritic ulcers can be produced which in a few cases can lead to scarring and blindness. Reactivation can also pose problems in patients whose immune system is depleted, either through immunosuppressive therapy (e.g. organ transplant patients) or through disease that depletes the host immune system like infection with HIV.

VZV: Varicella-zoster virus causes two different diseases: Varicella- the primary infection and zoster, a recurrent manifestation of the primary infection. Varicella more commonly known as chicken-pox is a common childhood fever with a characteristic rash. Zoster or shingles is a reactivation of the latent virus from the dorsal root or cranial nerve ganglia, mainly affecting adults and resulting in the eruption of painful vesicles.

HCMV: Diseases caused by cytomegalovirus are mainly opportunistic infections in patients with lower than normal resistance to infection. Asymptomatic infections are also common, however more serious problems are occasionally encountered. An asymptomatic infection in the mother can lead to infection in the newborn, with about one fifth of these infants going on to develop severe generalised infection (cytomegalic inclusion disease), surviving infants usually suffer from deafness and mental retardation. HCMV has also been associated with the European form of Kaposi's sarcoma (Giraldo *et al.*, 1975). In addition, HCMV can also cause problems in immunocompromised patients and is at present a particular problem with HIV patients.

EBV Epstein-Barr Virus infects B-lymphocytes and is the causative agent of infectious mononucleosis or glandular fever. It has also been linked to nasopharyngeal carcinoma and Burkitt,s lymphoma (Epstein *et al.*, 1964; de-The *et al.*, 1978; de-The, 1982).

HHV-6 Initially termed human lymphotrophic virus, human herpesvirus-6 can infect T-cells *in vitro* and was first isolated from patients with lymphproliferative disorders (Salahuddin *et al.*, 1986), it has been associated with the condition exanthem subitum, a common disease of infancy, characterised by high fever and the appearance of a rash (Yamanishi *et al.*, 1988).

HHV-7 Recently isolated from $CD4^+$ T-cells; human herpesvirus 7 has yet to be connected with any specific disease (Frenkel *et al.*, 1990).

2.2 TRANSFORMATION AND ONCOGENESIS

Herpesviruses have for some time been implicated with tumourogenic activity. In particular HSV-2 has been found to have an association with squamous cell carcinoma of the cervix; although a direct link between HSV infection and subsequent carcinoma development is unclear (reviewed by Rawls, 1985; Macnab, 1987), with evidence somewhat conflicting. There does seem to be some association between previous infection with HSV-2 and a later development of carcinoma, although the presence of HSV gene products and DNA in tissues from cervical carcinoma is detected to a much lesser extent than those from human papilloma virus (Macnab, 1987).

Three regions of the HSV genome which induce cellular transformation have been identified *in vitro* by transfection assays:

Camacho and Spear (1978), identified a transforming region present in HSV-1 which is termed MTR-I (morphological transforming region). This region has been mapped in the Xba I f (0.29-0.45 map units [m.u.]) and Bgl II i (0.311-0.415 m.u.)(Reyes *et al.*, 1979;

Galloway and McDougall, 1983).

MTRs II and III are present in the HSV-2 genome; these are found in restriction fragments BglII n and c respectively (Reyes *et al.*, 1979; Macnab and McDougall, 1980; Galloway and McDougall, 1981; Cameron *et al.*, 1985; Peden *et al.*, 1982). Both of which are required for oncogenesis (Jariwalla *et al.*, 1983, 1986). The minimal transforming region of MTR-II has been localized to a small, putative stem-loop structure of 737bp which is bounded by direct repeats and located in the left-hand portion of the BglII n fragment (Galloway *et al.*, 1984). These authors proposed that a similar structure, present in HCMV, may induce morphological transformation. It was proposed that such structures may be important as they can also be formed by insertion sequences (IS) that are capable of integrating into the cellular genome.

However the evidence in support of this proposal is not strong: Since the 737bp sequence does not conform to the standard description of an IS; it does not appear to be capable of moving around the genome and does not seem to encode a transposase (Lewin, 1990). Although this enzyme could be supplied by other regions of the genome no evidence for this exists. In addition, the significance of these potential secondary structures is unclear as it is possible to derive similar structures from many random DNA sequences (Macnab, 1987), indeed computer scanning has demonstrated many such structures elsewhere in the HSV genome (Shillitoe, 1988).

It is however still unclear what role is played by this region as the evidence is somewhat confusing; other experiments have observed that the left-hand portion of the BglII n fragment caused neither transformation nor mutagenesis in NIH3T3 cells whereas the right-hand end was transforming but not mutagenic (Pilon *et al.*, 1989)

The minimal transforming region of MTR-III that is capable of causing transformation has been mapped to a 486bp fragment located on the PstI c restriction

fragment (Jones *et al.*, 1986). Recent experiments have shown that the 486bp region has been shown to function as a transcriptional promoter when fused to the bacterial CAT gene (Jones, 1989), it was therefore suggested that this fragment might act to alter the expression of a cellular gene. It has been further suggested that the 486bp fragment in combination with cellular factors may initiate unscheduled rounds of cellular chromosonal replication which may ultimately result in cellular transformation (Zhu and Jones, 1990, Abstracts: 15th International Herpesvirus Workshop). In addition BglII c also contains most of the coding sequences for R_1, including a region with protein kinase activity (Chung *et al.*, 1989) and although transformation can be achieved with the 486bp fragment alone a role for R_1 has not been dismissed.

As HSV does not seem to carry a viral oncogene analogous to those found in retroviruses or DNA tumour viruses such as papova or adenoviruses, it is thought that HSV oncogenesis may develop from a disruption of normal cellular events to which HSV may have been involved (Galloway and McDougall, 1983; Macnab *et al.*, 1987). It has been proposed that HSV may act via a "hit and run" type mechanism where activation of a viral or cellular oncogene might occur before the HSV DNA is rapidly exised from the host cell (Skinner, 1976; Galloway and McDougall, 1983).

The failure of HSV-transformed cells *in vitro* to retain viral sequences is in contrast to the small but significant number of cervical cancer biopsies where it has been detected. This difference may however be due to the fact that cervical cancers are generally of epithelial origin whereas the rat cells examined *in vitro* are predominantly fibroblastic (Macnab, 1987). In fact, it is of doubt whether transformation experiments carried out under *in vitro* conditions have any direct correlation to the role played by HSV in causing tumours *in vivo*. However if HSV does play any role in cervical

carcinoma, it is probable that it acts in conjunction with a number of other factors like human papillomavirus (Zur Hausen, 1982; Durst *et al.*, 1983; Boshart *et al.*, 1984).

2.3 LATENCY

A characteristic feature of infection with HSV is the persistance of viral genetic information in a non-infectious state in neurons of the peripheral nervous system (Stevens and Cook, 1971; Baringer and Swoveland, 1973; Galloway *et al.*, 1974; McLennan and Darby, 1980; Hill, 1985). It is this ability to remain associated with their hosts following primary infection that plays an important role in the pathogenic potential of herpesviruses.

Puga *et al.*, (1978) using hybridisation techniques detected HSV DNA in the sensory ganglia of latently infected mice. The precise nature of the viral DNA in latently infected cells is unclear, however it is probably not present as a linear molecule as it has not been possible to detect any terminal genomic fragments (Rock *et al.*, 1983, 1985; Efstathiou *et al.*, 1986). Studies with mouse models of HSV latency have shown that latent DNA does not seem to be integrated into the cellular DNA, rather it appears it is maintained in an episomal state (Rock and Fraser, 1983; Mellerick and Fraser, 1987).

Recently transcription of HSV-1 DNA during latency has been shown to occur, with latency associated transcripts (LATs) detected in latently infected ganglia (Deatly *et al.*, 1987; Stevens *et al.*, 1987; Rock *et al.*, 1987; Spivak and Fraser, 1987; Steiner *et al.*, 1988). Three transcripts of 2.0, 1.5 and 1.45 Kb are present in latently infected neurons and originate from one area of the viral genome located in the repeat regions IR_L and TR_L (Spivak and Fraser, 1987; Wagner *et al.*, 1988a). The 1.5 and 1.45 Kb LATs being spliced (Wagner *et al.*,

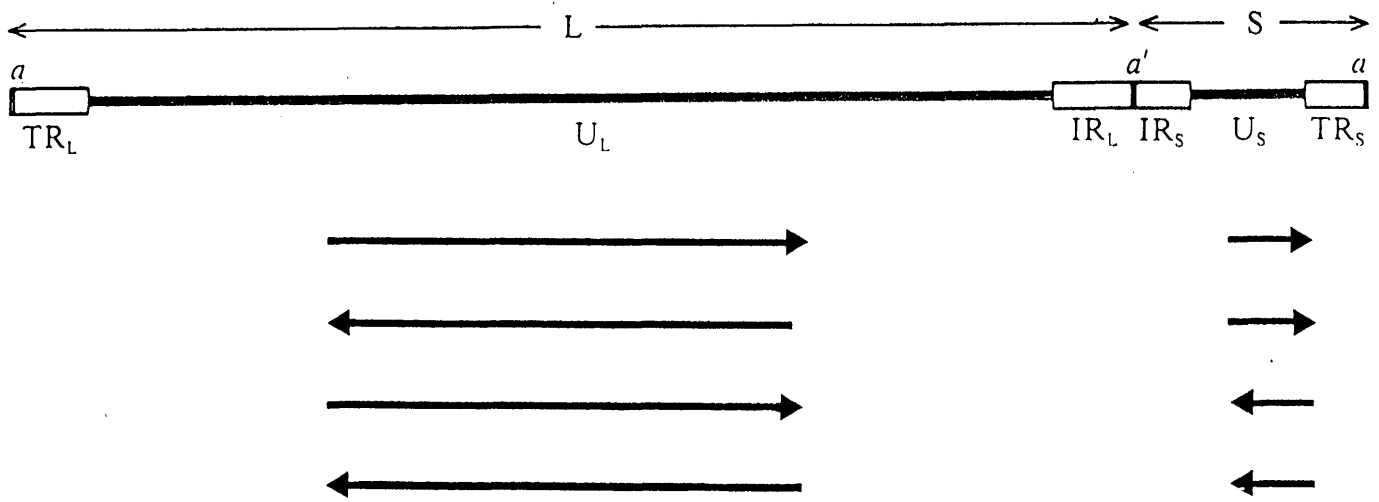
1988b; Wechler *et al.*, 1988). These LATs map to a region of the the genome that partially overlaps with immediate early gene 1 (Vmw 110), although they are transcribed from the opposite strand of the viral DNA (Spivak and Fraser, 1987; Stevens *et al.*, 1987). Open-reading frames have been identified in this region, however no evidence exists for any convincing coding sequences or associated proteins (McGeogh *et al.*, 1988; Weschler *et al.*, 1988, 1989).

It seems likely however, that the LATs are not essential for the establishment of HSV-1 latency, as an HSV-1 x HSV-2 recombinant has been isolated which is unable to express LATs while retaining the ability to establish a latent infection in mice (Javier *et al.*, 1988). Although this recombinant has recently been shown to be impaired in its ability to reactivate *in vivo* using a rabbit eye model (Hill *et al.*, 1990). Additional studies with an HSV-1 deletion mutant 1704 (MacLean and Brown, 1987) which fails to express LATs in tissue culture or in latently infected mice (Steiner *et al.*, 1989) have shown that although 1704 establishes latent infection in mice, reactivation of virus from explanted ganglia was slower than that of wild-type (Steiner *et al.*, 1989). In addition a LAT^- deletion mutant has been isolated that reactivates less efficiently than the parent LAT^+ virus (Leib *et al.*, 1989). The data from these experiments suggests that LATs, although not essential for establishment, play some role in mediating reactivation of latent virus. Recently experiments with a mutant that fails to express LATs during latency, but expresses truncated LATs *in vitro* have indicated that the first 838bp of the transcript seem to be important for biological function (Block *et al.*, 1990).

Both *in vivo* and *in vitro* models have been used to study HSV latency. These studies have been used in attempts to identify the viral functions necessary or dispensible for latent infection, although no gene products have as yet been shown to be individually

FIGURE 1

Organization of the HSV-1 genome. A conventional representation of the HSV-1 genome is shown with unique sequences (U_L and U_S) as solid lines and the major repeat elements (TR_L and IR_L, IR_S and TR_S) as open boxes. Terminal *a* sequences and the internal, inverted orientation *a'* sequence are indicated. The arrows indicating the orientation of the four isomeric forms are also indicated, these isomers occur in equimolar amounts. This figure is modified from McGeoch *et al.*, (1988).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S
a
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U_L
IR_L
IR_S
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TR_S

required (Polvino-Bodnar *et al.*, 1987; Meigneir *et al.*, 1988; Russell *et al.*, 1987). However, *in vitro* studies have implicated immediate early gene 1 in the reactivation of latent HSV as a mutant in IE gene 1 (*d*l1403), fails to reactivate HSV-2 *in vitro* by superinfection (Russell *et al.*, 1987). In addition recent experiments have also indicated a role for the viral thymidine kinase: it is not essential for the establishment of a latent infection but may play a role in reactivation of the virus (Coen *et al.*, 1989; Efstathiou *et al.*, 1989; Tenser *et al.*, 1989). There has also been a report of a mutant in HSV ribonucleotide reductase which fails to establish a reactivatable latent infection (Jacobsen *et al.*, 1989).

3. THE HSV-1 GENOME.

3.1 THE STRUCTURE OF THE HSV-1 GENOME

The genome of HSV-1 is a large (153Kb), linear, molecule consisting of two components, termed L(long) and S(short). These components are made up of unique sequences termed U_L and U_S respectively, which are flanked by inverted repeat sequences TR_L TR_S, IR_L IR_S (Fig 1) (Sheldrick and Berthelot, 1975; Hayward *et al.*, 1975). Each end of the genome shares a small terminal repeat sequence (termed the *a* sequence)(Davison and Wilkie, 1981). A single copy of this *a* sequence is found at the S terminus, with multiple copies detected at the L terminus, and a variable number of copies (in the inverted orientation to the terminal repeats) located internally between the L and the S segments (Wadsworth *et al.*, 1975; Wagner and Summers, 1978).

During viral DNA replication, the two unique regions invert relative to each other (Hayward *et al.*, 1975). The resulting viral DNA consists of an equimolar mixture of four isomeric forms (Fig. 1) differing only in the relative orientation of U_L and U_S (Delius and

Clements, 1976; Wilkie, 1976). The isolation of HSV mutants with genomes "frozen" in all four isomeric arrangements, which are still capable of replicating independently, suggests that inversion is not essential for viral DNA replication (Jenkins and Roizman, 1986).

3.2 THE NUCLEOTIDE SEQUENCE OF HSV-1

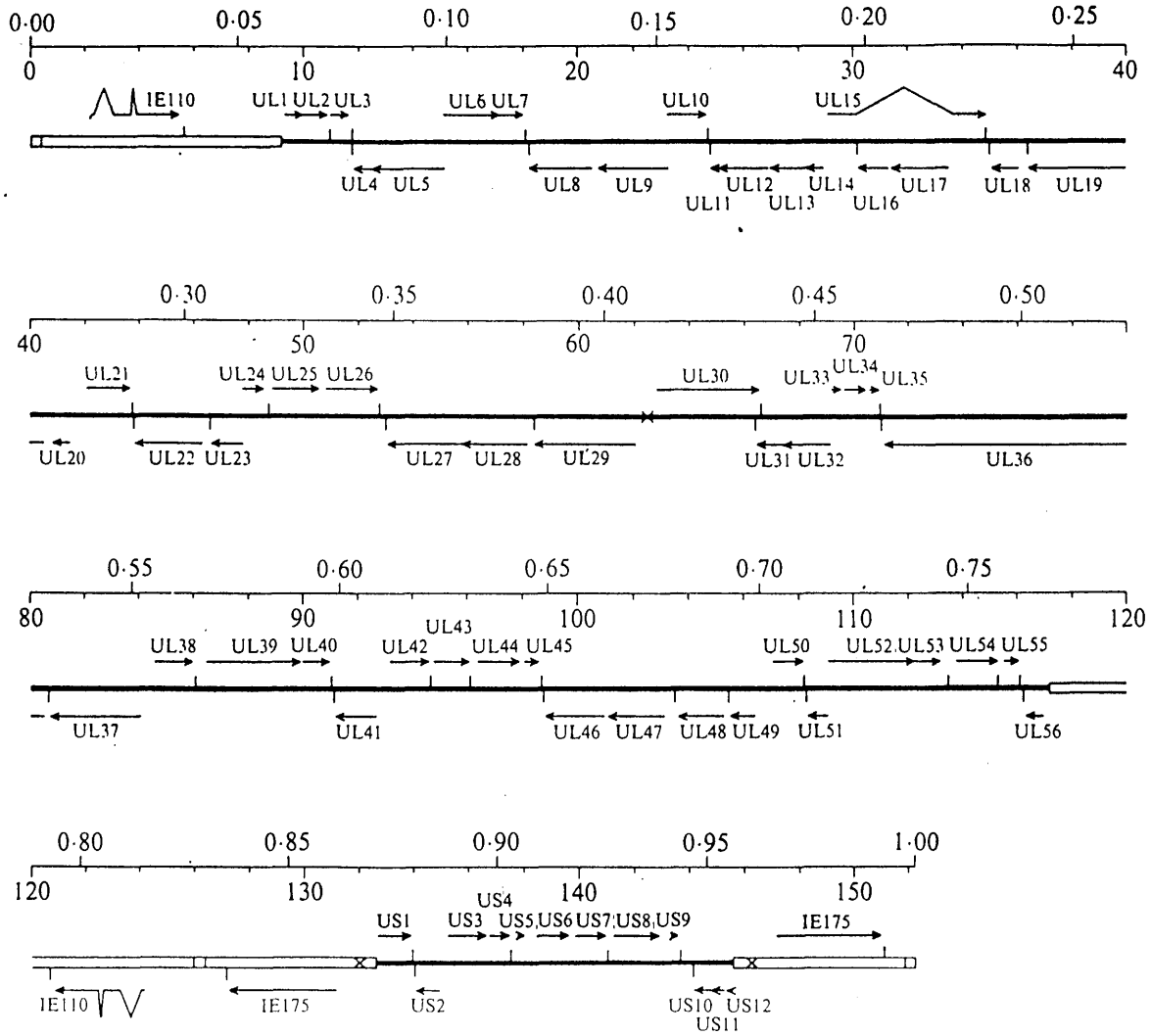
The nucleotide sequence of the entire genome of HSV-1 (strain 17) has now been determined (Davison and Wilkie, 1981; Murchie and McGeoch, 1982; McGeoch *et al.*, 1985; McGeoch *et al.*, 1986; Perry and McGeoch, 1988; McGeoch *et al.*, 1988). The genome contains 152260 residues, although this is slightly variable due to differences in the copy number of the *a* sequence and slight sequence reiterations.

A high percentage of the HSV-1 genome codes for proteins, with the genes densely packed on the genome. 89% of U_L and 79% of U_S is occupied by coding sequences, thought to specify proteins (Fig. 2) (Rixon and McGeoch, 1985; McGeoch *et al.*, 1988). The genome includes 56 genes in U_L (UL1-UL56) and a further 12 in U_S (US1-US12) with one in each copy of R_L and R_S. The analysis of McGeogh *et al.*, 1988, suggested a total of 72 genes encoding 70 distinct proteins. A further gene whose product is designated ICP 34.5, present in R_L upstream of immediate early gene 1 has been identified (Ackerman *et al.*, 1986; Chou and Roizman, 1986). In addition in latently infected cells a major virus-specific transcript, the latency associated transcript (LAT) has been show to initiate downstream of IE gene 1 (Stevens *et al.*, 1987; Wagner *et al.*, 1988; see earlier, section 2.3). It is possible that still further genes will be identified in the future.

Several of the genes encoded by HSV-1 in U_L have been shown to be non-essential for growth *in vitro*, including those genes encoding the viral thymidine kinase and the large sub-unit of ribonucleotide

FIGURE 2

Layout of genes in the genome of of HSV-1. The HSV-1 genome is shown on four successive lines with unique regions represented by solid lines and major repeat elements as open boxes. The lower scale represents kilobases numbered from the left terminus, and the upper scale represents fractional map units. The sizes and orientations of proposed functional ORF's are shown by arrows. Overlaps of adjacent, similarly orientated ORF's are not shown explicitly. Location of origins of DNA replication are shown as X. Locations of proposed transcription polyadenylation sites are indicated as short vertical bars. Genes are numbered UL1-UL56 and US1-US12 plus IE175 and IE110. This figure is taken directly from McGeoch *et al.*, 1988.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IE110
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130
140
150
US4
US1
US3 US5 US6 US7 US8 US9
IE175
IE110
IE175
US2
US10 US12
US11

reductase (Reviewed by McGeoch, 1988, see later), with almost all of U_S encoding genes which are dispensible for growth (Longnecker and Roizman, 1986, 1987; Weber *et al.*, 1987; Brown and Harland, 1987).

4. INFECTION OF CELLS WITH HSV-1

The pathway by which HSV-1 gains entry into host cells still remains to be defined. However HSV infections are thought to involve three distinct steps: Initial virion attachment, followed by fusion of the viral envelope with the cell's plasma membrane, and finally removal of the envelope with the nucleocapsid being released into the cytoplasm of the cell where it migrates to nuclear pores, disassembles, with the viral DNA being released into the nucleus.

4.1 ABSORPTION

Absorption of HSV-1 to the cell surface is a rapid process which may initially be of a non-specific nature, leading to an irreversible binding to the cell surface (Hochberg and Becker, 1968; Rosenthal *et al.*, 1984). It is as yet unclear which viral proteins are involved in binding to the cell surface. Of the identified glycoproteins only gB (Sarmiento *et al.*, 1979; Little *et al.*, 1981), gD (Ligas and Johnson, 1988), and gH (Weller *et al.*, 1983; McGeoch and Davison, 1986; Gompels and Minson, 1986; Desia *et al.*, 1988) are essential for infectivity. Of these three, only gH remains a candidate for a viral glycoprotein essential for absorption; as mutants are available in both gB and gD that retain the ability to absorb to the cell surface, but fail to synthesize viral polypeptides (Cai *et al.*, 1988; Ligas and Johnson, 1988).

It is however possible that any one, or a combination of the glycoproteins, could play a facilitating role in absorption. As absorption can occur

in the absence of normal glycosylation it is also possible that a non-glycoprotein virion component may play a role (Campadelli-Fiume *et al.*, 1982; Spivak *et al.*, 1982; Svennerholm *et al.*, 1982; Kuhn *et al.*, 1988). It seems likely that HSV types 1 and 2 have different receptors on the cell surface (Vahlne *et al.*, 1979; Addison *et al.*, 1984) although WuDunn and Spear (1989) have shown that an initial stage of virion attachment of both HSV-1 and HSV-2 to the cell surface is through an interaction between the virus and heparin-like cell-associated glycoaminoglycans (GAGs).

It has been demonstrated that the antibiotic neomycin inhibits the infection *in vitro* of HSV-1 while it fails to interfere with HSV-2 infection (Langeland *et al.*, 1986). As neomycin has to be present at the time of infection to exert maximum effect it is believed that the drug acts at the receptor attachment level. This effect is observed for other aminoglycosides and also to be dependent on the charge of the particular compound (Langeland *et al.*, 1987). This implies that phosphoinositides have a role in the type 1 receptor interaction, as the ability of aminoglycosides to bind phosphinositides is also related to their cationic charge (Marche *et al.*, 1983) and it has previously been shown that phosphinositides may be involved in the "anchoring" of plasma membrane glycoproteins at the cell surface (Low *et al.*, 1985, 1986). Similar inhibition effects to those exhibited by neomycin have also been demonstrated with polyamino acids such as polylysine and polyarginine which are also thought to be due to interference with the receptor (Langeland *et al.*, 1988; WuDunn and Spear, 1989).

Langeland *et al.*, (1990) took advantage of their observation that neomycin inhibits the binding of HSV-1 but not HSV-2 to map, using intertypic recombinants, the region encoding a protein or proteins involved in the absorption process blocked by neomycin. The region mapped from coordinates 0.580-0.687 which is predicted

to contain 2 partial (UL39 and UL48) and 8 complete (UL40-UL47) genes (McGeoch *et al.*, 1988) including gC and two others with potential transmembrane sequences: UL43 and UL45 (McGeoch *et al.*, 1988).

More recently Campadelli-Fiume *et al.*, (1990) also made use of intertypic recombinants to map neomycin resistance to within the same region. Both they and Langeland *et al.*, (1990) observed that HSV-1 gC$^-$ mutants absorbed more slowly to cells and were fully as sensitive to neomycin and polylysine as the parent virus and both groups concluded that gC-1 was not the virion component which was responsible for sensitivity to these drugs. Implicit in the analysis by Langeland *et al.*, (1990) was the assumption that both gC-1 and gC-2 attached to the same cellular receptor (though the possibility of low affinity type specific receptors was not excluded) and they concluded that whilst gC-1 seemed to facilitate absorption it was not the virion attachment component that was being mapped. However Campadelli-Fiume *et al.*, (1990) have more precisely defined the HSV-2 sequence that confers resistance of HSV-2 to neomycin to either gC-2 or the N-terminal 28 amino acids of gene UL45 and concluded that in BHK cells there exists in addition to the pathway blocked by neomycin and polylysine a pathway which is parallel and gC-2 dependent. That is, gC-1 and gC-2 attach to different receptors in BHK cells. This conclusion seems quite reasonable although insertion of a stop codon in gC-2 in the drug resistant construct of Campadelli-Fiume *et al.*, (1990) produced virus with an unexpectedly wide range of sensitivities to neomycin (4%-70% pfu compared with parent virus).

It is also possible that the N-terminal amino acids of UL45, which constitute the major portion of the region predicted to be external to a transmembrane region (see later) play a role in the putative alternative pathway. Precise DNA sequencing of the relevant regions of the various neomycin/polylysine

resistant constructs will be required to determine exactly what proteins are encoded.

4.2 PENETRATION AND UNCOATING

As previously mentioned gB and gD are non-essential for absorption of the HSV-1, although both have been shown to be required for penetration (Deluca *et al.*, 1982; Bond *et al.*, 1982; Bzik *et al.*, 1984; Johnson and Ligas, 1988), with neutralising monoclonal antibodies against gD not affecting attachment but blocking penetration (Highlander *et al.*, 1987). In addition experiments have shown that soluble forms of gD can inhibit virus entry into cells by binding to a limited number of cell surface receptors (Johnson *et al.*, 1990).

Recent experiments have indicated that following initial interaction with heparin-sulphate, HSV-1 may be able to use the high affinity receptor for fibroblast growth factor (FGF) to penetrate cells after absorption (Kaner *et al.*, 1990). Inhibitors of basic FGF binding to its receptor and competitive polypeptide antagonists of basic FGF have been shown to prevent HSV-1 uptake. In addition a cell line which fails to express basic FGF receptor, Chinese hamster ovary (CHO) cells were shown to be resistant to penetration by HSV-1, whereas following transfection of the gene encoding the basic FGF receptor, CHO cells become susceptible to HSV-1 uptake (Kaner *et al.*, 1990). Interestingly some homology exists between the basic FGF receptor and gD, although somewhat tenuous it does occur in the region of the receptor responsible for substrate binding (Kaner *et al.*, 1990).

Following penetration the viral capsids are then translocated to the nucleus, where, after uncoating the viral DNA then enters the nucleus at a nuclear pore. It is likely a viral-encoded protein(s) play a role in this process, as a *ts* mutant exists which is blocked in release of the DNA; this mutation has located between map units 0.501-0.503 (Knipe *et al.*, 1981; Batterson *et*

al., 1983); a position corresponding to the HSV-1 gene UL36 (McGeoch *et al.*, 1988).

4.3 THE EFFECT OF HSV INFECTION ON THE HOST CELL METABOLISM

Upon infection of cells, HSV causes a disruption of the metabolic processes of the host, in order to provide a suitable environment for the efficient replication of its DNA. There is a rapid decline in the synthesis of host macromolecules: cellular DNA and RNA synthesis is inhibited (Roizman and Roane, 1964), there is a termination of mitosis (Wildy *et al.*, 1961) and protein synthesis decreases rapidly, within 2-4 hours post infection (reviewed by Fenwick, 1984).

As a consequence of infection there is a decline in host mRNA accumulation (Pizer and Beard, 1976; Fenwick, 1984), although some cellular genes are activated, which in some cases is connected with expression of the product of IE gene 3-Vmw 175 (Latchman *et al.*, 1987; Kemp and Latchman, 1988). HSV infection induces efficient shut-off of the majority of host specified polypeptide synthesis (Sydiskis and Roizman, 1966, 1967), with HSV-2 generally more efficient at shut-off than HSV-1 (Powell and Courtney, 1975; Pereira *et al.*, 1977; Fenwick *et al.*, 1979; Schek and Bachenheimer, 1985), although HSV-2 strain HG52 is less efficient than HSV-1 strain 17 (Marsden *et al.*, 1978).

Cells infected in the presence of actinomycin D or with u.v. irradiated virus are still subjected to a rapid shut-off of host protein synthesis, a result which indicates that the shut-off process is controlled by a virion component(s) (Sydiskis and Roizman, 1967; Fenwick and Walker, 1979; Fenwick *et al.*, 1979; Schek and Bachenheimer, 1985). Using mutant viruses incapable of inducing host shut-off, it has been possible to identify the gene involved as UL41 (Read and Frenkel, 1983; Kwong *et al.*, 1988). When this gene was cloned and transferred

from HSV-2 strain G (which exhibits a particularily strong shut-off function) into the early shut-off deficient HSV-1 strain 17, efficient early shut-off was restored (Fenwick and Everett, 1990). Recently HG52 has now been found to encode a truncated UL41 product which most likely accounts for its poor shut-off (Everett and Fenwick, 1990).

In addition, recent experiments which have inserted the rabbit beta-globin gene under the control of its own promoter, into the HSV genome. Have demonstrated that this gene was activated upon infection by HSV immediate-early polypeptides whereas the expression of the endogenous cellular beta-globin gene was strongly suppressed, with beta-globin mRNA degraded (Simbert and Smiley, 1990). This effect was abolished by a LacZ insertion into the reading frame of the UL41 gene. The results of these experiments suggest that the preferential expression of HSV genes that occurs during infection is not ach ved solely through a sequence specific differentation between viral and cellular promoters or mRNAs.

5. HSV-1 DNA REPLICATION

The mechanism of HSV DNA replication is at present poorly understood due primarily to the size and structural complexity of the genome. Most viral DNA molecules have been shown to lo se their free ends shortly after infection, which is thought to be due to circularisation of the genome (Jacob and Roizman, 1977; Davison and Wilkie, 1983; Poffenberger and Roizman, 1985), possibly as a result of direct ligation of the ends (Marks and Spector, 1988). It is likely that ligation is carried out by either a host cell enzyme or a component of the virion particle as fusion of the termini shows no requirement for *de novo* protein synthesis (Poffenberger and Roizman, 1985).

Newly replicated viral DNA probably exists as

circular molecules or more likely large head-to-tail concatamers as examination with restriction enzyme digest shows that it fails to possess any detectable termini, (Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1981). Late in viral infection the ease with which the DNA can be sedimented is thought to be due to the presence of extensive concatamers (Jacob *et al.*, 1979). It is proposed that these molecules are generated via a rolling-circle type replication mechanism, with the HSV DNA intermediates generated, then being processed and packaged into capsids in the nucleus (Becker *et al.*, 1978; Frenkel *et al.*, 1981; Vlazny *et al.*, 1982). This theory has been supported by recent experiments utilising an *in vitro* HSV DNA replication system at a preformed replication fork (Rabkin and Hanlon, 1990). These workers have used electron microscopy and alkaline agarose gel electrophoresis to show that DNA replication in this system is via a rolling circle type mechanism.

5.1 ORIGINS OF DNA REPLICATION

Analysis of defective HSV genomes generated during serial passage of the virus at high multiplicity infections, provided evidence for the existence of *cis*-acting origins of replication (Frenkel *et al.*, 1975, 1976; Schroder *et al.*, 1975; Frenkel *et al.*, 1980; Kaerner *et al.*, 1981). It was demonstrated that monomeric units from defective genomes could be amplified, regenerating tandemly repeated defective DNAs, when co-transfected with wild-type HSV DNA which provides essential helper functions *in trans* (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982) so providing evidence for the presence of replication origins.

These origins of replication were fine mapped by identifying DNA fragments capable of inducing replication of plasmid sequences, when vectors containing the sequence of interest were introduced into

HSV-infected cells (Stow, 1982). Two copies of ori_S are located in the R_S segments of the genome (Stow and McMonagle, 1983) containing a 90bp fragment with a 45bp palindromic sequence featuring a central AT rich region, which by comparison with other known origin sequences is potentially a site of protein induced unwinding (Challberg and Kelly, 1989). It has been demonstrated that disruption of sequences making up the palindrome abolishes origin function (Stow, 1985). The single copy of ori_L is located in U_L between the genes encoding the DNA polymerase and the major DNA binding protein (Weller *et al.*, 1985) and contains a 72bp palindrome which shows 85% homology to ori_S (Gray and Kaerner, 1984; Quinn and McGeoch, 1985).

6. POLYPEPTIDES ENCODED BY HSV-1

6.1 GENES INVOLVED IN HSV-1 DNA REPLICATION

Proteins involved in the replication of HSV DNA fall into two catagories. Firstly those which are required directly for DNA synthesis and also those factors involved in nucleotide metabolism.

6.1.1 Genes required directly for DNA synthesis

The complete set of viral genes which are essential for DNA replication were identified by the use of a transient complementation assay (Challberg, 1986; Wu *et al.*, 1988). This was used to identify loci on the HSV genome which were required to replicate a bacterial plasmid containing the HSV-1 origin of replication ori_S. Comparison with the DNA sequence of McGeoch *et al.*, (1988) identified two sets of genes: Those which are essential for the replication of the origin containing plasmid and those genes which, although not absolutely required were able to increase the amplification of the plasmid, this latter group containing IE genes 1, 2 and 3 and the genes specifying ribonucleotide reductase.

There are seven HSV genes that have been shown to be essential for replication of viral DNA, all of which are located in the long segment of the genome: UL5, UL8, UL9, UL29, UL30, UL42 and UL52. Of these the genes encoding the major DNA binding protein (MDBP) and the viral DNA polymerase, encoded by genes UL29 and UL30 respectively had already been identified and shown to be essential by genetic studies of mutant viruses (Hay and Subak-Sharpe, 1976; Chartrand *et al.*, 1979; Conely *et al.*, 1981; Weller *et al.*, 1983; Coen *et al.*, 1984; Gibbs *et al.*, 1985). Although genetic studies suggest that no other HSV gene products are essential for DNA replication, it is howevever a possibility that one or more as yet unidentified host cell proteins might also play an essential role.

6.1.1.1 MDBP

This protein is encoded by gene UL29. The protein binds preferentially to single stranded DNA (ssDNA) with no detectable sequence specificity (Bayliss *et al.*, 1975; Powell and Purifoy, 1976; Conely *et al.*, 1981; Knipe *et al.*, 1982). MDBP also seems to have a role in gene regulation since viral *ts* mutants in MDBP overexpress gC, with the MDBP also playing a role in the repression of the IE 3 and major capsid protein (Vmw 155) genes (Godowski and Knipe, 1983, 1985, 1986). It is believed that the MDBP may bind to ssDNA formed at a replication fork, by the unwinding of the parental DNA, so facilitating the use of these strands as templates for DNA polymerase (Challberg and Kelly, 1989).

6.1.1.2 Viral DNA polymerase(UL30)

Extracts of HSV-infected cells contain a DNA polymerase (*pol*) which is biochemically distinct from host DNA polymerases (Keir and Gold, 1963; Purifoy *et al.*, 1977). Experiments with *ts* and drug-resistant mutants has shown that the polymerase is virally encoded and essential for DNA synthesis (Aron *et al.*, 1975; Hay

et al., 1976; Purifoy and Powell, 1981). *pol* shows several regions of homology with both prokaryotic and eukaryotic DNA polymerases, including the mammalian replicative DNA polymerase (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985; Wong *et al.*, 1988; Knopf, 1986; Larder *et al.*, 1987), although it differs from most eukaryotic polymerases by possesion of a 3' to 5' exonuclease (proof reading) activity (Knopf, 1979; O'Donnell *et al.*, 1987b; Marcy *et al.*, 1990). The *pol* has also recently been shown to posses a 5'-3' exonuclease activity capable of functioning as a RNaseH (Crute and Lehman, 1989; Marcy *et al.*, 1990).

Experiments showed that the 140kd polymerase product of HSV-2 co-purified with a 55Kd polypeptide (Vaughan *et al.*, 1984) and this has since been demonstrated to be the HSV-2 serotype equivalent of the product of the UL42 gene of HSV-1 (Gallo *et al.*, 1988)(see below). In addition the 226 carboxy-terminal amino acids of *pol* have been found to be necessary and sufficient for the interaction with UL42, which maps the UL42 binding site to a region distinct from that thought to be involved in substrate recognition (Digard and Coen, Abstracts: 15th International herpesvirus workshop). Other regions of *pol* could be involved in binding UL42 but isolation of the 226 amino acid fragment and comparison of its affinity for UL42 with that of intact *pol* will be needed to test this possibility.

6.1.1.3 UL42

The product of gene UL42 is a 65Kd DNA binding protein ($65K_{DBP}$)(Marsden *et al.*, 1987; Parris *et al.*, 1988; Gallo *et al.*, 1988). Immunoaffinity purification of the UL42 protein has demonstrated an interaction between $65K_{DBP}$ and *pol* (Gallo *et al.*, 1988) although this protein is not required for the catalytic activity of the HSV DNA polymerase. The $65K_{DBP}$ binds strongly in a sequence-independent manner to double-stranded DNA

(Bayliss *et al.*, 1975; Gallo *et al.*, 1988). Experiments have further shown that $65K_{DBP}$ excerts a 4-10 fold specific stimulatory activity on the HSV-1 polymerase, an effect that was blocked by addition of a $65K_{DBP}$ specific monoclonal antibody (Gallo *et al.*, 1989).

Recent experiments with sedimentation and gel filtration analysis have indicated that the complex formed between UL42 and *pol* is a heterodimer (Gottlieb *et al.*, 1990). The data indicates that UL42 acts as an accessory sub-unit of the DNA polymerase: the results demonstrate that the UL42 acts to increase the processivity of polymerisation, with the UL42 increasing the affinity of the polymerase for primer termini (Gottlieb *et al.*, 1990). An earlier report (O'Donnell *et al.*, 1988a) had suggested that the MDBP (ICP8) may be required for processivity, however the work of Gottlieb *et al.*, (1990), using more purified proteins demonstrates no such requirement.

6.1.1.4 UL9

The polypeptide encoded by gene UL9 binds specifically to *ori* DNA sequences (Olivo *et al.*, 1988; Weir *et al.*, 1989). Purified UL9 protein binds to ori_S at two sites located on each arm of the palindrome (Elias and Lehman, 1988; Olivo *et al.*, 1988). The role, played by the product of UL9 in DNA replication is unknown, although by analogy with other replication origin recognition sequences the binding to ori_S and ori_L may initiate the assembly of a multiprotein replication complex (Challberg and Kelly, 1989).

6.1.1.5 UL5, UL8 and UL52

The three proteins encoded by genes UL5, UL8 and UL52 have been shown to form a complex in HSV-1 infected cells which exibits DNA dependent ATPase activity, DNA dependent GTPase activity, DNA helicase and DNA primase activities (Dodson *et al.*, 1989; Crute *et al.*, 1988, 1989). Although it is not yet known which of these

activities are associated with which of the subunits of the complex it has been demonstrated that the product of the UL8 gene is not required for DNA-dependent ATPase or DNA-helicase activity; as co-expression of the UL5 and UL52 products in a baculovirus expression system, is sufficient for production of these activities (Calder and Stow, 1990), in addition recent experiments using immunofluoresence have indicated that the UL8 protein may be involved in transporting the helicase-primase complex into the cell nucleus (Calder and Stow, 1990; Abstracts: 15th International herpesvirus workshop).

6.1.2 Viral enzymes involved in nucleotide metabolism

6.1.2.1 Ribonucleotide reductase (RR)

Ribonucleotide reductase (RR) catalyses the reduction of all four ribonucleotides to deoxyribonucleotides and as such plays an essential role in DNA synthesis. Cohen, (1972) provided the first evidence that HSV induced a novel RR activity; with Averett *et al.*, (1983) providing the first good biochemical evidence for an enzyme in infected cells with properties distinct from that of the cell enzyme.

HSV-1 and 2 have been shown to specify two sub-units of RR, R1 and R2, with M_rs 136K and 38K for HSV-1 and 138K and 36K for the HSV-2 enzyme (Dutia, 1983; McLauchlan and Clements, 1983; Preston *et al.*, 1984; Cohen *et al.*, 1985; Frame *et al.*, 1985; Bacchetti *et al.*, 1986; Ingermarson and Lankinen, 1987; Darling *et al.*, 1988). The HSV-1 R1 and R2 polypeptides are translated from two mRNAs of 5Kb and 1.2Kb which share a common 3' terminus (McLauchlan and Clements, 1982, 1983) and are encoded by genes UL39 and UL40 respectively (McGeoch *et al.*, 1988). Direct evidence that these sub-units formed a complex was provided by experiments with specific antisera and *ts* mutants (Frame *et al.*, 1985; Bacchetti *et al.*, 1986; Preston *et al.*, 1984, 1988). Later, Ingermarson and Lankinen, (1987), demonstrated

that the complex contained two molecules of R1 and two of R2.

Experiments with mutants in RR have shown that HSV-1 RR activity is largely dispensible for virus growth and DNA replication in exponentially growing cells *in vitro* at 34° C; although it is required for optimal growth in resting cells or in cells at high temperature (39.5° C) (Goldstein and Weller, 1988a, 1988b; Preston *et al.*, 1988) . This suggests that a cellular factor capable of complementing HSV RR is present in growing cells at lower temperatures, but is somehow inactive at 39.5° C. Indicating that the viral requirement for its own RR is largely dependent on the conditions at which infection occurs.

Observations that a short synthetic oligopeptide corresponding to the carboxyl 9 amino acids of the small sub-unit, specifically inhibited the viral but not the cellular enzyme activity led to the belief that this enzyme may be a possible target for antiviral chemotherapy (Dutia *et al.*, 1986, Coen *et al.*, 1986). It was proposed that the inhibition of activity resulted from the ability of the nonopeptide to competitively inhibit binding of the small sub-unit with the binding site on R1. This proposal has now been confirmed (McClements *et al.*, 1988; Paradis *et al.*, 1988; Darling *et al.*, 1990). These findings lead to the interesting possibility that small peptides could be selected on the basis of sequence and structural information for their potential to inhibit sub-unit interaction. This view is supported by a similar finding with peptides from the thymidylate synthetase enzyme of VZV (Marsden *et al.*, 1990; Abstracts, 15th International Herpesvirus Workshop).

Support for the view that HSV RR might be a valid antiviral target comes from the observation that the virulence of RR mutants in mice is highly attenuated (Cameron *et al.*, 1988). Another mutant (in R1) was shown to be severely impaired in its ability to replicate in

the mouse eye model and failed to establish reactivatable latent infection (Jacobson *et al.*, 1989). In contrast RR mutants have been demonstrated to replicate in a guinea-pig model (Turk *et al.*, 1989). It is not clear however, how appropriate either of these models are in relation to the situation encountered in human infection.

6.1.2.2 Thymidine kinase

The HSV-encoded Thymidine kinase (TK)(Kit and Dubbs, 1963), phosphorylates thymidine (Dubbs and Kit, 1964) as well as deoxycytidine (Jamieson and Subak-Sharpe, 1974) and a variety of nucleoside analogues (Elion *et al.*, 1977). The TK gene has been mapped to around 0.3 on the HSV-1 genome (Wigler *et al.*, 1977; Halliburton *et al.*, 1980; Reyes *et al.*, 1982) a position corresponding to gene UL23 (McGeoch *et al.*, 1988). Studies with *tk*⁻ mutants have indicated that HSV TK is dispensible for growth in growing tissue culture cells (Dubbs and Kit, 1964) but not in resting cells (Jamieson *et al.*, 1974).

Recent results have suggested that virus-encoded TK, although not essential for the establishment of latent infection, may be necessary for reactivation of the virus (Coen *et al.*, 1989; Efstathiou *et al.*, 1989; Tenser *et al.*, 1989). Several anti-HSV drugs, principally acyclovir become activated following phosphorylation by HSV TK (Elion *et al.*, 1977; Fyfe *et al.*, 1978; Field *et al.*, 1980).

6.1.2.3 dUTPase

The HSV-1 induced enzyme dUTPase catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab and Franke, 1980; Caradonna and Cheng, 1981; Williams, 1984). The gene encoding the dUTPase has been mapped on the HSV-1 genome to a region corresponding to the UL50 gene (Preston and Fisher, 1984, McGeoch *et al.*, 1988). The viral dUTPase is not essential for growth in

dividing cells in tissue culture (Fisher and Preston, 1986).

6.2 OTHER ENZYMES ENCODED BY HSV-1

6.2.1 Uracil-DNA glycosylase

The uracil-DNA glycosylase is an enzyme involved in DNA repair which removes uracil residues from DNA. The presence of a HSV-induced uracil-DNA glycosylase has been detected in HSV-infected cells (Caradonna and Cheng, 1981) and has been mapped to a position corresponding to the UL2 gene on the HSV-1 genome (Worrad and Caradonna, 1988; Mullaney *et al.*, 1989). A mutant in this gene has also been shown to behave identically to wild type HSV, indicating this gene is dispensible for growth in tissue culture (Mullaney *et al.*, 1989).

6.2.2 Alkaline exonuclease

HSV induced alkaline exonuclease activity has been described in both HSV-1 and HSV-2 infected cells (Keir and Gold, 1963; Morrison and Keir, 1968; Hay *et al.*, 1971). It seemed likely that alkaline exonuclease activity was virally encoded as a *ts* mutant has been isolated that affected the activity of the enzyme (Franke *et al.*, 1978) and microinjection of HSV DNA fragments into *Xenopus laevis* ooctyes resulted in the production of the nuclease activity (Preston and Cordingly, 1982). The gene responsible for the nuclease activity has been mapped on the HSV-1 genome between 0.168-0.175 map units (Preston and Cordingly, 1982; Costa *et al.*, 1983; Wathen and Hay, 1984; Banks *et al.*, 1985). A position which corresponds to the UL12 gene (McGeogh *et al.*, 1988).

Early experiments with a HSV-2 *ts* mutant suggested that the alkaline exonuclease was essential for viral DNA replication (Moss, 1986). However, recent experiments with a lacZ insertion mutant in this gene

(Weller *et al.*, 1990) have shown that although the mutant is severely compromised for growth in Vero cells, it was able to produce wild-type levels of DNA and late proteins, indicating that it is not essential for viral DNA synthesis. Electron microscopy of this mutant has revealed that it accumulated large numbers of partially cored capsids. It therefore seems likely that the alkaline exonuclease is involved at a later stage possibly in the processing of the viral DNA into capsids (Weller *et al.*, 1990).

6.2.3 Protein kinase

Following infection of cells with HSV-1 a novel protein kinase activity is induced (Blue and Stobbs, 1981; Purves *et al.*, 1986). Comparisons of eukaryotic protein kinase sequences with the published sequences of HSV-1, HSV-2 (McGeoch *et al.*, 1985, 1987) and VZV (Davison and Scott, 1986) has revealed homology with the US3 gene of HSV-1 (McGeoch and Davison, 1986).

Experiments with antisera raised against a synthetic peptide from the predicted DNA sequence and purification of the kinase activity from HSV infected cells has confirmed that the protein kinase is indeed encoded by gene US3 (Frame *et al.*, 1987) although this activity is dispensible for viral growth in tissue culture (Frame *et al.*, 1987; Purves *et al.*, 1987). The product of gene UL13 has also been proposed as being a protein kinase (Smith and Smith, 1989), however the protein remains to be identified.

6.3 HSV-1 IMMEDIATE EARLY PROTEINS

HSV-1 encodes five IE polypeptides: Vmw110, Vmw63, Vmw175, Vmw68 and Vmw12 encoded by IE genes 1-5 respectively (Reviewed by Everett, 1987). Although recent work of Wymer *et al.*, (1989) with HSV-2 has demonstrated a TAATGARAT-like element in the promoter of the HSV-2 R1 gene, it therefore seems likely that R1

should also be classified as an IE gene; as in addition it is expressed very early in infection and in the presence of cyclohexamide and the absence of immediate early transactivation (Preston, 1979a; DeLuca *et al.*, 1985; Roizman and Batterson, 1985).

During infection in the presence of inhibitors of protein synthesis, there is an accumulation of IE mRNAs (Preston, 1979a). These transcripts expressed in the absence of *de novo* protein synthesis (Kozak and Roizman, 1974; Clements *et al.*, 1977; Jones and Roizman, 1979) have been mapped on the HSV-1 genome (Clements *et al.*, 1979). All the immediate genes with the exception of IE gene 2 are located in the vicinity of the repeat regions of the genome, with IE genes 1 and 3 situated within the long and short inverted repeats respectively, resulting in the presence of two copies of each (Watson *et al.*, 1979; Anderson *et al.*, 1980; Mackem and Roizman, 1980; Marsden *et al.*, 1982; Rixon *et al.*, 1982).

6.3.1 Vmw175

This is an essential protein and a major transactivator of both early and late genes. A number of viral *ts* mutants in IE gene 3 have been isolated: These fail to synthesize DNA or to produce early or late proteins at the NPT (Beyyesh-Melnick *et al.*, 1974; Marsden *et al.*, 1976; Preston *et al.*, 1979a; Dixon and Schaffer, 1980; Watson and Clements 1978, 1980). These *ts* mutants overproduce IE transcripts at the NPT (Preston *et al.*, 1979a; Dixon and Schaffer, 1980) implicating Vmw175 in the autoregulation of IE genes: Infact plasmids that express Vmw175 can repress transcription from the IE-3 promoter, (O'Hare and Hayward, 1985b). The other IE promoters can also be repressed by Vmw175 under certain conditions in transfection assays (Deluca and Schaffer, 1985; Gelman and Silverstein, 1987a, b).

Vmw175 binds specifically to the target consensus sequence 5'-ATGCTG-3', which is present at the

transcription start site of its own gene (Beard *et al.*, 1986; Faber and Wilcox, 1986; Kristie and Roizman, 1986; Muller, 1987) and it was thought that this binding mediated autoregulation (Gelman and Silverstein, 1987b; Muller, 1987; Roberts *et al.*, 1988). However recent work has suggested that the Vmw175 binding site in the IE-1 promoter has no apparent role in the expression of Vmw110. Although a mutation in the Vmw175 binding site reduced the ability of Vmw 175 to repress the IE-1 promoter in transfection assays, it had no effect on the levels of Vmw110 expression during normal HSV-1 infection (Everett and Orr, 1990).

6.3.2 Vmw63

This protein is also essential for viral growth *in vitro*, with *ts* mutants over-producing Vmw175 and Vmw110 at the NPT (Sacks *et al.*, 1985). It is thought that this protein is implicated in the regulation of late gene expression, as although Vmw63 mutants exhibit normal levels of early protein and DNA synthesis they have a severe reduction in production of late proteins. (Rice and Knipe, 1988).

6.3.3 Vmw110

This protein is involved in activation of both viral and cellular promoters *in vitro* (Everett 1984; O'Hare and Hayward, 1985a,b; Quinlin and Knipe, 1985; Gelman and Silverstein, 1985; Mavromara-Nazos *et al.*, 1986). However, experiments with a Vmw110 deletion mutant *dl*1403 (Stow and Stow, 1986, 1989), have shown that the polypeptide is not absolutely essential for growth in tissue culture, with a high moi able to surmount the absence of the polypeptide. However the virus did show reduced growth with low moi.

The transactivation effect of Vmw110 on viral and cellular promoters has been shown to occur with Vmw110 by itself or in association with Vmw175 (Everett 1984b, 1986; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985;

Gelman and Silverstein, 1986). It also seems likely that Vmw110 is unable by itself to activate the expression of early genes without the presence of Vmw175, as a deletion mutant in IE gene 3 lacked the ability to grow in normal cells (DeLuca *et al.*, 1985).

6.3.4 Vmw68

The role of the product of the IE gene 4 is at present unclear and no *ts* mutants in this gene have been isolated at present. However experiments have linked Vmw68 with a possible role in the expression of late genes, as a mutant virus has been constructed which lacks part of the polypeptide (Post and Roizman, 1981). The mutant grows poorly in some tissue culture cells and is deficient in the expression of L genes (Sears *et al.*, 1985).

6.3.5 Vmw12

This is the least characterised IE protein. Its function is unclear and is unique among the IE genes in that it lacks phosphorylation and localises predominantly to the cytoplasm instead of the nucleus (Preston 1979b; Marsden *et al.*, 1982; Palfreyman *et al.*, 1984). Vmw12 is non-essential for growth in tissue culture as recombinant viruses have been constructed which exhibit normal growth characteristics, while lacking the entire IE gene 5 (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

6.4 HSV-1 STRUCTURAL PROTEINS

Greater than 30 proteins have been detected in virions of HSV-1 (Spear and Roizman, 1972; Heine *et al.*, 1974; Marsden *et al.*, 1976; reviewed by Dargan, 1986). These can be grouped into either capsid proteins, tegument proteins or glycoproteins.

6.4.1 Capsid proteins

Capsids isolated from infected cells are either "empty" or those which contain viral DNA. Empty capsids are made-up of at least 5 proteins,VP5 (Vmw155), VP19 (Vmw53), VP23 (Vmw36), VP24 (Vmw24)(Gibson and Roizman, 1972) and 12K (Vmw12) (Heilman *et al.*, 1979; Cohen *et al.*, 1980).

VP5 is commonly referred to as the major capsid protein (MCP). By use of polyvalent antisera to assay *in vitro* translation products, (Costa *et al.*, 1984) VP5 has been shown to be encoded by gene UL19 (Davison and Scott, 1986b; McGeoch *et al.*, 1988). VP19C is a DNA binding protein (Braun *et al.*, 1984) and was initially mapped using intertypic recombinants to a region of the HSV-1 genome between UL39 and UL42 (Braun *et al.*, 1984). However recent work involving direct amino acid sequencing of purified capsid proteins has demonstrated that VP19C is infact encoded by gene UL38 (Rixon *et al.*, 1990). This work of Rixon *et al.*, (1990) additionally assigned VP23 to gene UL18.

In addition to those five polypeptides, capsids containing HSV DNA also posess two further proteins, one of which is polypeptide VP21, a major component of the cylindrical protein plug which in combination with the viral DNA forms the virion core (Gibson and Roizman, 1972). The second is VP22a (p40): This was thought to be a component of full not empty capsids (Gibson and Roizman, 1972, 1974; Heilman *et al.*, 1979; Preston *et al.*, 1983) and was intially thought to be a capsid protein which may have undergone processing to form the abundant virion protein VP22 that forms a loose association with the nucleocapsid (Gibson and Roizman, 1974).

Preston *et al.*, (1983) showed that a mutant containing a temperature sensitive lesion in gene UL26 is defective in the processing of VP22a and proposed that UL26 encodes VP22a. Experiments with this *ts* mutant, *ts*1201, have shown that during infections at the

NPT viral DNA is not packaged into capsids, suggesting a possible role for this protein in DNA packaging (Preston *et al.*, 1983. Further experiments with *ts* mutants (Rixon *et al.*, 1988) have indicated that VP22a is indeed present in empty capsids, but does not form a major component of full capsids. These experiments indicate that VP22a probably forms a transient association with capsids during their assembly, however its removal from capsids seems to be connected with the DNA packaging process (Rixon *et al.*, 1988).

Map locations have as yet not been assigned to proteins VP24, VP21 and 12K.

6.4.2 Tegument proteins

The virion tegument is a poorly defined structure which lies between the envelope and the capsid and comprises approximately 65% of the virion by volume (Schrag *et al.*, 1989). Tegument proteins (in addition to glycoproteins) can be identified when released from virions in the presence of non-ionic detergents. However some proteins seem to associate more strongly with capsids than others (Lemaster and Roizman, 1980; Roizman and Furlong, 1974; Spear, 1980). It is unclear how many proteins are located within this region or how they are orientated, however a major component of the tegument is the protein involved in transinduction of IE genes Vmw65 (VP16), the product of gene UL48 (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell *et al.*, 1984).

6.4.3 Glycoproteins

HSV-1 encodes at least seven distinct glycoproteins: gB, gC, gD, gE, gG, gH and gI (Spear, 1976; Marsden *et al.*, 1978, 1984; Bauke and Spear, 1979; Buckmaster *et al.*, 1984; Roizman *et al.*, 1984; Gompels and Minson, 1986; Frame *et al.*, 1986; Longnecker *et al.*, 1987; Johnson and Feenstra, 1987; McGeoch, 1987). These genes are encoded by the genes UL27, UL44, US6, US8, US4, UL22 and US7 respectively (McGeoch *et al.*, 1985,

1988). Sequence analysis has also suggested an additional glycoprotein potentially encoded by gene US5 (McGeoch *et al.*, 1985). Recent experiments with an antisera raised against a US5-fusion protein have identified a 40K virion associated protein, which is somewhat larger than that predicted by the DNA sequence (Gao and Spear, 15th International herpesvirus workshop). This new glycoprotein will be designated gJ.

Of these glycoproteins mutations in gC have shown that it is non-essential for infectivity (Heine *et al.*, 1974; Holland *et al.*, 1984; Draper *et al.*, 1984., Homa *et al.*, 1986), in addition glycoproteins, gE, gG, gI and the product of the US5 gene have all also been shown to be dipensible for growth in tissue culture (Longnecker and Roizman, 1986, 1987; Longnecker *et al.*, 1987; Weber *et al.*, 1987; Harland and Brown, 1988).

Only three of the glycoproteins therefore have been shown to be essential for infectivity *in vitro*, namely gB, gD and gH (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Ligas and Johnson, 1988; Weller *et al.*, 1983; Gompels and Minson, 1986; McGeoch and Davison, 1986b; Desia *et al.*, 1988). Of these only gH is possibly essential for absorption (Buckmaster *et al.*, 1984; Gompels and Minson, 1986; Desia *et al.*, 1988) as virus mutants in both gB and gD have been shown to bind efficiently to the cell surface (Cai *et al.*, 1988; Ligas and Johnson, 1988; see earlier), while failing to synthesis viral polypeptides.

7. CONTROL OF HSV-1 GENE EXPRESSION

Lytic infection of cells *in vitro* by HSV-1 leads to expression of its genes in a defined pattern which can be divided into three main phases: Immediate early (IE), early (E) and late (L), alternatively termed alpha, beta and gamma (Honess and Roizman, 1974; Clements *et al.*, 1977). Experiments with inhibitors of viral DNA and protein synthesis have shown that this expression occurs

in a temporal cascade (Honess and Roizman, 1974, 1975; Clements *et al.*, 1977; Jones and Roizman, 1979), with expression of IE polypeptides being essential for the induction of early genes and functional E proteins subsequently being required for expression of late proteins.

7.1 IMMEDIATE-EARLY GENES

IE proteins are first detected about 1h after absorption with gene expression initially thought to peak at about 2-3 hours, however these observations were usually made on measurements of transcription in the absence of viral protein synthesis or functional Vmw175 (Honess and Roizman, 1975, Preston *et al.*, 1978), rather than on observations of time course in a normal infection.

Infact, where studies have been carried out in the course of a normal infection, high levels of IE polypeptide accumulation have been detected in the middle and late stages of infection (Reviewed by Everett and Orr, 1990). Indeed recent experiments have shown that Vmw110 steadily accumulates throughout infection with no obvious evidence of repression (Everett and Orr, 1990).

The promoter regions of the IE genes, consists of two components, a "TATA" box sequence necessary for basal and induced levels of transcription, and upstream regulatory sequences containing multiple *cis*-acting elements conferring enhancer function and responsivity to IE regulation (Mackem and Roizman, 1982a,b; Cordingley *et al.*, 1983; Preston *et al.*, 1984; Kristie and Roizman, 1984; Bzik and Preston, 1986).

All the IE promoters contain GC-rich regions (Sp1 binding sites) and one to three copies in either orientation of the element TAATGARAT (where R is a purine), which is a *cis*-acting motif (Mackem and Roizman, 1982c; Whitton *et al.*, 1983; Whitton and Clements, 1984; Preston *et al.*, 1984; Galloway *et al.*,

1985), which has been shown to mediate the stimulation of IE transcription by the major virion tegument protein Vmw65 (VP16), the product of gene UL48, upstream of the mRNA initiation sites (Post *et al.*, 1981; Mackem and Roizman, 1982; Campbell *et al.*, 1984; Preston *et al.*, 1984).

Vmw65 does not form a direct interaction with DNA (Marsden *et al.*, 1987) but instead interacts with one or more cellular factors to form a ternary complex (IEC) that binds to TAATGARAT (McKnight *et al.*, 1987; Preston *et al.*, 1988; O'Hare *et al.*, 1988). A cellular component of this complex appears to be a ubiquitous protein with a variety of names NFIII (Pruijn *et al.*, 1986), OBP100 (Baumruker *et al.*, 1988; Strum *et al.*, 1987), OTF-1 (Gerster and Roeder, 1988), TRF (O'Hare and Goding, 1988) and alpha-H1 (Kristie and Roizman, 1987).

This component recognizes the TAATGARAT element and also an octomer consensus sequence ATGCAAAT. Although NFIII is capable of binding to a range of sequences, there is only efficient formation of the IEC complex when the TAATGARAT element is present (O'Hare *et al.*, 1988; Gerster and Roeder, 1988; ApRhys *et al.*, 1989). IEC complex formation facilitates an acidic activating domain, which is located within the c-terminus of Vmw65 to interact with a transcription complex and so amplify gene expression (Dalrymple *et al.*, 1985; Triezenberg *et al.*, 1988; Sadowski *et al.*, 1988; Cousens *et al.*, 1989).

7.2 EARLY GENES

Synthesis of early genes is initiated after the appearance of immediate-early polypeptides (Honess and Roizman, 1974; Wagner, 1985; Weinheimer and McKnight, 1987; Zhang and Wagner, 1987), as early genes require prior synthesis of IE gene products for their expression (Honess and Roizman, 1974; Clements *et al.*, 1977).

However there seems to be a greater deal of variation in the kinetics of expression of early genes in comparison to IE genes. gD although expressed as an

early gene, is not maximally produced until the onset of viral DNA replication (Gibson and Spear, 1983: Johnson *et al.*, 1986) and as such has been described as a beta-gamma or early-late (EL) gene (Roizman and Batterson, 1985; Wagner, 1985; Harris-Hamilton and Bachenheimer, 1985). This increase in transcription of EL genes does not neccesarily show a direct requirment for viral DNA synthesis, as increased expression may be due soley to an increased template copy number (Everett and Johnson, 1986a).

The promoter regions of HSV early genes have been well studied and no viral specific sequences involved in transactivation have been detected. A typical HSV early promoter has been found to contain a "TATA" box element upstream of the transcriptional start site in combination with other elements, including "CCAAT" boxes, GC-rich regions (for Sp1 binding) and G-rich elements (McKnight *et al.*, 1985; Everett, 1983; Eisenberg *et al.*, 1985; El Kareh *et al.*, 1985). Binding of various cellular transcription factors such as the "CCAATT" box binding factor and Sp1 has been demonstrated for the promoter of the HSV tk gene (Jones *et al.*, 1985; Graves *et al.*, 1986). Although many HSV promoters contain homologies to TATA, CCAAT and GC-rich elements, there has been found to be a great deal of diversity between promoters of the same class (Mackem and Roizman, 1982c; Wagner, 1985). This may explain the differential rates of expression described previously for genes of the same class (Honess and Roizman, 1974; O'Hare and Hayward, 1985a, Harris-Hamilton and Bachenheimer, 1985).

7.3 LATE GENES

Late gene product accumulation can initially be detected at about 3 hours post absorption, reaching a peak at around 10-16 hours. this is approximately 2 hours after the peak of viral DNA synthesis at 8 hours post absorption (Munk and Sauer, 1964; Roizman, 1969;

Wilkie, 1973). The expression of late genes is dependent on the presence of a functional Vmw175 protein (Watson and Clements, 1980) and is also dependent upon the replication of the viral DNA. As demonstrated by experiments with *ts* DNA$^-$ mutants and inhibitors of DNA synthesis (Swanstrom and Wagner, 1974; Honess and Roizman, 1974; Powell *et al.*, 1975; Marsden *et al.*, 1978; Jones and Roizman, 1979; Holland *et al.*, 1980; Conely *et al.*, 1981; Pederson *et al.*, 1981).

Late genes can be split into two classes; either gamma$_1$ or gamma$_2$, otherwise termed "leaky-late" and "true-late" respectively. "Leaky-lates" are those genes whose expression is detectable in the absence of viral DNA replication, although they require DNA synthesis for maximal expression. Whereas "true-late" genes have an absolute requirement for DNA synthesis for expression (Wagner, 1985; Roizman and Batterson, 1985; Johnson *et al.*, 1986). An example of a "leaky-late" gene is the product of gene UL19, Vmw155-the major capsid protein. Whereas a protein which exhibits "true-late" characteristics is the product of gene US11 (Johnson *et al.*, 1986).

Experiments which linked an HSV origin of replication with the US11 promoter found that only the "TATA" box and sequence downstream to the transcription start site and replication of the host plasmid in *cis* were all that was required for full expression (Johnson and Everett, 1986a, b). It is unclear what connection exists between the replication of viral DNA and late gene expression, although an increase in template copy number is unlikely to be responsible, as some effect would be detectable on early genes also.

A possible role has been indicated for IE proteins Vmw63 and Vmw68 in late gene induction, as mutants in these genes exist which are deficient in expression of certain late polypeptides (Sacks *et al.*, 1985; Sears *et al.*, 1985).

8. CLEAVAGE AND PACKAGING OF VIRAL DNA

Two steps are involved in the maturation of HSV DNA: The processing of the concatameric DNA into unit lengths and the assembly of capsids containing a genome equivalent of the viral DNA.

It is thought likely that the maturation of the viral genome and its subsequent packaging involves site specific cleavage of viral DNA concatamers. The *cis*-acting sequence required for this cleavage is located within the *a* sequence (Vlazny and Frenkel, 1981; Mocarski and Roizman, 1982; Spaete and Mocarski, 1985; Stow *et al.*, 1983); with two seperate *cis*-acting sequences within the *a* sequence appearing to be essential for the cleavage and package process (Varmuza and Smiley, 1985; Deiss *et al.*, 1986). The cleavage signal has now been further defined to a 179-bp fragment across a *a*-*a* junction (Nasseri and Mocarski, 1988). Some common mechanism seems to be involved in the maturation of herpesvirus genomes, as structural conservation exists in the signals involved in processing and packaging among many of the herpesviruses (Davison, 1984; Albrecht *et al.*, 1985; Spae te and Mocarski, 1985; Hammerschmidt *et al.*, 1988; Marks and Spector, 1988).

Studies have been aided by isolation of viral mutants that are capable of replicating viral DNA but are deficient in processing and packaging. The viral alkaline exonuclease, has been implicated in this process, as an insertion mutant in this gene produces wild-type levels of DNA synthesis and late proteins while failing to process and package its DNA (Weller *et al.*, 1990, see previous section 4.7). At least five other genes have been implicated; UL6, UL26, UL28, UL32 and UL54: with viral mutants existing in these genes which are defective in DNA processing and encapsidation. suggesting that capsid formation and DNA packaging are dependent on several viral gene products (Weller *et al.*, 1983, 1987; Preston *et al.*, 1983; Rixon *et al.*, 1988;

Matz *et al.*, 1983; Sacks and Schaffer, 1987; Sherman and Bacchenheimer, 1987, 1988; Addison *et al.*, 1990). It is unclear at present what particular role in processing/packaging is played by each of these genes.

9. GENERATION OF ANTISERA TO SYNTHETIC OLIGOPEPTIDES

During the last decade the rapid development of DNA sequencing methods has produced a convenient way of determining the amino-acid sequence of proteins which are predicted to be encoded by identified open-reading frames. Although it proved relatively easy to generate primary sequence data for genes it was then necessary to link gene sequences with specific proteins. One obvious solution was to produce an antibody probe against the putative protein.

Due to size and cost limitations, chemical synthesis of complete predicted proteins is generally not practical. As an alternative short peptide sequences were one suggestion as possible mimics for antigenic sites on proteins which would allow the useful development of antibodies of pre-determined specificity. Early results from studies with the bacteriophage MS2 provided evidence that indicated peptides may be able to function as immunising antigens (Langebeheim *et al.*, 1976). These experiments showed that a fragment of the coat protein of MS2 and the corresponding synthetic peptide with the same sequence elicited antibody which reacted with the intact virus particle.

However work on the immunogenicity of proteins (reviewed by Lerner, 1982; Benjamin *et al.*, 1984) predicted that most immunogenic sites were composed of conformational determinants, formed by complex tertiary interactions between amino acid residues that although proximal in the protein tertiary structure, were distal in relation to the primary linear amino acid sequence. Therefore it was thought that small portions of a protein would, in general, be unlikely to elicit

antiserum reactive against an intact protein. It was believed that for a peptide to elicit antibodies that could react with an intact protein containing the peptide sequence, it would be neccessary to construct the peptide in such a way as to attempt to reproduce the tertiary confirmation of an antigenic site on the intact protein (Arnon, 1980).

This theory was challenged by experiments with chemically synthesised protein fragments from the amino or carboxy terminals of viral proteins whose amino acid sequences had been predicted from analysis of nucleic acid sequences. Two groups were first to describe protein reactive antibodies generated by synthetic peptides: antibodies to the amino and carboxy terminal regions of the simian virus 40 (SV40) transforming protein were found to react with the native protein (Walter *et al.*, 1980), providing indirect confirmation that the DNA sequence and the presumed open reading frame were correct. In addition antisera to an oligopeptide corresponding to a region of an unknown suspected gene, predicted by analysis of Moloney Leukemia virus (MoLv) DNA, allowed identification of a previously unidentified gene product (Sutcliffe *et al.*, 1980).

These initial observations were followed by numerous other reports of oligopeptide induced antisera acting as probes for polypeptides (e.g. Lerner *et al.*, 1981; Baron and Baltimore, 1982; Bittle *et al.*, 1982; Green *et al.*, 1982; Sutcliffe *et al.*, 1983).

9.1 UTILISATION OF ANTIPEPTIDE ANTIBODIES

As antipeptide antibodies possess the advantage of having pre-determined specificity they have also proved useful for answering a variety of biological questions e.g. For establishing the orientation of proteins in plasma membranes (Schneider *et al.*, 1983), for distingushing between closely related proteins that

differ by only a single or few amino acids (Alexander *et al.*, 1983), for designing immunodiagnostic reagents (Gerin *et al.*, 1983) and for analysing the molecular basis of viral and microbially induced immunity (Dryberg and Oldstone, 1986).

In recent years the availability of regions of, and subsequently the complete, DNA sequence of HSV-1 (Davison and Wilkie, 1981; Murchie and McGeoch, 1982; McGeoch *et al.*, 1985, 1986, 1988; Perry and McGeoch, 1988) has allowed this technique to be applied to the attempted identification and characterisation of the predicted products of the identified HSV-1 open-reading frames. Palfreyman *et al.*, (1984b) using synthetic oligopeptides conjugated to BSA to produce rabbit antisera reactive with the products of HSV-1 immediate early genes, 5 and 3, namely Vmw12 and Vmw175 were the first to successfully generate antisera to synthetic peptides which was reactive with HSV-1 gene products. This led to numerous successful uses of the technique with HSV-1 polypeptides.

To date some 14 HSV-1 genes have either had their products identified or known proteins assigned to them using this method: The 21K product of gene US11, a DNA binding protein which interacts with the *a* sequence, and is localised in nucleoli (Dalziel and Marsden, 1984; MacLean *et al.*, 1987); the 38K product of gene UL40, the small sub-unit of ribonucleotide reductase (Frame *et al.*, 1985); the products of genes US3, US4 and US9, which encode a protein kinase, glycoprotein G and a 10K tegument phosphoprotein respectively (Frame *et al.*, 1986a, 1986b and 1987). Confirmation of the products of the genes required for DNA replication namely, UL5, UL8, UL9, UL42 and UL52 was also made by this technique (Olivo *et al.*, 1989). Identification of the products of genes UL31, UL45, UL46 and UL47 using antisera generated with synthetic oligopeptides is described in this thesis.

In recent years the majority of interest

surrounding the production of immune responses to synthetic peptides has focused on the possibility that they may provide useful immunogens in the development of synthetic vaccines to a range of viral and microbial diseases (reviewed by Brown, 1990). The first description of a protective effect induced by immunisation with a synthetic peptide was with Foot and Mouth Disease Virus (FMDV) (Bittle *et al.*, 1982), this group demonstrated a protective immune response in Guinea-Pigs using the amino acid sequence from 141-160 of the VP1 protein of FMDV, other examples of demonstrated neutralising antibodies have been described to poliovirus (Eminin *et al.*, 1983), in cows with FMDV (DiMarchi *et al.*, 1986), protection against experimental infection of malaria in monkeys (Patarryo *et al.*, 1987), and also to gp41 of a bovine rotavirus (Frenchick *et al.*, 1987) so raising hopes that synthetic epitopes in the form of peptides may provide some answers to as yet unsolved vaccine problems.

Before peptides can be considered as suitable vaccines a number of problems will have to be surmounted: It is unlikely that any peptide based vaccine that contained a carrier protein (necessary to enhance immunogenicity, see section 9.2) would be acceptable for use in humans or animals due to the undefinable nature of the immunogen and the possibility of adverse reactions that might be induced by such a carrier. Also, many of the conjugation methods used contain compounds that would be unsuitable for human use or introduction into the food chain because of their toxic or carcinogenic properties. There would be a particular problem if a series of injections proved necessary as it has been demonstrated that pre-existing immunity to carrier proteins can severely reduce their efficiency in stimulating a response (Schultze *et al.*, 1985; Frenchick *et al.*, 1987). Furthermore a peptide-protein conjugate would be almost impossible to quantitate accurately in terms of dose and alternative

adjuvants would also have to be developed as those currently available are too harsh for vaccine development.

9.2 FACTORS INFLUENCING THE ANTIGENICITY OF PEPTIDES

There are a large number of factors which have been shown to influence the success of antipeptide antibody production. The choice of peptide sequence was thought to play an important role in determining the response against the antigen; with the inclusion of a variety of amino acids on the basis of charge or size suggested as influencing immunogenicity (Lerner,1984). In particular inclusion of a proline residue or residues was shown to enhance significantly the antigenicity of synthetic peptides. Peptides containing proline residues in addition to polar or uncharged residues routinely produce antibodies that react with the native protein (Sutcliffe *et al.*, 1983). This may be due in part, to the the fact that proline residues form imide bonds instead of amide bonds creating bends in the polypeptide chain. Such regions are often accessible to antibodies and may enhance mimicking of the native conformation (Sutcliffe *et al.*, 1983).

The length of the immunising sequence is also critical: to raise antibodies to a peptide a minimum of six amino acids is required (Wilson *et al.*, 1984) and peptides of more than ten amino acids generally induce antibodies that bind with high frequency to the native protein (Palfreyman *et al.*, 1984a; Tanaka *et al.*, 1985).

Furthermore, the region of the protein from which the immunising sequence is derived is thought to be significant. Antibodies raised against an accessible epitope have a better chance of binding to the native protein than antibodies to an inaccessible site, although by its very nature, the process of antibody binding itself may also render inaccessible sites accessible (Wilson *et al.*, 1984). It would be expected

that more hydrophilic amino acid residues would be located on the surface of the molecule and therefore analysis using hydrophilicity plots (Hopp and Woods, 1981, 1983) and hydropathicity plots (Kyte and Doolittle, 1982) make it possible to predict which parts of the protein are likely to be surface orientated. These sites therefore have a better chance of corresponding to antigenic determinants and so peptides can be chosen accordingly. In addition peptides containing hydrophilic amino acids have the added advantage of being more likely to be soluble in water and are thus much easier to work with.

Terminal peptides, either amino or carboxy, are also good candidates as choices for immunising peptides. The surface location of many chain termini in proteins (Thornton and Sibanda, 1983) provides a rational for their inclusion as immunogens. It has also been suggested that the higher than average antigenicity of chain termini could be due to the fact that they are less constrained than other sections of the polypeptide chain and show a high relative flexibility (Van Regenmortel *et al.*, 1986). Peptides corresponding to highly mobile regions of the protein, when used as immunogens are able to elicit antibodies which react strongly with native proteins (Tainer *et al.*, 1984). Other factors such as immunisation dose, choice of adjuvant and immunisation schedules can also influence the result, but usually to a lesser extent (Shinnick *et al.*, 1983; Lerner, 1984; Palfreyman *et al.*, 1984a). Although these variables are unlikely to have an influence over the success of a given antibody's potential to recognise a native protein, their alteration can have an strong influence over the titre of the resulting response (Shinnick *et al.*, 1983).

As has been mentioned, care is usually taken to choose the most antigenic peptide sequence when attempting to raise antibodies. However it has been suggested, (Lerner, 1984) that unless all that is

required is to access a protein predicted by a nucleic acid sequence, the overriding consideration when choosing a peptide should be the precision with which a given antibody will be able to answer the experimental question posed. It is thought that even if an area adjacent to a primary target seems more attractive (i.e. more hydrophilic) one should (within reason) serve the needs of the experiment rather than relying on the theoretical "rules" governing peptide immunogenicity (Lerner, 1984).

Studies with the haemaglutinin protein of influenza virus (Green *et al.*, 1982; Sutcliffe *et al.*, 1983) have provided the basis for support of these observations. Synthesis of peptides covering the majority of the HA1 chain showed that the majority of the antibodies produced reacted with the intact molecule (Green *et al.*, 1982). Because the complete DNA sequence was available (Min Jou *et al.*, 1982) and its crystallographic structure was available to high resolution (Wilson *et al.*, 1981) it was known that in its folded state the HA1 molecule displays a number of secondary structures including alpha-helices, extended chains and beta-sheets. It was therefore evident that reactivity of antipeptide antibodies is in general, independent of secondary structure or location in the molecule. These observations have been further supported by experiments with MuLv: 12/12 peptides selected at random across the *pol* gene of MuLv produced antibodies reactive with the native protein (Sutcliffe *et al.*, 1983). Furthermore 18/18 peptides selected from the rabies virus glycoprotein gene were also successful in eliciting an antiprotein response (Sutcliffe *et al.*, 1983). Perhaps more important influencing factors may be the relative abundance of the target protein and the titre of the antibody probe, rather than its sequence.

To function as a successful immunogen, antigens must be presented in such a way that they are

effectively taken up by macrophages (i.e. not lost by excretion or rapidly removed by irrelevant cells such as granulocytes or hepatocytes) and can also be processed so as to be recognised by T-cells. They must also be in a form that makes them stable to enzymes which could destroy their immunogenic configuration (Humphery, 1986).

Although some reports indicated that it was possible to immunise animals with "free" peptides without the need for a macromolecular carrier (Beachy *et al.*, 1981; Lerner *et al.*, 1981; Dreesman *et al.*, 1982; Jackson *et al.*, 1982; Atassi and Webster, 1983; Young *et al.*, 1983; Francis *et al.*, 1987a), it is generally necessary to immumise animals with peptide-carrier conjugates. With the use of suitable proteins such as KLH, thyroglobulin, ovalbumin and BSA (although if it is intended to use antibodies to react with the products of cultured cells, it is probably best to avoid BSA as a carrier, as the anti-albumin antibodies can give spurious results, as so many ligands and proteins bind to the albumin contained in the bovine serum in cultured cells). Recently there has also been a description of the utilisation of the core antigen of Hepatitis B virus as a carrier molecule for synthetic peptides (Clarke *et al.*, 1987; Francis *et al.*, 1990). These peptide conjugates have produced titres of antibody to a FMDV protein almost equal to that elicited by the intact protein (Clarke *et al.*,1987). The coupling of peptides to carriers may provide T-cell help to antipeptide producing B-cells (Mitchison, 1971) and also help improve immunogenicity of the peptide by increasing its size, thus enhancing its chances of capture by antigen presenting cells and generally delaying its clearance from the immune system (Borras-Cuesta *et al.*, 1988).

Although this method has produced many successful antisera, there are a number of disadvantages in the use of carrier proteins to produce an immune response to synthetic peptides:

1. It is impossible to accurately quantitate the dose of peptide as it is only possible to make an estimation at coupling efficiencies.

2. Only a very small percentage of the immunogen will be the desired antigenic sequence, with the remainder being accounted for by sequences derived from the carrier.

3. Depending on the peptide sequence it can be a time consuming excercise to find suitable reaction conditions to couple peptides to carrier proteins and these sometimes employ highly carcinogenic compounds (e.g. DAB).

4. Most conjugation methods employed to attach peptides to carrier proteins: through free amino groups using gluteraldehyde (Avrameas and Terynck, 1969; Korn *et al.*, 1972); through either amino or carboxy positioned cysteine residues using MSB (Liu *et al.*, 1979; Green *et al.*, 1982); through tyrosine residues using DAB (Gordon *et al.*, 1958; Basseri *et al.*, 1979); or employing carbodiimides (Goodfriend *et al.*, 1964), have all been shown to have a detrimental effect on the antigenicity of either the peptide or the carrier protein by altering conformations or blocking important side-chains.

Briand *et al.*(1985) carried out a systematic study of the use of different carrier proteins and a variety of conjugation methods and demonstrated that although the choice of carrier protein did not appear critical the choice of conjugation method did, with all conjugation methods tested affecting the antigenicity of the test peptides to a greater or lesser extent and conjugation with gluteraldehyde abolishing completely the antigenicity of a lysine containing peptide. It has also been shown that in certain circumstances exposure of the carrier protein itself to the coupling agent can have a detrimental effect on its ability to provide help to the antipeptide response (Briand *et al.*, 1985),

resulting in a dose dependent suppression against the synthetic epitope to which it is attached (Jacob *et al.*, 1985).

5. Carrier protein-peptide conjugates are also unacceptable for development of vaccines based on synthetic peptides for a variety of reasons described earlier.

6. Although many antibodies have been produced using peptides conjugated to carrier proteins not all attempts have been successful (Nestorowicz *et al.*, 1985; Tanaka *et al.*, 1985) and many failures occur despite attention to pick sequences of the appropriate size and charge. Antibodies that are generated are often of low titre placing severe restrictions on the range of experiments that can be undertaken.

9.3 ALTERNATIVE METHODS OF ANTIPEPTIDE ANTIBODY GENERATION

Due to the constraints mentioned above, much recent work has been directed at producing suitable alternatives to the use of carrier proteins in eliciting responses against synthetic peptides. A variety of approaches have been investigated which have been successful to a greater or lesser extent in producing an immune response to a peptide in the absence of a macromolecular carrier.

Attempts have been made to produce large repeating polymers by polymerising the short peptides of interest into a long chain (Audibert *et al.*, 1982; Jacob *et al.*, 1985; Bittle *et al.*, 1984; DiMarche *et al.*, 1986; Borras-Cuesta *et al.*, 1988). In all cases this method has produced good levels of antibody, however it still possesses some of the problems associated with the use of carrier proteins: There is still the need for a coupling reaction during the polymerisation which has the problems mentioned earlier, also it is difficult to predict the resulting peptide structure after polymerisation, as a homogeneous product is rarely

formed. In addition the procedure may alter the immunogenic epitopes of the peptide and in certain cases the compound used for polymerisation may be immunogenic itself (Briand *et al.*, 1985).

The association of peptides with lipids has also been investigated with the effect of long chain fatty acids of particular interest. Already Rude *et al.*, (1971) have shown that attachment of a lipid moiety such as lauric acid causes an enhancement of the immunogenicity of soluble antigens. Stark *et al.*, (1980) also showed enhanced immunogenicity of lipid-conjugated proteins. Efficient anti-hepatitis B surface antigen antibodies have been produced using synthetic peptides conjugated to dipalmityl-lysine (Hopp, 1984). The results indicated that that the dipalmityl conjugates were as effective at antipeptide antibody generation as the peptide conjugated to KLH (Hopp, 1984; Jacob *et al.*,1985). Successful responses have also been demonstrated to FMDV peptides incorporated into membrane liposomes (Francis *et al.*, 1985 and 1987a) although the reponse was no greater than that achieved by peptide-carrier conjugates.

Jacob *et al.*, (1985), compared the effects of using protein carriers, lipid conjugated peptides and polymerisation of peptides and demonstrated that although all methods were successful, no one method performed any better than another.

The generation of an antibody response requires that T-helper (T_H) cells recognise antigenic peptides associated with the major histocompatability complex (MHC) class II molecules and these T_H cells interact with antigen specific B-cells. To achieve a response to peptides in the absence of a carrier-protein, it is thought that the immunising peptide must contain antibody recognition sites (B-cell epitopes) and sites capable of eliciting T-cell help for antibody production (T_H-cell epitopes)(Mitchison, 1971). These T_H- epitopes must possess the ability to bind to MHC class II

molecules on the surface of host cells and subsequently form a complex with the T-cell receptor on the surface of T_H-cells (Rosenthal, 1978; Babbit, 1985).

It was proposed therefore that assocciation of the target sequence (B-cell epitope) with a sequence known to stimulate T_H-cells in the animal of choice might enhance immunogenicity. Two groups first demonstrated that conjugation with "natural" T_H-cell epitopes (Good *et al.*, 1987) or co-polymerisation using gluteraldehyde, with "foreign" T-cell epitopes (Leclerc *et al.*, 1987) could enhance the immunogenicity of peptides containing B-cell epitopes. More recently, successful linear synthesis of B and T_H-cell epitopes has also been demonstrated (Borras-Cuesta *et al.*, 1987; Cox *et al.*, 1988; Milich *et al.*, 1988; Palker *et al.*, 1989).

Francis *et al.*, (1988) demonstrated that addition of a variety of "foreign" T_H-cell epitopes to the FMDV VP1 peptide 141-160 by co-linear synthesis could overcome genetic restricted non-responsiveness in inbred mice. Polymers containing repeating units of both B and T_H-cell epitopes have also been shown to be successful in eliciting responses in the absence of carriers (Borras-Cuesta *et al.*, 1988). The method by which the T_H-cell epitope is incorporated into the immunogen seems to have a marked effect on the subsequent response. Linear synthesis of both epitopes produces high titres to the B-cell epitope , whereas chemical conjugation of the two epitopes using bismaleimidohexane (BMH) produced only a moderate to low response to the B-cell epitopes (Nicholas *et al.*, 1990).

Although most of the previously described methods have been successful at generating responses to synthetic peptides in the absence of carriers, some still have the disadvantage of requiring one or more conjugation reactions which, as described previously, can interfere with antigenicity and make it difficult to accurately define the antigen.

Tam (1988), first described a system which provided

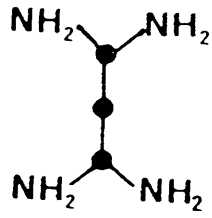
FIGURE 3

Diagrammatic representation of the core matrix of the branched peptide molecule constructed from lysine residues. (A) First level, divalent; (B) second level, tetravalent; (C) third level, octavalent; and (D) fourth level, hexadecavalent. The black dots represent lysine residues except for the free amino groups (NH_2). This figure is taken directly from Tam, (1988).

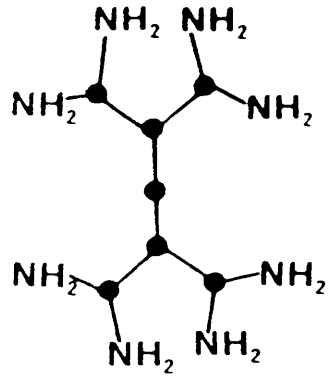
A


NH2
NH2

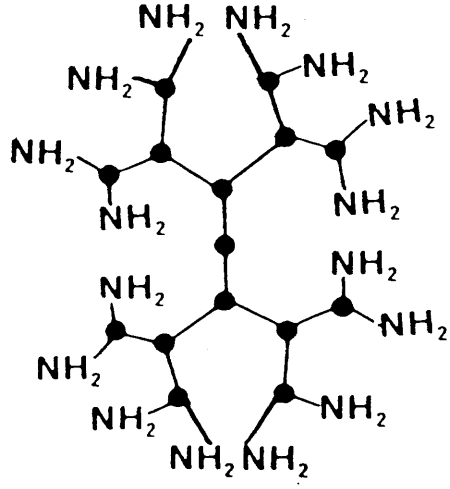
B


NH2
NH2
NH2
NH2

C


NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2

D


NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2
NH2

an accurately definable immunogen. Termed the multiple antigen peptide system (MAP), this utilises a simple "back-bone" of a low number of sequential levels (n) of a trifunctional amino acid (in this case lysine) as the core matrix and 2^n peptide antigens to form a macromolecule with a high density of peptide antigens. He described a small core of lysine molecules built up by solid phase synthesis of peptide bonds through both the alpha and the epsilon carbons of lysine. This enabled the construction of a branched lysine core (Fig. 3) of theoretically unlimited size. In practice a structure of 7 lysines were used which provided 8 free amino groups onto which copies of the peptide immunogen could be synthesised. For a fifteen amino acid peptide sequence the resulting structures had M_rs in the region of 12,000-15,000 and were therefore thought to be large enough to dispense with the need for a carrier protein.

Following immunisation these molecules produced high titre antisera in both mice and rabbits, the sera having a considerably higher titre of antibodies than sera prepared from the same peptides anchored covalently to keyhole limpet haemocyanin (KLH) as a carrier. (Tam, 1988; Posnett *et al.*, 1988). It was further shown that none of the antibodies produced were cross-reactive. This indicates that no response was produced to the hepta-lysine core, which would have reduced the usefulness of such immunogens. In fact, most of the antipeptide response has been shown to be directed against residues located distal from the core at the amino terminal end of the molecule (Posnett *et al.*, 1988). These results raise the possibility that the MAPs may take up a configuration in solution whereby the lysine backbone is "buried" within the molecule and as such is innaccessable to the immune system.

These branched peptide molecules produce immunogens that can be prepared to give a chemically unambiguous, accurately defined dose and if necessary can be purified to homogeneity. In addition they contain a very high

density of the immunogenic sequence of interest (>95%) in contrast to the low antigen densities exihibited by most peptide-protein conjugates (<5%). They are also of predetermined and reproducible structure, and have therefore been suggested as suitable candidates for synthetic vaccine development (Tam, 1988).

Recently, preliminary experiments with these structures in which the peptide sequence corresponding to the neutrilizing epitope of FMDV (amino acids 141-160 from the FMDV protein VP1) were reported. The branched structures elicited a 50-fold increase in neutralising antibody titre over that achieved with the equivalent monomeric peptide (Brown, 1990). It has also been suggested that a combination of these branched structures with T_H-cell epitopes, as has been previously shown for monomeric peptides (Section 9.3), may further enhance their immunogenic potential (Tam, 1988). Clearly, branched peptides potentially have advantages over previously used techniques for eliciting a useful immune response.

A detailed investigation of the immunogenic properties of branched peptides, with and without T_H cell epitopes, is contained within this thesis.

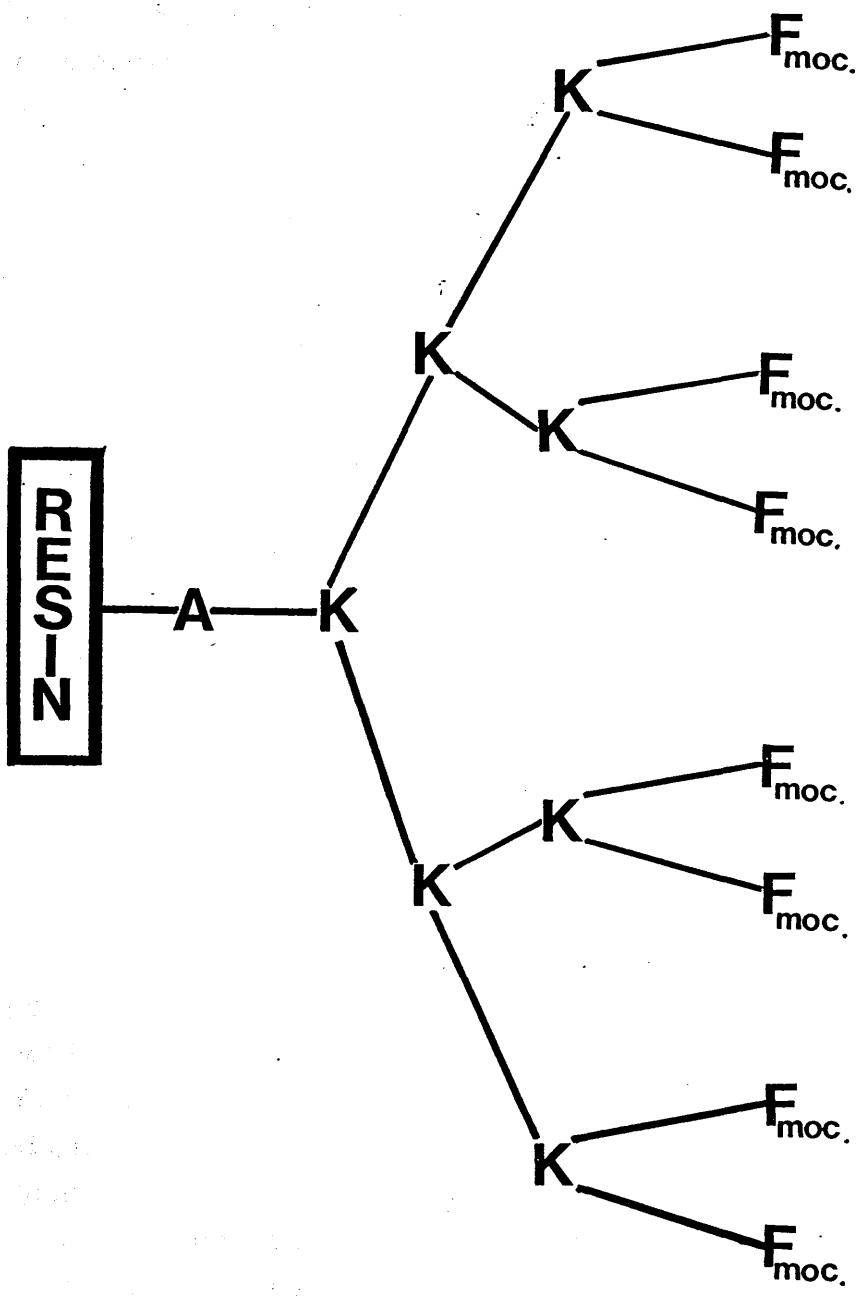
10. THE AIMS OF THE WORK PRESENTED IN THIS THESIS

The work covered in this thesis falls into two distinct areas: Firstly to investigate the potential of branched peptides for producing antisera to synthetic peptides. Secondly, to use the antisera generated to identify and characterise putative HSV gene products, for which no information existed.

As described earlier in this introduction, generation of antisera to synthetic oligopeptides has provided a very powerful tool in modern biology. These antibodies have many uses, not least of which is the ability to detect proteins which previously were only predicted to exist by the interpretation of a DNA

FIGURE 4

A diagrammatic representation of the octavalent lysine core used for synthesis of branched peptides. Amino acids are represented by the one letter code.


RESIN
A
K
K
K
K
K
K
K
F moc.
F moc.
F moc.
F moc.
F moc.
F moc.
F moc.
F moc.

sequence and also to assign previously identified polypeptides to known open-reading frames. However, as discussed, numerous problems are associated with use of carrier proteins to stimulate an immune response to a peptide. Moreover, even if successfully produced many oligopeptide antisera are of low titre, placing severe limitations on experimentation.

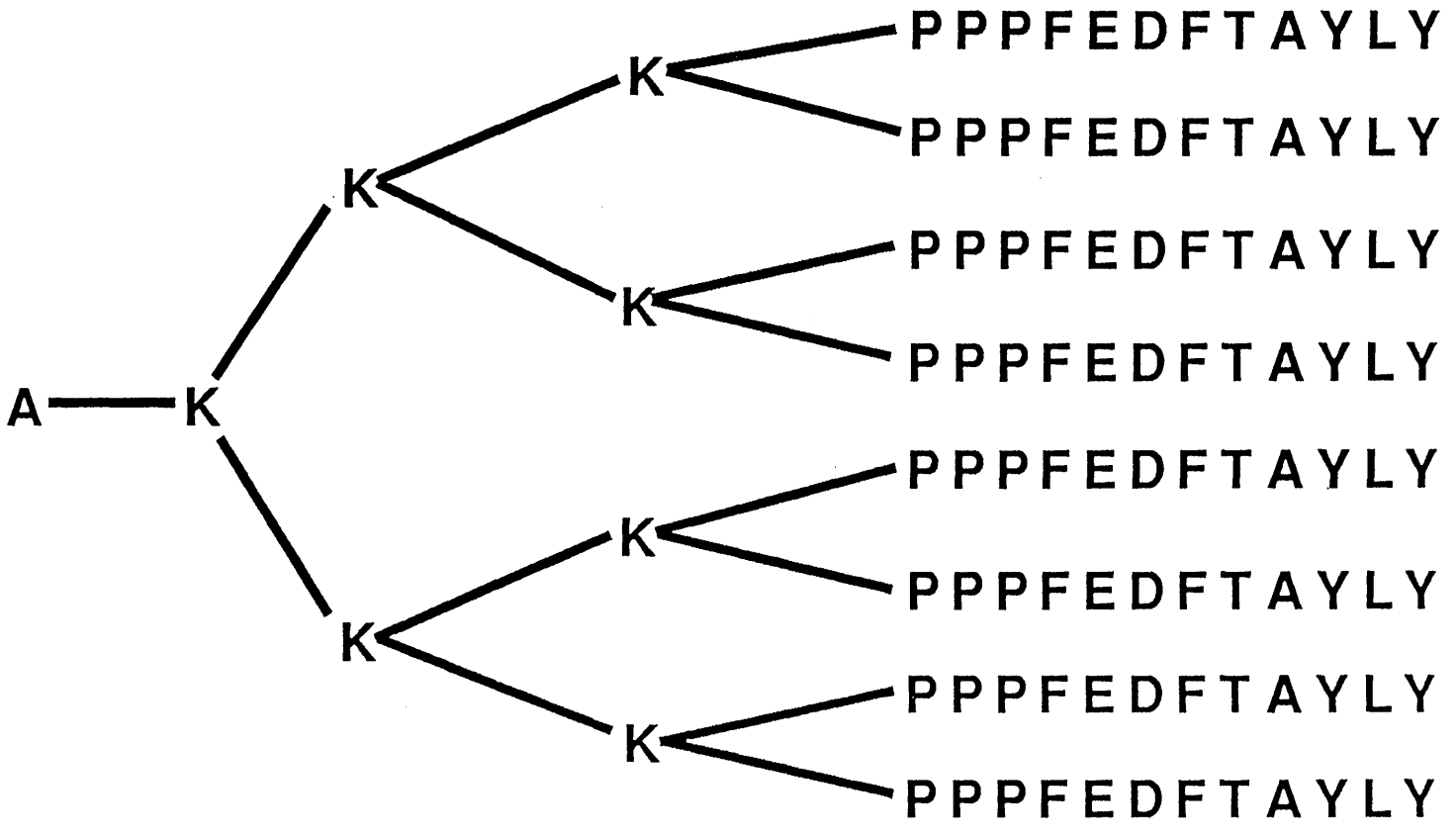
Therefore, experiments described in this thesis compare the efficacy of the "carrier-protein" method with some alternatives that were available i.e. the branching peptide immunogens described by Tam (1988)[see section 5.3]. This involved modifying the synthesis protocols described to use Fmoc chemistry to produce batches of the polylysine core (Fig. 4). On the branched core immunogens containing the sequence of interest were synthesised (Fig. 5). Another method tested was retention of the oligopeptide on the synthesis matrix of Kieselgur resin, which it was hoped, would then subsequently substitute for the macromolecular carrier. In addition the potential for transfer of the production of these antisera from outbred rabbit strains to Balb/c inbred mice was investigated, in order that a system for the production of monoclonal antibodies might be developed. It was anticipated that conjugation of the oligopeptides to synthetic "foreign" T_H-cell epitopes (section 5.3) would overcome the genetic restriction which might be expected to be encountered in such an inbred population.

It was decided to use the techniques described above to generate sera against peptides corresponding to regions of putative HSV-1 polypeptides. Five such gene products were chosen for investigation namely, UL31, UL41, UL45, UL46 and UL47. Identification and characterisation of the encoded proteins within the assigned open-reading frames of these genes would confirm the accuracy of the DNA sequence and increase our understanding of the molecular biology of HSV.

UL45: Identification of the predicted protein

FIGURE 5

Diagrammatic representation of branched peptide no.161. Amino acids contained in the molecule are represented by the one letter code.


A
K
K
K
K
K
K
K
K
PPPFEDFTAYLY
PPPFEDFTAYLY
PPPFEDFTAYLY
PPPFEDFTAYLY
PPPFEDFTAYLY
PPPFEDFTAYLY
PPPFEDFTAYLY
PPPFEDFTAYLY

product of the UL45 gene is described in this thesis. The UL45 gene product is potentially membrane associated as it contains a highly hydrophobic N-terminus with a potential transmembrane spanning region and a predicted signal sequence from the second translational start site (McGeoch *et al.*, 1988).

UL46 and UL47: The DNA sequence of McGeoch *et al.*, (1988) has predicted polypeptides of Mr 78,239 and 73,812 potentially encoded by genes UL46 and UL47 respectively. It has been suggested that the products of these genes are involved in the modulation (positively and negatively respectively) of the activity of the product of gene UL48 (the 65K trans-inducing factor) (McKnight *et al.*, 1987) although these results remain to be confirmed. There are also considerable sequence differences between the published sequences of genes UL46 and UL47 (McKnight *et al.*, 1987; McGeoch *et al.*, 1988) and it was anticipated that generation of protein-reactive antisera made against synthetic peptides would help to establish the accuracy of these sequences.

Recently reported experiments demonstrate that UL47 and perhaps UL46 are dispensible for viral growth on tissue culture as deletion mutants have been isolated that lack the complete UL47 gene and the carboxy terminal one third of the UL46 gene, which grow *in vitro* indistinguishably from wild-type, (Barker and Roizman, 1990). Additionally *in vivo* studies with two UL46 deletion mutants have shown the mutants display pathogenicity similar to that of wild-type virus in a rabbit eye model, although they show a reduced neuropathogenicity in mice (Gordon *et al.*, 1990; Abstracts: 15th International Herpesvirus workshop).

UL31: DNA sequence analysis indicates that the UL31 gene encodes a polypeptide of Mr 33,951. The sequence information indicates that there is an overlap of 19 codons, in a "tail-to-tail" fashion with gene UL30 (HSV-1 DNA polymerase), as well as a 3 codon overlap with gene UL32 (Quinn and McGeoch, 1985; McGeoch *et al.*,

1988). However this interpretation remains tentative, due to the uncertainty of the position of translational start sites. It was anticipated that by using a peptide sequence contained wholly within the proposed overlap to generate an antiserum, it would be possible to confirm the existance of the predicted overlap.

UL41: DNA sequence analysis indicates that the UL41 gene encodes a polypeptide of M_r 54,914. The product of this ORF has been identified as being involved in shut-off of host macromolecular synthesis (Read and Frenkel, 1983; Kwong *et al.*, 1988). However no specific antisera existed to the UL41 protein. It was hoped that generation of an antipeptide serum directed against the product of this gene would facilitate the further study of its involvment in host shut-off.

MATERIALS

Unless specifically stated all chemicals were obtained from BDH chemicals Ltd., Poole, Dorset or Sigma (London) Ltd. and were of analytical grade or better.

A. CHEMICALS AND REAGENTS

TISSUE CULTURE: All reagents used for tissue culture were supplies by Gibco-Biocult, Paisley, Scotland.

ELECTROPHORESIS: Acrylamide was supplied by Koch-Light Laboratories Ltd., Suffolk, UK. Ammonium persulphate and TEMED were obtained from Biorad Laboratories, Richmond, California. .

AUTORADIOGRAPHY: Kodak X-OMAT XS-1 and duplicating film were obtained from Kodak (London) Ltd. En^{3}hance was obtained from New England Nuclear, Boston, Mass., USA.

IMMUNOBLOTTING: Sheets of nitrocellulose were obtained from Schleicher and Schull, Dassel, West Germany. Gelatin was supplied by Biorad Laboratories. Non-immune rabbit serum was obtained from the Scottish Antibody Production Unit (SAPU), Scotland.

ELISA: Horseradish peroxidase-protein A conjugate and horseradish peroxidase-antimouse IgG conjugates were supplied by Biorad Laboratories.

OLIGOPEPTIDE SYNTHESIS: dimethylformamide, acetic acid, diethylether, t-amyl-alcohol and piperidine were all obtained from Rathburn Chemicals Ltd. F-moc amino acids were obtained from LKB Biochrom. Trifluoroacetic acid was supplied by Aldrich Chemical Company, England. Fmoc amino acids were supplied by LKB Biochrom, England; with the exception of Fmoc-Lys (Fmoc) and resin with the first amino acid attached which were obtained from Peptide and Protein Research, England.

HIGH PRESSURE LIQUID CHROMOTOGRAPHY: HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals Ltd., Walkerburn, Scotland. Dynamax 300A columns were obtained from Dynamax, England.

MISELLANEOUS: Ficoll 400, CNBr-activated sepharose and PD10 columns were supplied by Pharmacia, Upsalla, Sweden. NP-40 was obtained from Pierce Chemical Company, Chicago, Illinois. All radiochemicals were supplied by Amersham International PLC, Buckinghamshire, England.

B. SOLUTIONS

Electrophoresis buffer
52mM Tris, 53mM glycine, 0.1% SDS.

Transfer (Towbin) buffer
25mM Tris pH 8.3, 192mM glycine, 20% (v/v) methanol.

Tris buffered saline (TBS)
20mM Tris pH 7.5, 500mM NaCl.

Phosphate buffered saline (PBS)
170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4 pH 7.2 supplemented with 6.8mM $CaCl_2$ and 4.9mM $MgCl_2$.

Versene
6mM EDTA dissolved in PBS containing (w/v) phenol red.

Trypsin-Versene
One volume 0.25% (w/v) Difco trypsin (dissolved in 25mM Tris, 140mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 1mg/ml dextrose, 0.0015% phenol red pH 7.4 supplemented with 100 units/ml penicillin and 100 micrograms/ ml streptomycin plus 4 volumes versene.

Giemsa

1.5% (v/v) suspension of Giemsa in glycerol, heated at 56° C for 90–120 minutes and diluted with an equal volume of methanol.

Denaturing buffer

50mM Tris–HCL pH 6.7, 2% SDS, 700mM 2–mercaptoethanol, 10% glycerol, bromophenol blue (to colour).

Blocking buffer

1 x TBS plus 3% gelatin heated to 37° C.

Antibody buffer

1 x TBS + 1% BSA + 0.01% Sodium azide.

METHODS

11. GROWTH OF CELLS AND VIRUS STOCKS

11.1 GROWTH OF CELLS

Cells were grown in 80oz roller bottles in Eagle's medium supplemented with 5% v/v tryptose phosphate and 10% newborn calf serum (ETC-10),in an atmosphere of 90% air and 10% carbon dioxide. Once confluent the cells were harvested by washing twice with 20ml of trypsin/versene (1:4). The cells were then harvested in 20ml of ETC-10 and resuspended at a concentration of $1x10^7$ cells/ml and stored at 4° C for up to 3 days. Petri dishes were seeded at $3x10^6$ cells/50mm dish or $8x10^6$ cells/90mm dish for use the following day.

11.2 GROWTH OF VIRUS STOCKS

HSV-1 strain 17 syn^+ (Brown *et al.*, 1973) was used in these studies. In addition the phosphonoacetic acid resistant mutant PAA^r-1 was used, this was derived from HSV-1 17syn^+ (Hay and Subak-Sharpe, 1976); the mutation has been mapped within the DNA polymerase gene (Crumpacker *et al.*, 1980). Elite virus stocks (virus with a low serial passage number) was used to infect BHK monolayers which were approximately 90% confluent at a mutiplicity of 1 plaque forming unit (pfu) per 300 cells in 40ml of ETC-10. Virus was grown at 31° C and harvested when maximum cytopathic effects (cpe) were observed.

Infected cells were pelleted at 2,000rpm for 10 min in a Fisons Coolspin. The supernatant was spun in a Dupont Sorvall GSA rotor at 12,000rpm for 2h and the virus containing pellet resuspended in 5ml of supernatant and son icated until homogeneous. This was termed the supernatant virus stock (SV).

The cell pellet was sonicated until homogeneous, freeze thawed, resonicated and then spun at 2000rpm for 10min. The supernatant was kept and the pellet resuspended in 5ml of fresh ETC-10 and the above procedure repeated. The two supernatants were pooled and this represented the cell-associated virus stock (CV).

Virus stocks were checked for sterility by streaking on brain heart infusion blood agar plates and incubated at 37° C for 7 days. If no contamination was evident stocks were stored at -70° C.

Virus stocks were also titrated at both 31° C and 38.5° C : Virus was serially diluted 10-fold in PBS and used to infect BHK monolayers on duplicate plates. Virus was allowed to absorb for 1hr at 37° C then overlayed with 3% methyl cellulose and ETC-10 (1:1) to prevent extracellular spread of the virus and the plates incubated at 31° C for 3 days or 38.5° C for 2 days. The monolayers were fixed and stained with Giemsa stain and plaques counted using a dissection microscope.

12. PREPARATION OF INFECTED CELL EXTRACTS

12.1 PREPARATION OF LABELLED EXTRACT FROM CELLS LATE IN INFECTION

Monolayers of BHK cells at about 90% confluency were infected with stock virus at a multiplictiy of 20 pfu per cell in ETC-10. Virus was allowed to absorb at 37° C for 1hr and the plates were then washed twice in Eagle's medium containing one fifth the normal concentration of methionine and 2% calf serum. They were then further incubated in 5ml (90mm dish) of this medium for two hours. ^{35}S-methionine was then added at 50uCi/ml and the cells then reincubated at 37° C. Infected cells were harvested at 16-24hpost-infection.

12.2 PREPARATION OF EARLY LABELLED EXTRACTS

Cell extracts prepared in the absence of viral DNA replication were prepared by first pre-treating the cell monolayer with phosphonoacetic acid (PAA) at 300ug/ml for 1 h, then infected, maintained and labelled as for a normal infection but with the continuous presence of PAA.

12.3 HARVESTING OF INFECTED CELL EXTRACTS

Infected cell extracts were harvested in 2.5ml (for a 90mm dish) and 20ml (for a roller bottle) of denaturing buffer containing 50mM Tris.HCl pH 6.7, 0.1% SDS, 700mM 2-mercaptoethanol and 10% glycerol, containing bromophenol blue. Samples were heated to 70° C for 5 min then alliquoted and stored at -70° C until required.

13. PREPARATION OF PURIFIED LABELLED VIRIONS

Monolayers of BHK cells in 80oz roller bottles were infected when 90% confluent with virus at a multiplicity of 0.05 in ETC-10. Following overnight incubation at 37° C the culture medium was removed and replaced with Eagle's medium containing one fifth the normal concentration of methionine and reincubated for a further 5-6 hours. ^{35}S-methionine was then added to each roller bottle to give a final concentration of 25uCi/ml, the cells were then reincubated for a further 3-4 days until maximum cpe was observed.

The cell culture was clarified by centrifugation at 2500rpm for 30 min, the supernatant was then collected and the free virus was pelleted by centrifugation in a Sorvall GSA rotor for 2 h at 12,000rpm and 4° C. The pelleted virus was resuspended in a minimal volume of Eagle's medium without Phenol-red and stored at -70° C until required.

5-15% Ficoll 400 gradients were prepared with Eagle's medium without phenol red in cellulose nitrate

centrifugation tubes (Beckman AH627) and chilled to 4° C. Virus samples were then layered gently onto the top of the gradients, and spun at 12,000rpm for 2 h at 4° C in a Sorvall OTD0-50 ultracentrifuge using an AH627 rotor.

Following centrifugation the virion band was visualised by shining a high intensity light from above and removed by insertion of a 18 gauge needle through the wall of the tube. The virion particles were then pelleted in Eagle's medium without phenol-red by centrifugation in a AH627 rotor for 2 h at 21,000 rpm. (J.F. Szilagyi and C. Cunningham, personal communication). The virion pellet was resuspended in 500ul of Eagle's medium without phenol-red and stored at -70° C until required. For electron microscopy of virions, the purified virions (2ul) were applied to a parlodion-coated copper grid, allowed to absorb for 5min., blotted dry and stained with 3% phosphotungstic acid pH 7.0. Electron microscopy was carried out by Dr.F.J. Rixon.

13.1 IODINATION OF VIRION PROTEINS

Virion proteins were iodinated essentially as described by Markwell and Fox, (1978). Briefly, 2mg Iodo-gen (Pierce)[1,3,4,6-tetrachloro-3,6,-diphenylglycouril] was dissolved in 2ml chloroform immediately before use, 25ul was transferred to an Eppendorf tube, dried and stored under nitrogen. ^{125}I (200uCi)(Amersham; IMS.30) was added, followed by 30ul of Percoll-purified virions which had been diluted in phosphate buffer (pH 7.4) to a final buffer concentration of 10mM. Incubation was performed for 10min at between 0 and 4° C and the reaction stopped by transferring the solution to another tube without Iodo-gen. Iodination of virion proteins was carried out by Dr. N.Langeland and Dr. L. Haarr.

14. DETERGENT EXTRACTION OF VIRION PARTICLES

For detergent extraction of virion particles, NP-40

was diluted to the desired concentration in a buffer containing, 20mM Tris/ 5M NaCl pH 7.5. Virion particles in Eagle's medium without phenol-red were added to 100ul of a solution of NP-40 of the desired concentration , covered and incubated for 30 min at 31° C. Samples were then gently mixed and spun for 1 h. at 50,000 rpm and 4° C in a TLA 100.2 rotor using a Beckman table top ultracentrifuge. Following centrifugation both pellet and supernatant were resuspended in denaturing buffer at a ratio of two parts sample to one part buffer and boiled at 100° C for 5 min. Samples were then loaded on an SDS-polyacrylamide gel and separated by electrophoresis. For experiments involving the iodination of virion proteins the centrifugation step was ommited.

15. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

15.1 GEL ELECTROPHORESIS

For protein analysis vertical acrylamide gels were used: These were either single concentration or 5-12.5% gradient gels. Stocks of 30% acrylamide were prepared in water, the ratio of acrylamide to the crosslinking agent (N, N'-methylene bisacrylamide or N, N'-Diallyltartardiamide) was 20:1 and 40:1 for gradient and single concentration gels respectively. After filtering through Whatman No.1 filter paper these solutions were used to prepare the appropriate gel concentration in a buffer of final concentration of 375mM Tris.HCl, pH 8.9, 0.1% SDS.

For gradient gels the higher concentration acrylamide solution also contained 15% glycerol to stabilise the gradient. The gel solutions were polymerised with a final concentration of 3.65mM ammonium persulphate and 2.75mM TEMED. Following polymerisation of the acrylamide, a stacking gel consisting of 5% acrylamide in 122mM Tris.HCl, pH6.7, 0.1% SDS was added, along with a teflon comb to form the sample wells. Before

electrophoresis protein samples were boiled for 5 min in buffer of a final concentration of 50mM Tris.HCl, pH 6.7, 2% SDS, 700mM 2-mercaptoethanol and 10% glycerol, with sufficient bromophenol blue to visualise the dye front. Electrophoresis was carried out in a buffer containing 52mM Tris.HCl, 53mM glycine and 0.1% SDS, at either 10mA overnight at room temperature or 70mA for 3-4 h at 4° C.

15.2 STAINING OF SEPARATED PROTEINS

15.2.1 Coomassie brilliant blue staining

Following electrophoresis gels were fixed for 30 min. in methanol: acetic acid :water (50:7:50) containing 0.2% Coomassie brilliant blue R250, followed by destaining for at least 1h, in 5% methanol and 7% acetic acid. If required gels were the dried under vacuum onto Whatman 182 filter paper and exposed to Kodak XS-1 film at -70° C.

15.2.2 Silver staining

Following electrophoresis gels were fixed for 30 min in 30% ethanol/ 10% acetic acid. Gels were then removed from the fix solution and incubated for a further 30 min in a solution containing: 30% ethanol, 0.5M acetic acid, 0.5% gluteraldehyde and 0.2% sodium thiosulphate. The fixed gels were then rinsed thoroughly in water for 3x10 min, and then soaked in 0.1% silver nitrate/ 0.02% formaldehyde for 15-30 min. Gels were then developed in a solution containing 2.5% sodium bicarbonate/ 0.01% formaldehyde, pH 11.8, for 5-15 min. until all bands were visible. Development was then stopped by addition of 0.05M EDTA for 5 min followed by a further wash in dionised water. All solutions for siver staining were prepared using high purity filtered HPLC grade water.

Sequences of peptides described in this thesis

PEPTIDE NUMBER	PEPTIDE SEQUENCE [a,b] (McGeoch et al., 1988)	HSV-1 GENE	POSITION OF PEPTIDE IN HSV-1 GENE	TOTAL NO. OF AMINO ACIDS.
161	(Y)LYATFDEFPPP	UL31	290-301	301
177	(Y)THHLVKRRGLGA	UL41	12-23	480
172	(Y)GGFVQFVTSTRNA	UL45	156-168	172
202	YRPLGPTPPMRARLPA	UL45	10-26	172
258	MPLRASEHAYRPLGPTPPMRARLPAAAWVGVGTII	UL45	1-35	172
175	(Y)LTDANLIRGDNA	UL46	17-28	718
173	(Y)GAAALRAHVSGRRA	UL47	671-684	693

(a) Tyrosine residues in parentheses (Y) were added to facilitate coupling.

(b) Branched peptides consisted of eight copies each synthesised onto a branching lysine core, general formula: $(peptide)_8K_7A$.

TABLE 1

16. SYNTHESIS AND PRODUCTION OF SYNTHETIC OLIGOPEPTIDES.

16.1 SYNTHESIS OF SYNTHETIC OLIGOPEPTIDES.

Synthetic oligopeptides were synthesised, using continuous flow Fmoc chemistry (for reviews, Atherton *et al*, 1979, Sheppard 1983) using a LKB Biolynx automated peptide synthesizer. This involved sequential addition of of F-moc protected amino acids, in line with the required sequence onto a polyamide resin to which the first amino acid had been coupled via an acid labile bond (obtained from Peptide and Protein Research, Reading, UK) or directly onto Ultrasyn C resin (Pharmacia).

The peptides were synthesised both directly onto the resin and onto a branching lysine core (Fig. 4) to generate a peptide with eight identical branches (Fig. 5; Possnett *et al.*, 1988; Tam 1988) using two columns of the synthesiser in series. The lysine core was synthesised (onto an alanine residue coupled onto the resin) using Fmoc-Lys (Fmoc) pentafluorophenyl ester (purchased from Peptide and Protein Research, Reading, UK). The protecting F-moc group was removed at the end of every cycle using 20% piperidine in dimethylformamide (DMF) for 15-20 min. Following synthesis the peptide containing resin was dried down using alternate washes of DMF, t-amylalcohol, acetic acid (Sequencer grade), t-amylalcohol, DMF and finally diethyl ether and stored at -20° C until cleavage.

16.2 CLEAVAGE OF PEPTIDES FROM SYNTHESIS RESIN.

Following final deprotection and removal of the terminal F-moc group, the oligopeptides were cleaved from the synthesis resin using different protocols according to the types of amino acids in the oligopeptide. These protocols were taken from the Biolynx Peptide Synthesis Manual and were based on advice from Dr. P White (Pharmacia), Mr. P.Seale (Glaxo Group Research Ltd.), Dr.

M.Munns (Peptide and Protein Research Ltd.) via Miss A.Owsianka.

16.2.1 Cleavage of peptides attached to the resin via an acid labile linkage.

16.2.1.1 Cleavage of peptide 161 (Table 1).

This peptide contained no arginine residues: it was simoultaneously cleaved from the resin and side-chain protecting groups removed with a mixture of 95%TFA/5%H_2O. The dried down peptide bound resin was added to the TFA/H_2O mixture in a 100ml round bottom flask, stoppered and left at room temperature for 2 h. The mixture was then filtered slowly through a scintered glass funnel and then washed through with a further 25ml of 95%TFA/H_2O The peptide containing solution was then rotary evaporated at 35° C to remove the TFA. The remaining peptide containing solution was mixed with 50ml of water and shell-frozen in an acetone/dry ice bath, followed by freeze drying overnight. The resulting peptide was weighed and then stored at -20° C until required. The linear, monomeric form of peptide 161 was purchased from Cambridge Research Biochemicals Ltd.

16.2.1.2 Cleavage of peptides 172, 173, 175, 177 and 202 (Table 1)

These peptides contained one or more arginine residues. Removal of the Mtr (4-methoxy-2,3,6-trimethylphenylsulphonyl) protecting group is relatively slow and phenol is added as a scavenger to protect against unwanted side-chain reactions. The dried down peptide bound resin was added to 25ml of the TFA/phenol solution in a 100ml round bottom flask, stoppered and left at room temperature overnight. The solution was then filtered slowly through a scintered glass funnel and then washed through with a further 25ml of the cleavage

solution. The TFA was then removed by rotary evaporation at 35° C and the flask attached to a vacuum pump for two hours until the peptide solution was a crystalline solid. 100ml of 50% diethylether was then added to the flask, the solution was thoroughly mixed and added to a 500ml separation flask from which the aqueous phase was collected and subjected to a further round of ether extraction, the resulting aqueous phase was then rotary evaporated to remove any excess ether. The peptide mixture was then mixed with 50ml of H_2O, shell frozen and freeze dried as before.

16.2.1.3 Cleavage of peptide 258 (Table 1)

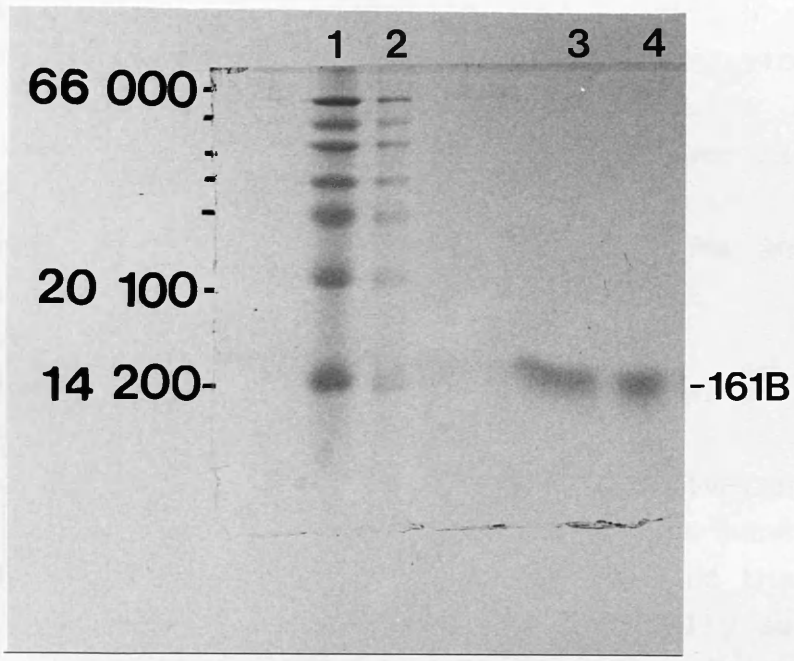
Peptide no.258 contains within its sequence both tryptophan, and arginine residues. This combination poses special problems as the Mtr side-chain protecting group from arginine can covalently attach to tryptophan especially during the long deprotection time needed for removal of the Mtr group. Furthermore methionine needs protection against oxidation. Finally tryptophan can irreversibly, covalently attach to the resin during cleavage of the peptide.

Cleavage and deprotection strategy involved blocking the reactive group on tryptophan with trimethylsiyl bromide (tmsb), which could later be removed. In addition the peptide was first cleaved from the resin under conditions in which side-chain protecting groups were not removed. The detailed protocol was as follows: The resin used for synthesis was Ultrasyn C., therefore the protected peptide was removed using 5% TFA in DCM, which was slowly dripped through the resin for 60 mins. The TFA and DCM was then removed by evaporation using nitrogen.

Following cleavage the side-chain protecting groups were removed using: TFA + 1M thioanisole, 1M trimethylsiyl bromide (tmsb), 20 molar eqiv. cresol and 20 molar equiv. ethanedithiol (EDT)[5mls of TFA +

FIGURE 6

Gel of molecular weight estimation of branched peptide 161. Peptides were separated on an SDS-polyacrylamide single concentration (15%) gel. Lanes 1 and 2 represent molecular weight markers (1ug and 0.5ug) respectively. Lanes 3 and 4 contain 5ug and 1ug of peptide 161B respectively. Peptides were visualised by staining with coomassie brilliant blue.


1
2
3
4
66 000
20 100
14 200
-161B

scavengers per 10mg resin]. The above mixture was added to the protected peptide at 0° C, under an atmosphere of nitrogen and incubated at 0° C for 25mins. Following incubation the TFA + scavengers were removed using nitrogen evaporation. Sufficient diethylether was added to precipitate the peptide, following centrifugation ether extraction was repeated followed by freeze-drying. (Note: tmsb is a highly reactive chemical, special precautions were taken during the use of this chemical, including use of protective clothing and full face-protection and carrying out all manipulations under an atmosphere of nitrogen).

16.2.2 Cleavage of peptides attached via base-labile linkage agent

Side-chain protecting groups of peptides covalently attached to the synthesis resin via a base-labile handle were removed while leaving the peptide attached to the synthesis resin. Cleavage was performed identically as descibed in section 16.2.1 except following reaction with the cleavage solution, a futher 100ml of cleavage solution was dripped through the resin very slowly in a scintered glass funnel to ensure complete removal of all side-chain protecting groups.

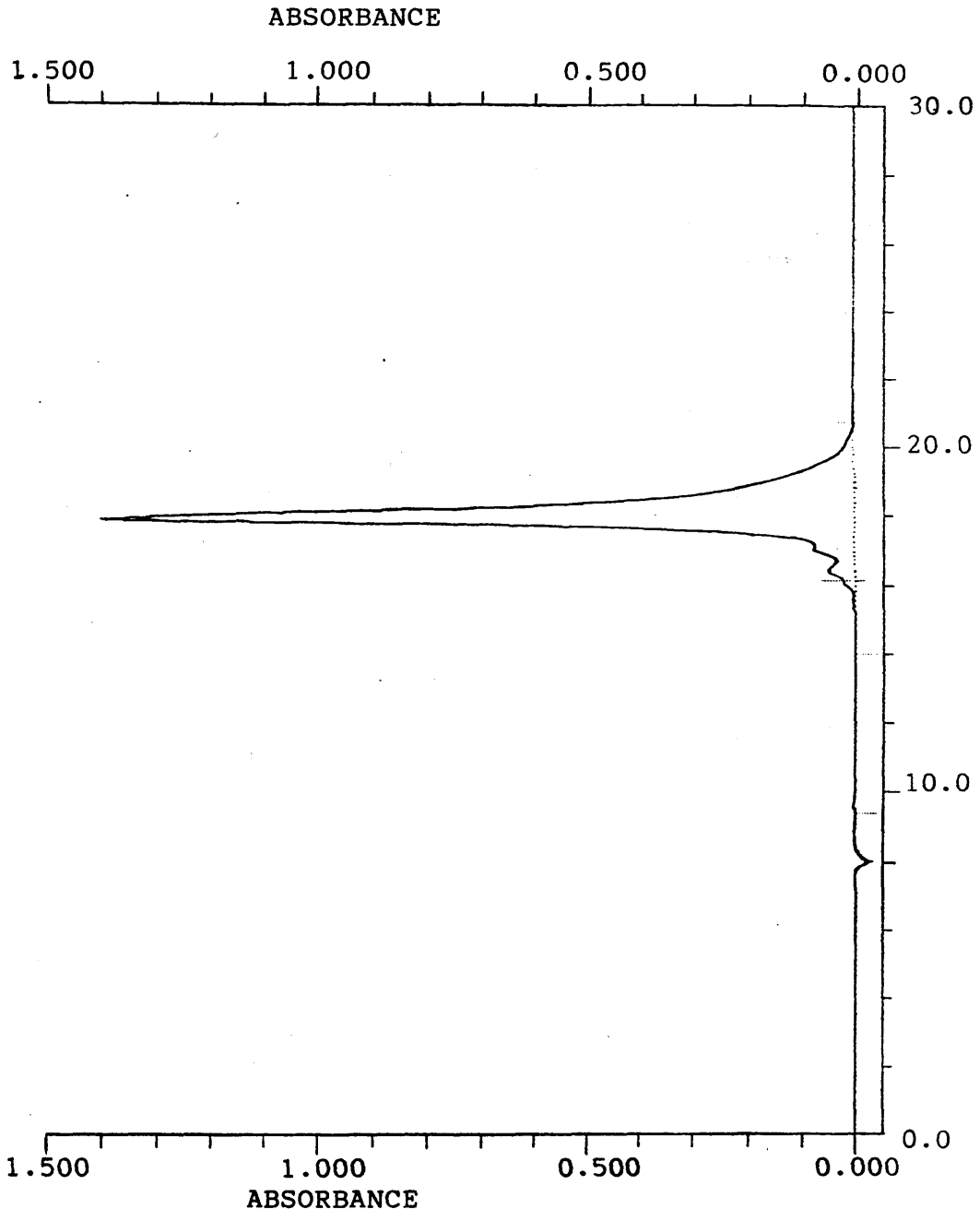
16.3 ANALYSIS OF SYNTHETIC OLIGOPEPTIDES

Following continuous-flow sythesis in three columns linked in series, peptides were cleaved from the synthesis resin as described above and subjected to analysis in order to determine their composition with respect to homogeneity, amino acid content and M_r as follows:

Monomeric, linear, peptides were subjected to HPLC analysis using a Beckman System Gold HPLC and a Dynamax analytical column (4.6mm internal diameter x 25cm length, 300A C8 resin, catalogue no. 83-3-03-C), and a gradient of 0% to 95% acetonitrile (Rathburn Chemicals) in water,

FIGURE 7

HPLC profile of peptide No.258 (Table 1). 50ug of peptide 258 was analysed using a Beckman System Gold HPLC and a Dynamx analytical column as described in Methods section 16.3. The peptide is represented by the large single peak which was 94% of the material analysed. The Y-axis represents absorbtion (225nm) while the X-axis represents time in minutes.


ABSORBANCE
1.500
1.000
0.500
0.000
30.0
20.0
10.0
0.0
1.500
1.000
0.500
0.000
ABSORBANCE

of duration 20 minutes and flow rate 0.5ml/min (wavelength 225nm). In addition they were also routinely tested for the correct M_r using Fast atom bombardment spectroscopy (Fab-mass) by M-Scan Ltd. The results of these analysis are summerized in Table 2.

Branched peptides, because of their high molecular weight can be visualised using polyacrylamide gel electrophoresis as an initial test of homogeneity and estimation of M_r, an example of which is shown in Fig. 6, where the branched form of peptide 161 (Table 1) with a predicted M_r of 12,641 was shown to have a mobility of around M_r 14,000 and an appearance of a single homogeneous band. All other branched peptides were also analysed in this way and gave the expected results. Branched peptides were also subjected to HPLC analysis as described (an example of which is shown in Fig. 7) above with the results summarised in Table 2. Finally all branched peptides were subjected to amino acid analysis to determine their amino acid composition (carried out by Cambridge Research Biochemicals Ltd.). All branched peptides gave the expected amino acid compositions.

Analysis was not carried out on the resin-linked peptides. As synthesis was carried out on three columns of the synthesiser in series, peptides which remained attached to the sythesis resin by the base-labile linkage were assumed to be identical in composition to the monomeric peptides. By the nature of the structure of resin-linked peptides HPLC and Fab-mass analyses were not possible.

Throughout this thesis peptides are referred to by their number as given in Table 1. In addition, branched peptides are referred to by 'B' e.g. 161B, whereas 'R' represents resin-linked peptides and 'E' represents peptides conjugated to the T_HCE e.g 161R and 161E respectively.

TABLE 2

Analysis of peptides as cleaved from the resin: M_r values (a) determined by fast-atom bombardment spectroscopy (M-Scan Ltd.); % purity (b) determined by HPLC analysis as described in Section 16.3; amino acid composition (c) determined by amino acid analysis (Cambridge Research Biochemicals Ltd.)

TABLE 2

Analysis of peptides

PEPTIDE NUMBER	EXPECTED M_r	OBSERVED M_r[a]	%PURITY[b]	AMINO ACID[c] COMPOSITION
161	1459	1459	90	NA
161B	12641	NA	84	CORRECT
177	1507	1507	69	NA
177B	13025	NA	74	CORRECT
172	1546	1546	82	NA
172B	13337	NA	79	CORRECT
202	1850	1850	89	NA
202B	15769	NA	77	CORRECT
258	3582	3582	94	NA
175	1435	1435	90	NA
175B	12449	NA	72	CORRECT
173	1555	1555	77	NA
173B	13409	NA	81	CORRECT

16.4 COUPLING OF PROTECTED PEPTIDES TO RESIN-BOUND BRANCHING PEPTIDES.

Protected 17-mer SWM-1 peptide (Peptide and Protein Research, Reading; Fig. 11) was coupled to resin bound branching peptides using diisopropylcarbodiimide (DIPCI): 0.1 mM Of peptide bound resin was mixed with a solution containing 0.2mM of protected peptide and 0.22mM 1-hydroxylbenzotriazole (HOBT) in a minimal volume of DMF, (in practice 1 ml) in a 10ml glass stoppered test tube. This mixture was then added to an equal volume of dichloromethane (DCM) and placed on ice. 0.22mM of DIPCI was added dropwise by syringe and the solution agitated for 60 sec. then allowed to stand on ice for 15 min. The mixture was then rotated gently overnight using the motor of a rotary evaporator at room temperature. The resulting peptide bound resin was cleaved as described previously (Section 16.2).

17. GENERATION OF ANTIBODIES

17.1 LINKAGE OF PEPTIDES TO CARRIER PROTEINS

17.1.1 Preparation of bis-diazotised benzidine(DAB)

DAB was prepared by Dr.Howard S. Marsden according to the method of Likhite and Sekar (1967). Briefly, 0.23g benzidine hydrochloride was dissolved in 45ml 0.2M HCl, 5ml of 0.5M sodium nitrate was added and the solution stirred slowly at 0° C for 1 h. The DAB was then aliquoted into 2ml amounts and stored at -70° C.

17.1.2 Coupling reaction

The coupling reaction was carried out according to the method of Basseri *et al.*, (1979). 30mg of appropriate carrier protein: either bovine thyroglobulin, bovine serum albumin, were added to 10mg of appropriate peptide dissolved in 0.16M borate pH 9.0, 0.13M NaCl. 1ml of DAB was added slowly dropwise stirring continuously and the

solution was left on ice for 1 h. The resulting solution was dialysed against PBS-A to remove excess DAB and stored at -70° C.

17.2 PRODUCTION OF ANTISERA TO SYNTHETIC OLIGOPEPTIDES

17.2.1 Quantitation of peptide used to immunise animals

The amount of peptide used for immunisations was quantitated in the following ways. For protein-conjugated peptides the extent of coupling was determined in a series of independent experiments in which peptides were first iodinated via the tyrosine residue (Hunter and Greenwood, 1962) and the radioactivity both attached to the carrier protein and remaining as free peptide was measured following separation on a Sephadex G25 column (Pharmacia). The extent of coupling varied between 20% and 30%. Branched peptides were weighed directly. For resin-linked peptides the amount of peptide was estimated by first measuring colorimetrically the amount of the first amino acid attached per gram of resin as described in the Pharmacia peptide synthesis manual and then assuming 100% coupling of each amino acid during the synthesis.

17.2.2 Preparation of antisera in rabbits

Female New Zealand White and Sandy-Half Lop rabbits were used for all immunisations. Rabbit sera was prescreened for low reactivity on western blots before immunisation. Initial screening was performed for each animal on 1ml of serum obtained from the supplier (HyLine) who tagged each animal. Animals with low reactive serum were then purchased for the study and 10ml of pre-immune serum obtained and stored as described in section 17.3. Rabbits were given an intramuscular immunisation on day 0 and then a further three injections on days 10, 30 and 40. Test bleeds were taken 7 days after every injection and rabbits were bled out on day 50. All immunisations contained 100ug peptide emmulsified

in either Freund's complete adjuvant (FCA) for the primary injection or Freund's incomplete adjuvant (FIA) for all subsequent injections.

17.2.3 Preparation of antisera in mice

Female Balb/c mice were used for all immunisations. Mice were immunised either intramuscularly, intraperitoneally or subcutaneously on day 0 and day 30 and test bleeds taken 7 days after each injection. Immunisations contained either 1ug or 50ug of peptide emmulsified in FCA, for the primary and FIA for the subsequent immunisations.

17.3 PREPARATION OF ANTISERA

Following collection, rabbit and mouse serum was placed at 37° C for 1h, in order to aid clotting. Released serum was then removed by aspiration using a glass pipette. The blood clot was then broken down using a sterile glass rod and the blood subjected to centrifugation (2000rpm for 10min); followed by removal of the remaining serum by aspiration. The resulting serum was aliquoted and stored at -20° C.

For antiserum generated against peptides conjugated to BSA, the collected serum was passed through a BSA-Sepharose column to remove any BSA reactive antibodies. To do this BSA was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Laboratories) according to the manufacturers instructions. 1g of dried beads was swollen for 15-30 min using 1mM HCl, in a scinted glass funnel and then rinsed with coupling buffer (0.1M $NaHCO_3$ pH 8.3, 0.5M NaCl). BSA (5-10mg/ml of gel) was dissolved in coupling buffer adjusted to a volume of 5ml and mixed end-over-end with the Sepharose for 2 h at room temperature. The beads were then washed with coupling buffer.

Any remaining active groups on the beads were blocked by incubating end-over-end with 15ml of 1M

ethanolamine pH 8.0 for 2h at room temperature. Non-covalently absorbed proteins were removed by washing three times in 0.1M acetate, 0.5M NaCl, pH 4.0, then three times in 0.1M $NaCO_3$, 0.5M NaCl, pH 8.3. The beads were then given a final rinse in PBS and stored at 4° C in PBS containing 0.01% sodium azide until required. Following passage through the column the resulting serum was aliquoted and stored as described previously.

18. IMMUNOASSAYS

18.1 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

ELISA assays were carried out to determine the titre of antipeptide sera obtained from mice and rabbits. The appropriate antigenic peptide was diluted in PBS to a final concentration of 20ug/ml. 100ul of antigen solution was added to each well of a 96 well microtitre plate giving 2ug of peptide per well.

Peptides were allowed to adsorb to the plate at 37° C overnight. Antigen solution was then removed and the plates blocked in a solution containing 1% BSA for 1 h. at 37° C, the plates were washed three times in PBS containing 0.01% Tween-20 (PBS-Tween) and incubated with 100ul of the the appropriate antibody dilution at 37° C for 1 h. For detection of bound antibody plates were washed five times in PBS-Tween and incubated with 100ul per well of either horseradish peroxidase-protein A conjugate (for rabbit antisera) or horseradish peroxidase-antimouse IgG conjugate (for mouse antisera) at a 1/1000 dilution in PBS for 1h at 37° C. Plates were then washed seven times in PBS-Tween and incubated in a 50mg/ml solution of 2'2 azino-bis(3-ethylbenzthazoline-6-sulphonic acid) [ABTS] in citrate phosphate buffer containing 0.01% hydrogen peroxide. After 15 min of colour development reaction the plates were read on a Titretek Multiscan plate reader at 405nm. All ELISA results were the means of duplicate parallel

determinations. Appropriate controls of pre-immune sera and unrelated peptides were included in all ELISA experiments.

18.2 IODINATION OF PROTEIN A

Protein-A was reconstituted in PBS at a concentration of 0.5mg/ml, aliquoted into 20ul amounts and stored at -70° C until required. To 10ug (20ul) of protein A was added, 10ul of 1M potassium phosphate buffer pH 7.5, 200uCi of ^{125}I (NaI^{125}, Amersham International) and 20ul of chloromine T (0.5mg/ml in PBS). This mixture was allowed to react for 20sec. at room temperature, then 20ul of sodium metabisulphite (1mg/ml in PBS) was added to stop the iodination reaction. Removal of free NaI^{125} was achieved by passage through a Sephadex G25 PD10 column (Pharmacia).

Pre-packed PD10 columns containing 9ml of Sephadex G25 were obtained from Pharmacia laboratories. Non-specific protein binding sites were blocked with 20ml of buffer containing 0.1% BSA, and then equilibriated with the required buffer.

The iodinated protein A was eluted from the column using PBS, 0.5ml fractions were collected and 10ul was assayed for radioactivity in a gamma counter. Two peaks were observed, the first containing the protein A and the second the free iodide. Fractions containing the iodinated protein A were pooled and stored at 4° C, in the presence of 0.1% sodium azide.The iodination reactions were carried out by Mrs. Mary Murphy.

18.3 IMMUNOBLOTTING

The immunoblotting technique was that of Towbin *et al.* (1979), with several modifications. Cells were harvested in denaturing buffer at a concentration of 10^7 cells/ml.(Methods section 12.3). Samples were boiled for 5 min and were loaded onto SDS-polyacrylamide gels,

at approximately 10^6 cell equivalents per cm. Following electrophoretic separation at 10mA overnight, proteins were then transfered to nitrocellulose using a Biorad transblot apparatus. Three foam pads and two sheets of Whatman no. 182 filter paper were pre-soaked in transfer buffer (192mM glycine, 25mM Tris.HCl pH 8.3 and 20% methanol) along with the nitrocellulose sheets to be used in the transfer. The gel was layed down on one sheet of the filter paper on top of two of the pre-soaked foam pads and then covered with nitrocellulose. This assemblage was rolled with a glass rod to exclude air bubbles. The nitrocellulose was then covered with the other sheet of filter paper and a further foam pad and the plastic holder was closed tightly over the sandwich. This sandwich was placed in the transfer tank with the gel towards the cathode and the nitrocellulose towards the anode. Proteins were transfered onto nitrocellulose electrophoretically in transfer buffer at 250mA for a minimum of 3 h. at room temperature.

Following electrophoretic transfer nitrocellulose was removed from the sandwich and blocked for 2 x 30 mins. at 37°C in blocking buffer (3% gelatin, 1mM Tris.HCL pH 7.4, 15.4mM NaCl, and 0.05% Tween-20). Sheets were then washed 2 x 5 min. in wash buffer (1M Tris.HCL pH 7.4, 15.4mM NaCl with 0.05% Tween-20). Antibody solutions were made up to the desired concentrations in wash buffer containing 1% BSA and 0.01% sodium azide and added to the nitrocellulose in perspex wells or plastic dishes, depending on the size of the nitrocellulose and incubated overnight at room temperature.

Following overnight incubation in the antiserum, nitrocellulose was washed thoroughly in wash buffer for 4 x 5 min. ^{125}I-protein A was diluted to 1.5 x 10^5 cpm/ml in wash buffer containing 3% BSA and incubated with the nitrocellulose for 2h at 37°C, then washed in wash buffer containing 1M potassium iodide for 2 x 60 min. The nitrocellulose was then given a final rinse in wash buffer without Tween-20 and dried on tissues, before

being taped to cardboard sheets for autoradiography.

For autoradiography the procedure of Haarr *et al.*, (1985) was used, to align the ^{35}S-methionine labelled protein tracks with the ^{125}I-protein A signals. This involves simultaneously exposing the nitrocellulose to 3 sheets of Kodak XS-1 film. The first film is placed directly on top of the membrane followed by a sheet of black paper then the further two sheets of film and an image intensifying screen (Dupont). The first film detects electrons from the decay of ^{35}S-methionine which are unable to transfer to the second film. The high energy gamma emmisions from the ^{125}I-protein A pass directly through all three films with little absorption and interact with the image intensifying screen producing photons which are directed mainly onto the third film and to a lesser extent onto the second film: the signal is prevented from reaching the first film by the sheet of black paper. Thus the ^{35}S-methionine signals on the first film remain largely independent of the ^{125}I signal. Radioactive ink containing ^{14}C and ^{125}I spotted onto the cardboard produces images on the three films allowing them to be aligned (^{14}C on the first and ^{125}I on the second and third).

18.4 VIRAL NEUTRILISATION ASSAY

Virus was diluted to the desired concentration and mixed with the antiserum under test at the desired concentration diluted with various concentrations of guinea-pig complement (SAPU). Virus was incubated with antibody at 31°C for 1 h. Following incubation, virus samples were then plated onto 80% confluent BHK cells in 50mm dishes (200pfu and 2000pfu) and overlayed with Eagle's medium containing methyl cellulose as described in section 11.2 . Plates were incubated for three days at 31°C and then plaques counted.

RESULTS

19. GENERATION OF OLIGOPEPTIDE INDUCED ANTISERA

For generation of antisera to put tive HSV polypeptides, oligopeptides were synthesised which ranged from 13-35 amino acids in length. The sequence and nomenclature of peptides is shown in Table 1. Three types of oligopeptides were synthesised:

A. Linear, monomeric peptides.

B. Branched peptides (Tam, 1988; Posnett *et al.*, 1988; see Section 9.3; Fig. 5).

C. Linear, monomeric peptides covalently attached to the synthesis matrix.

To investigate the immunogenic properties of the three peptide forms. Six different peptide sequences were presented in each of the three forms (monomeric, branched and resin-linked) to rabbits. Petides 161, 172, and 173, (Table 1) were used to immunise female New-Zealand White rabbits, whereas peptides 175, 177 and 202, (Table 1) were used to immunise female Sandy Half-lop rabbits. Immunisations of 100ug peptide were made intramuscularly to the schedule described in Methods section 17.2.1. Seven days after each injection approximately 20ml of blood was taken from rabbits, serum was prepared as described (section 17.2.3) and tested for antipeptide antibodies.

Table 1, shows the sequences of the synthetic peptides and the predicted HSV-1 gene products containing those sequences (McGeoch *et al.*, 1988). These peptides were selected from predicted protein sequences on the basis of their proximity to their amino- or carboxy-terminus or because they correspond to hydrophyillic regions, criteria which are considered favourable to generation of antisera reactive with the protein of which the peptide forms a part (Palfreyman *et al.*, 1984; Lerner, 1984; Introduction section 9.2). These six peptides, each protein-conjugated, resin-bound and in

FIGURE 8

ELISA determination of anti-peptide response. Rabbit serum was tested in 2-fold dilutions against the immunising peptide sequence. Results are plotted as A_{405nm} vs dilution of serum. Results shown are for the final sera obtained on day 50 of the experiment and represent the mean values of duplicate plates. Panels a-f represent the data obtained with the six peptide sequences, 161, 177, 172, 202, 175 and 173 respectively. ···○···○···○··· represents rabbits immunised with peptides coupled to a carrier protein, -■-■-■- represents rabbits immunised with branched peptides. Animals immunised with the resin-linked peptides are represented by -▲-▲-▲-

ANTIPEPTIDE ELISA TITRE

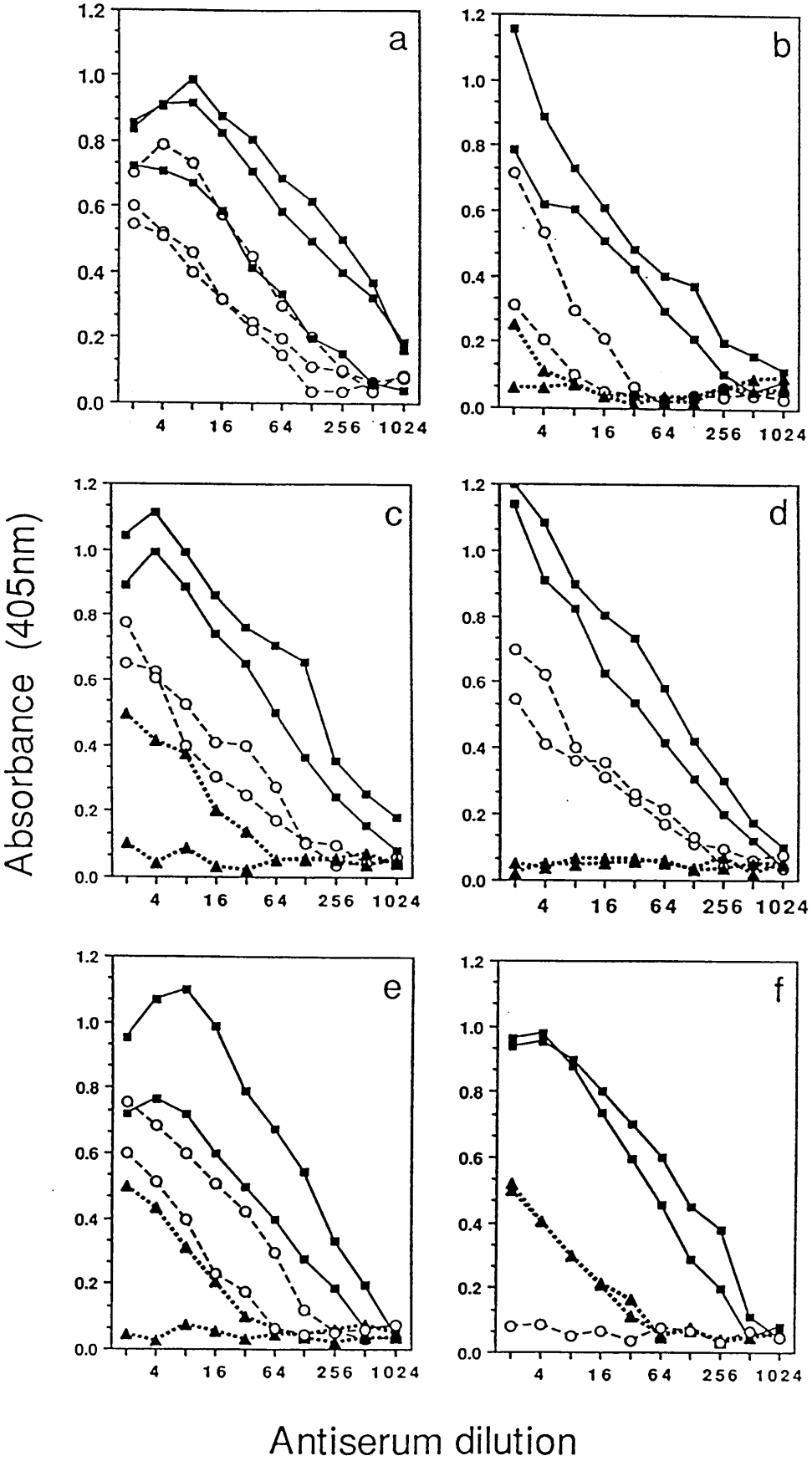


TABLE 3

Antipeptide titre of antipeptide rabbit sera[a]

PEPTIDE NUMBER	HSV-1 GENE CONTAINING PEPTIDE SEQUENCE	TITRE OF SERA FROM RABBITS IMMUNISED WITH		
		PROTEIN-CONJUGATED PEPTIDE	BRANCHED PEPTIDE	RESIN-LINKED PEPTIDE
161	UL31	128, 64, 64	1024, 512, 128	nd
177	UL41	64, 4	256, 128	2, 0
202	UL45	64, 32	1024, 256	16, 0
172	UL45	64, 32	256, 256	0, 0
175	UL46	64, 16	512, 256	16, 0
173	UL47	0, d	256, 256	16, 16
TOTAL:		596	5120	66
MEAN:		49.7	393.8	6.6

a Titre expressed as the greatest dilution giving an OD_{405} greater than 0.2 in ELISA assays.

0 No detectable reactivity

nd not done

d died during experiment

branched form, were used to immunise rabbits.

19.1 ANTIPEPTIDE TITRE OF THE RABBIT SERA

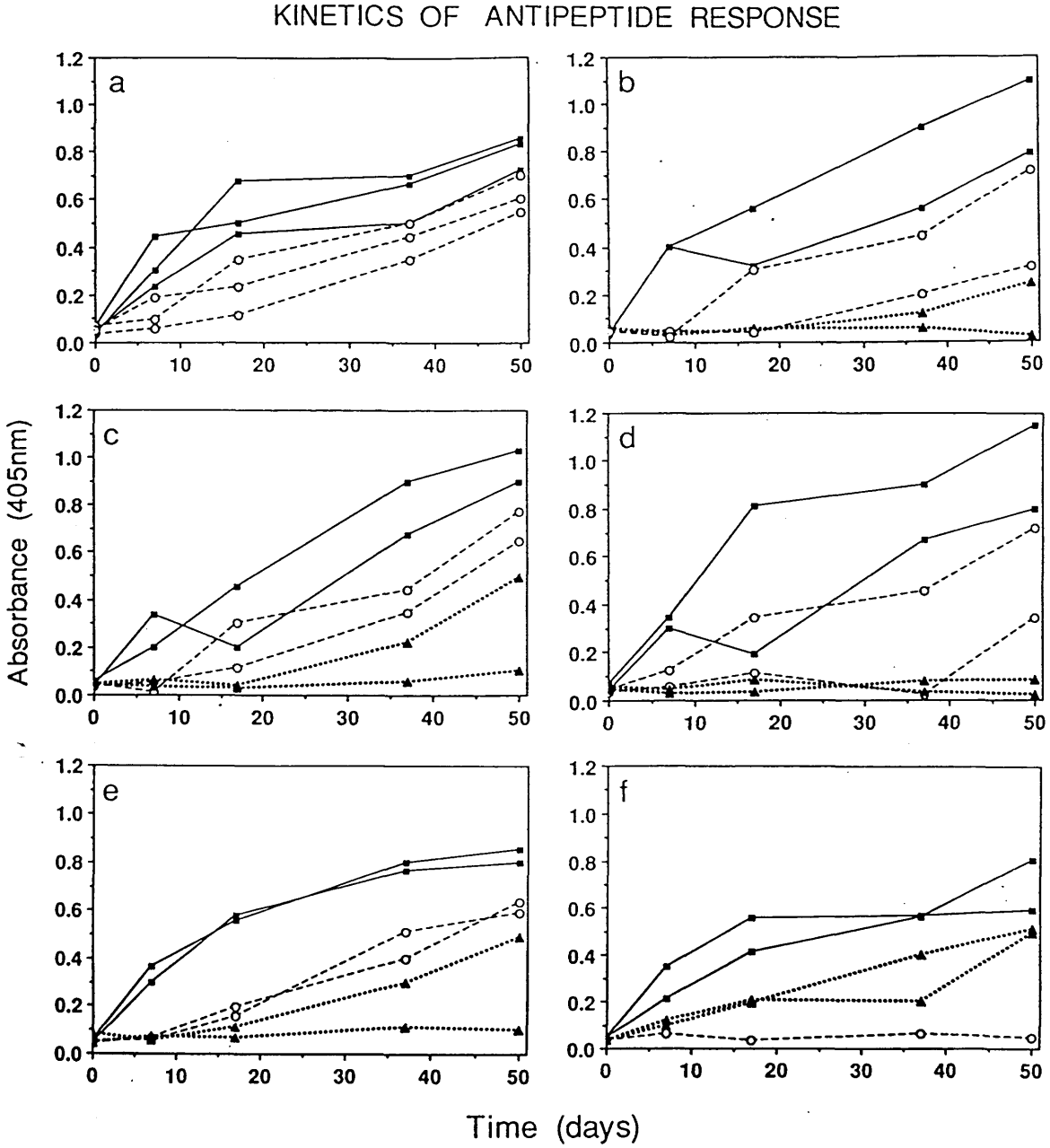
Enzyme-linked immunoabsorbant assay (ELISA) was used to determine the antipeptide titre of the five sera obtained from each of the 36 rabbits used in the experiment. Fig. 8, shows the titres of sera from the final bleed (day 50) taken from the rabbits. Each panel represents the sera from rabbits immunised with one peptide sequence.

Several interesting features are evident from this data. First, all rabbits immunised with branched peptides produced sera which was reactive with the immunising peptide. Whereas in contrast, some rabbits immunised with either the protein-conjugated peptides (Panel f) or the resin-linked peptides (Panels b, c, d and e) failed to give any detectable response. Secondly the sera of animals immunised with the branched peptides are consistently of higher titre than sera of animals immunised with protein-conjugated or resin-linked peptides. In fact, in only one case was a serum obtained (following immunisation with a protein-conjugated peptide) with a titre which was equal to that obtained using a branched peptide (Panel a).

The data were quantified (Table 3) in the following manner. First, the dilution of antiserum required to give an optical density of 0.2 was obtained from the data in Fig. 8. Non-reactive sera were given a value of zero. These values were summed for rabbits immunised with each of the three forms of peptide and this sum divided by the number of rabbits in each group. The values so obtained were 49.7, 394 and 6.6 for protein-conjugated, branched and resin-linked peptides respectively. This data shows that the antipeptide titre of sera generated using branched peptides was 9-fold higher than that generated by protein-conjugated peptides or about 58-fold higher than that generated by resin-linked peptides.

FIGURE 9

ELISA determination of anti-peptide response with time. Rabbits were immunised as described, on days 0, 10, 30, and 40. Results shown are a 1/10 dilution of serum collected on days 7, 17, 37, and 50. Results are plotted as A_{405nm} vs time (in days) and are the means of duplicate plates. Panels A-F represent the data obtained with the six peptide sequences, 161, 177, 172, 202, 175 and 173 respectively. The symbols are identical to those for Figure 8.


KINETICS OF ANTIPEPTIDE RESPONSE
a
b
c
d
e
f
Absorbance (405nm)
Time (days)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
10
20
30
40
50

Titre in Western blots of antipeptide sera[a]

Peptide	HSV-1 gene containing peptide sequence	Titre of sera from rabbits immunised with		
		protein-conjugated peptide	branched peptide	resin-linked peptide
161	UL31	40, 0, 0	160, 0, 0	nd
177	UL41	0, 0	0, 0	0, 0
202	UL45	40, 0	160, 0	0, 0
172	UL45	160, 0	160, 0	0, 0
175	UL46	0, 0	160, 160	0, 0
173	UL47	0, d	160, 160	0, 0
Total		240	1120	0
Mean		20	86.2	0

a titre expressed as the greatest fold dilution giving a band
0 no detectable reactivity
nd not done
d died during experiment

TABLE 4

To determine the rate at which the rabbits responded to immunisation with the differently presented peptides, a similar analysis was performed on sera obtained on days 0 (pre-immune), 7, 17, 37 and 50. Figure 9, shows a part of the data; namely the ELISA titre of ten-fold dilutions of antisera. Several features are obvious: First, the response against immunisation with the branched peptides is both more rapid and of greater magnitude than that against either protein-conjugated or resin-linked peptides. Second, the sera of all animals immunised with branched peptides has elevated peptide titres after only a single injection. This is in contrast to animals immunised with both protein-conjugated peptides (in which only 3 out of 12 sera had antipeptide antibodies and these were of low titre) and resin-linked peptides (in which only 2 out of 10 sera had antipeptide antibodies, again of low titre). Third, the titre of almost all positive sera increased with each immunisation suggesting that continued immunisations might produce more protein reactive sera.

19.2 ANTI-PROTEIN TITRE OF THE RABBIT SERA

Western blotting was used to determine the anti-protein titre of the final bleeds from each of the rabbits. Sera were serially four-fold diluted and screened together with the pre-immune sera on both mock-infected BHK cells and cells infected with HSV-1. The results for one serum 94497, from a rabbit immunised with peptide 173 are shown in Figure 10. The important features of this figure are the folowing: First, a polypeptide not recognised in extracts from uninfected cells or by pre-immune serum is recognised by the immune serum in extracts of infected cells. The M_r of this polypeptide is 82,000 and its properties are described in detail later in this thesis and elsewhere (Section 21.1; McLean *et al.*, 1990). Second, the polypeptide is recognised by this serum diluted up to 160-fold. Data for

FIGURE 10

Western blotting for determination of anti-protein titre of rabbit serum. HSV-1 infected cell polypeptides were separated by SDS-PAGE and western blotted. Lanes 1-4 are 1/10, 1/40, 1/160 and 1/640 dilutions respectively of antiserum 94497 raised against the branched peptide 173B, known to recognise a HSV-1 protein of M_r 82,000 (McLean *et al.*, 1990). Lane 5 shows the reactivity of the 94497 pre-immune serum and lane 6 shows the reactivity of 94497 with mock-infected cells.

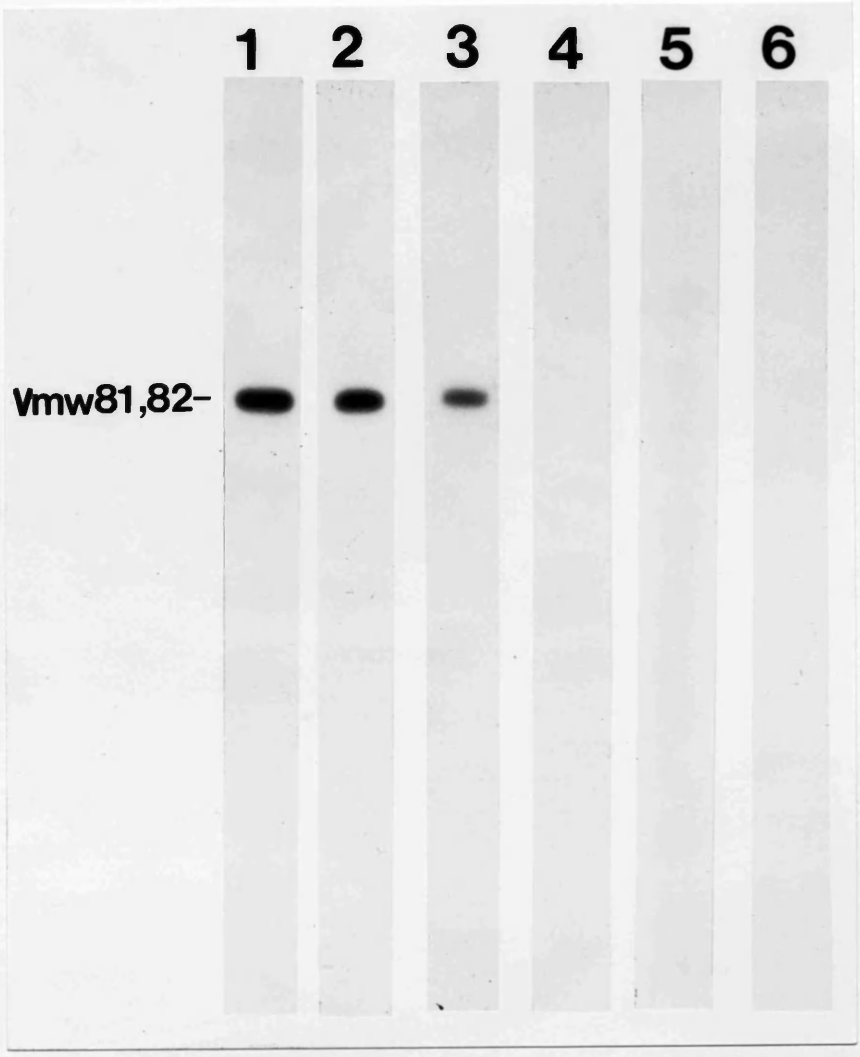

1
2
3
4
5
6
Vmw81,82–

FIGURE 11

Diagramatic representation of the 161 branched peptide following attachment of the SWM-1 T_H-cell epitope to all eight amino terminal tyrosine residues. The portion of the immunogen which represents the T_H CE is shown within the broken lines. All amino acids are represented by the one-letter code.

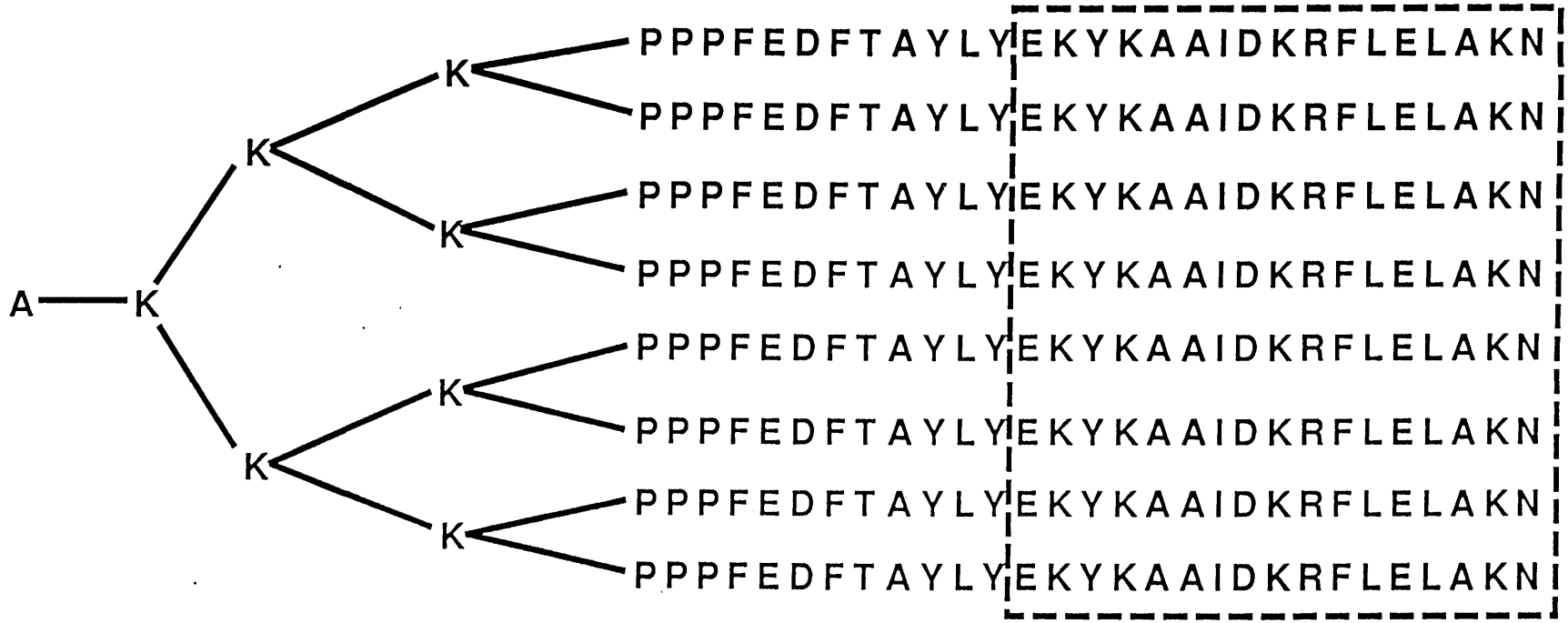

A
K
K
K
K
K
K
K
K
PPPFEDFTAYLYEKYKAAIDKRFLELAKN
PPPFEDFTAYLYEKYKAAIDKRFLELAKN
PPPFEDFTAYLYEKYKAAIDKRFLELAKN
PPPFEDFTAYLYEKYKAAIDKRFLELAKN
PPPFEDFTAYLYEKYKAAIDKRFLELAKN
PPPFEDFTAYLYEKYKAAIDKRFLELAKN
PPPFEDFTAYLYEKYKAAIDKRFLELAKN
PPPFEDFTAYLYEKYKAAIDKRFLELAKN

TABLE 5

Antipeptide response to SWM-1

PEPTIDE NUMBER	ANTIPEPTIDE TITRE TO T_HCE[a]
161	256, 256, 512, 512, 512
173	256, 1024, 1024, 1024, 1024
175	128, 512, 512, 512, 1024
177	0, 0, 0, 0, 0
242	256, 512, 1024, 1024, 1024
244	512, 512, 512, 1024, 2048

0 No detectable reactivity.

a Titre expressed as the greatest dilution giving an OD_{405} greater than 0.2 in ELISA.

this serum and that of the other rabbit sera were quantitated as described for the antipeptide sera and are shown in Table 4.

These data show that branched peptides produced a much better anti-protein response than did either protein-conjugated or resin-linked peptides. None of the resin linked peptides yielded a serum reactive with protein. The mean titres of the sera produced by the branched and protein-conjugated peptides were 86.2 and 20.0 respectively (Table 4). No sera from animals immunised with peptide 177, from the predicted product of gene UL41, was reactive with a virus-specific protein.

19.3 COUPLING OF "FOREIGN" T-CELL EPITOPE TO BRANCHED PEPTIDES

The 17 amino acid "foreign" T-cell epitope (T_HCE) SWM-1 (Berkower *et al.*, 1985), from sperm whale myoglobin was coupled via carbodiimide to the amino termini of the branched peptide molecules, to produce a large immunogen containing both B and T_H-cell epitopes (Fig. 11). This procedure is described in detail in Methods section 16.4. Four branched peptides, were used namely: 161B, 177B, 173B and 175B (Table 1). Two additional branched peptides were included in these experiments. Although unrelated to the other work described in this thesis they were used to further increase the number of sample antigens in the experiment and so provide a more comprehensive investigation. They were 242B: $(MATDIDMLIDLGLDLS)_8K_7A$ from HSV-1 gene UL54 (IE63), representing amino acids 1-16 and 244B: $(TGAPDVSALGAQGVL)_8K_7A$ from HSV-1 gene IE175, representing amino acids 985-999. These peptides were synthesised and coupled to the T_HCE by Miss A.M. Owsianka and Dr.H.S.Marsden.

To confirm that the T_HCE was attached and was being introduced into the mice; the resulting mouse sera were tested via an ELISA assay for the presence of

FIGURE 12

Titration of antiserum raised in ten Balb/c mice. The data presented is for mice immunised with branched peptide 173, either alone or conjugated to the SWM-1 T-cell epitope. Those sera raised against 173 alone are represented by —○—○—○— while those sera raised against 173 + SWM-1 are represented by ■—■—■

DATA FOR PEPTIDES 173 AND 173E

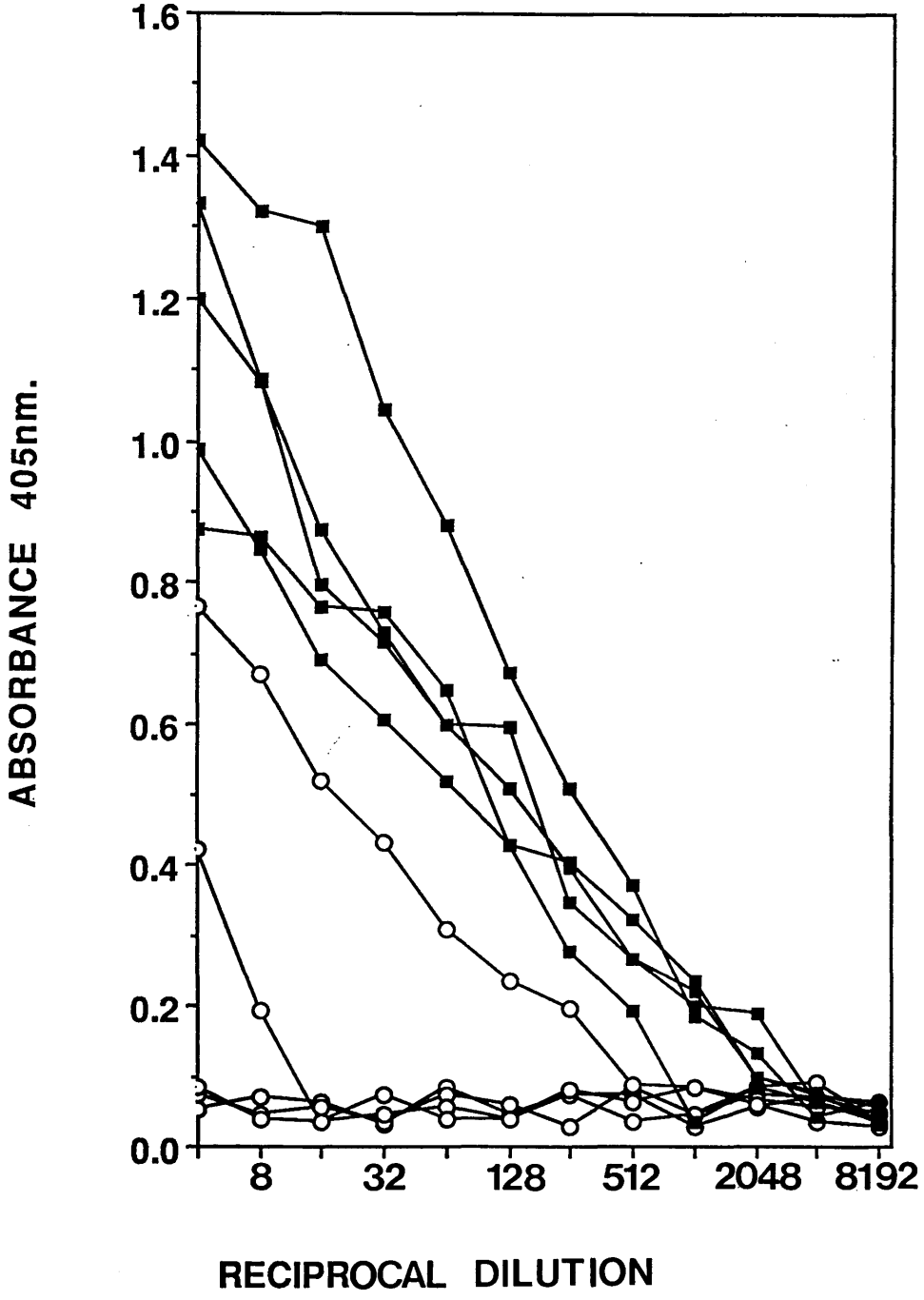


TABLE 6

Antipeptide titre of sera from mice immunised with branched peptide or branched peptide coupled to SWM-1

PEPTIDE NUMBER	TITRE OF SERA FROM MICE IMMUNISED WITH:[a]	
	BRANCHED PEPTIDE ALONE	BRANCHED PEPTIDE + SWM-1
161	0, 0, 0, 0, 1024	128, 128, 256, 1024, 1024
173	0, 0, 0, 4, 128	256, 512, 1024, 1024, 1024
175	0, 0, 0, 0, 0	512, 512, 1024, 2048, 4096
177	0, 0, 0, 4, 8	0, 0, 4, 4, 128
242	0, 0, 4, 4, 8	512, 512, 1024, 2048, 2048
244	4, 8, 32, 256, 256	1024, 1024, 2048, 2048, 2048
TOTAL:	1740	29064
MEAN:	58	968.8

0 No detectable reactivity

a Titre expressed as the greatest dilution giving an OD_{405} greater than 0.2 in ELISA

antibodies to the SWM-1 sequence. All mouse sera, with the exception of those mice injected with peptide 177E, contained high titres of antibodies to this sequence (Table 5), providing evidence of attachment of the T_HCE to at least one "arm" of the immunogen. This was confirmed by examination of the products of coupling reactions using SDS-PAGE analysis which showed coupling efficiencies ranging from 0-100% attachment of SWM-1 (not shown).

19.4 ANTIPEPTIDE TITRE OF BRANCHED PEPTIDES + SWM-1

Balb/c mice were immunised with two 50ug injections of the branched peptide, either alone or in combination with T_HCE molecules, as described in detail in Methods, section 17.2.2. Bleeds were taken 7 days after the second immunisation and tested for antipeptide titre in an ELISA assay. Sera were screened against the appropriate branched peptide in the absence of the T_HCE to exclude measurment of antibodies generated against the T_HCE sequence.

Figure 12, shows the titres of sera from the bleed taken 7 days after the second immunisation of one peptide sequence. The panel represents the sera from mice immunised with one peptide sequence. Several interesting features are evident from this data (Table 6). First, of the 30 mice immunised with branched peptides in combination with the T_HCE only two mice failed to respond to the immunogen, whereas when the corresponding branched peptides were used without the presence of the T_HCE, 17 mice out of a possible 30 failed to give any detectable antipeptide response. Second, using criteria to determine antipeptide titre identical to those described earlier (section 19.1), the data shows that of the 13 mice that responded to the branched peptides in the absence of the T_HCE only one achieved a titre of greater than 1000 (8%). Whereas, of the 28 positive antisera raised using the branched peptides in combination with the T_HCE, 15 had a

FIGURE 13

Bar graph representing the mean titres for 12 groups of 5 mice immunised with branched peptides either alone (−), or in combination with the T_HCE (+). Peptide numbers are shown along the X-axis while mean titres were determined from the data presented in Table 6.

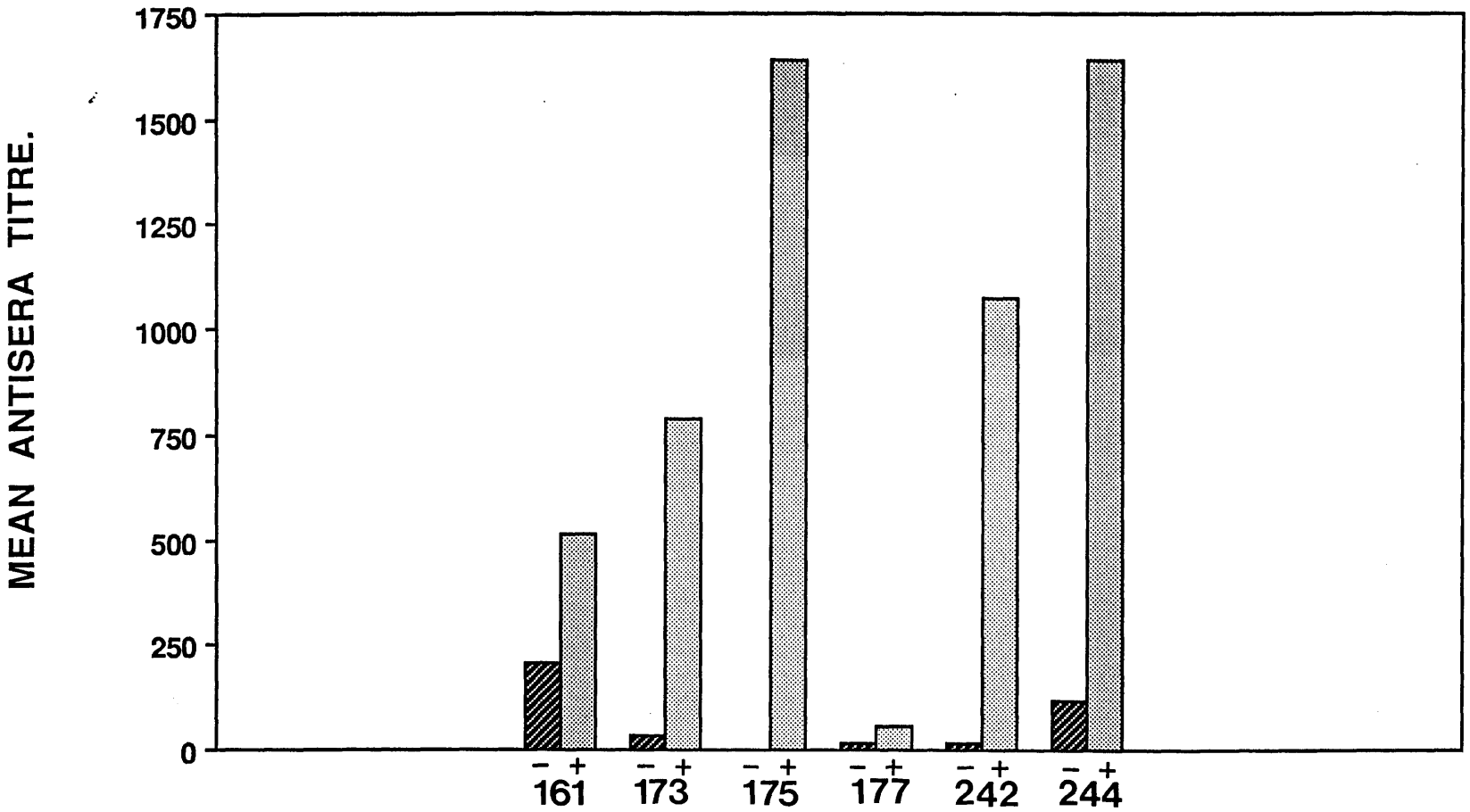
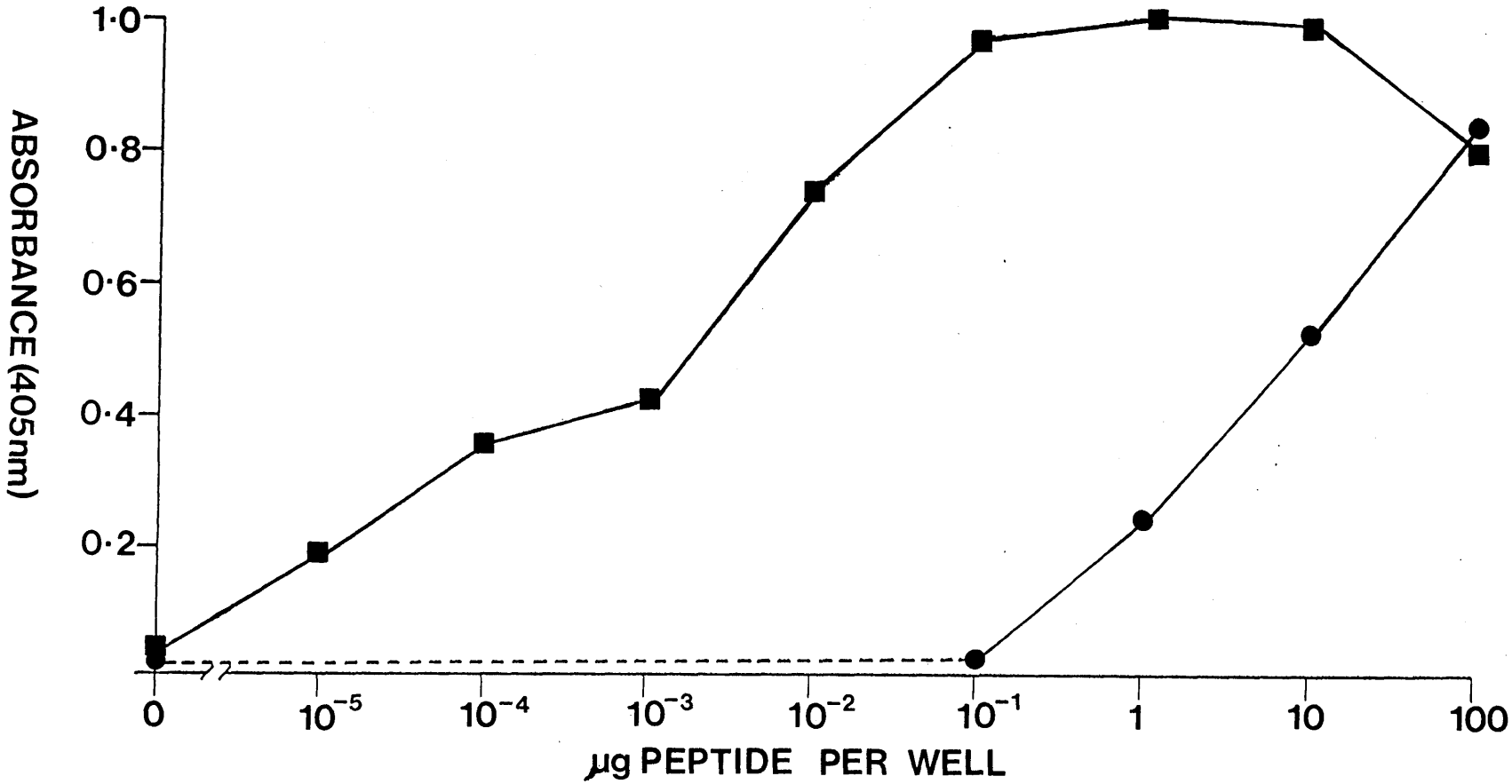

MEAN ANTISERA TITRE.
1750
1500
1250
1000
750
500
250
0
− +
161
− +
173
− +
175
− +
177
− +
242
− +
244

FIGURE 14

Graph representing increasing concentrations of both branched and monomeric peptide (X-axis), tested via an ELISA assay against one single concentration of antiserum 94497 (1/40). Branched peptide 173B is represented by  , while the monomeric peptide 173 is represented by 


ABSORBANCE (405nm)
1·0
0·8
0·6
0·4
0·2
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
10
100
µg PEPTIDE PER WELL

titre of greater than 1000 (54%) (Table 6). Third, the only branched peptide/T_H CE molecule that failed to produce 5 positive mice, giving only 3 positives of low titre, was 177 and this peptide produced a generally poor response in rabbits (Figures 8 and 9).

The antipeptide titre for mice immunised either with or without the T_H CE were summed, and these sums were divided by the total number of mice in each group (30) (Table 6). The values so obtained for branched peptide/T_H CE immunogens was 968.8, whereas the value for branched peptides alone was 58. Showing that the antipeptide titre of sera generated by branched peptides in combination with the SWM-1 T_H CE was about 17-fold higher than that generated when the T_H CE was absent. The mean titres of antisera raised with both immunogens either with or without the T_H CE are summerised in Figure 13.

20. SENSITIVITY OF BRANCHED AND MONOMERIC PEPTIDES IN ELISA ASSAYS

Both monomeric and branched peptides were tested in ELISA assays to determine the minimum amounts of each that was neseccary to detect a given antibody, the results from one example are shown in Figure 14. The results presented in this figure show that a concentration of 1ug of monomeric peptide per well of a microtitre plate was required to obtain an absorbance of >0.2. Whereas in contrast as little as 0.01ng of the branched peptide form was required. This result held true whether the antiserum had been raised against either the branched or monomeric peptide. The results presented in Figure 14, are typical of those observed for all peptide sequences tested; these results are summerized in Table 7 and the values demonstrate that the amounts of branched peptide required to elicit a given absorbance in an ELISA assay ranged from 10^2 to 10^4 less than the amount of monomeric peptide required.

TABLE 7

Sensitivity of branched and monomeric peptides to detect antibody in ELISA assays

PEPTIDE NUMBER	ug PEPTIDE REQUIRED FOR OD GREATER THAN 0.2 AT 405nm.			
	SERA RAISED AGAINST PROTEIN -CONJUGATED PEPTIDE		SERA RAISED AGAINST BRANCHED PEPTIDE	
TESTED AGAINST:	BRANCHED PEPTIDE	MONOMERIC PEPTIDE	BRANCHED PEPTIDE	MONOMERIC PEPTIDE
161	10^{-4}	10^{-1}	10^{-4}	10^{-1}
172	10^{-4}	10^{-1}	10^{-3}	1
202	10^{-3}	1	10^{-3}	10
174	10^{-4}	1	10^{-4}	1
173	10^{-4}	10^{-1}	10^{-4}	1
177	10^{-3}	1	10^{-4}	10

FIGURE 15

A schematic presentation of the predicted protein product of gene UL47 of HSV-1 strain 17 with the location and sequence of the peptide against which the antiserum 94497 was raised. The HSV-1 genome is shown with the unique long (U_L) and unique short (U_S) regions, the terminal repeats (TR_L and TR_S), internal repeats (IR_L and IR_S) and the locus of gene UL47 (McGeoch *et al.*, 1988). The fractional length of the genome is shown at the top of the figure. The numbers at the end of the open reading frame and peptide refer to amino acid numbers in the predicted sequence. The amino-terminal tyrosine (Y) residue in parenthesis is not part of the predicted amino acid sequence of UL47 (see text).

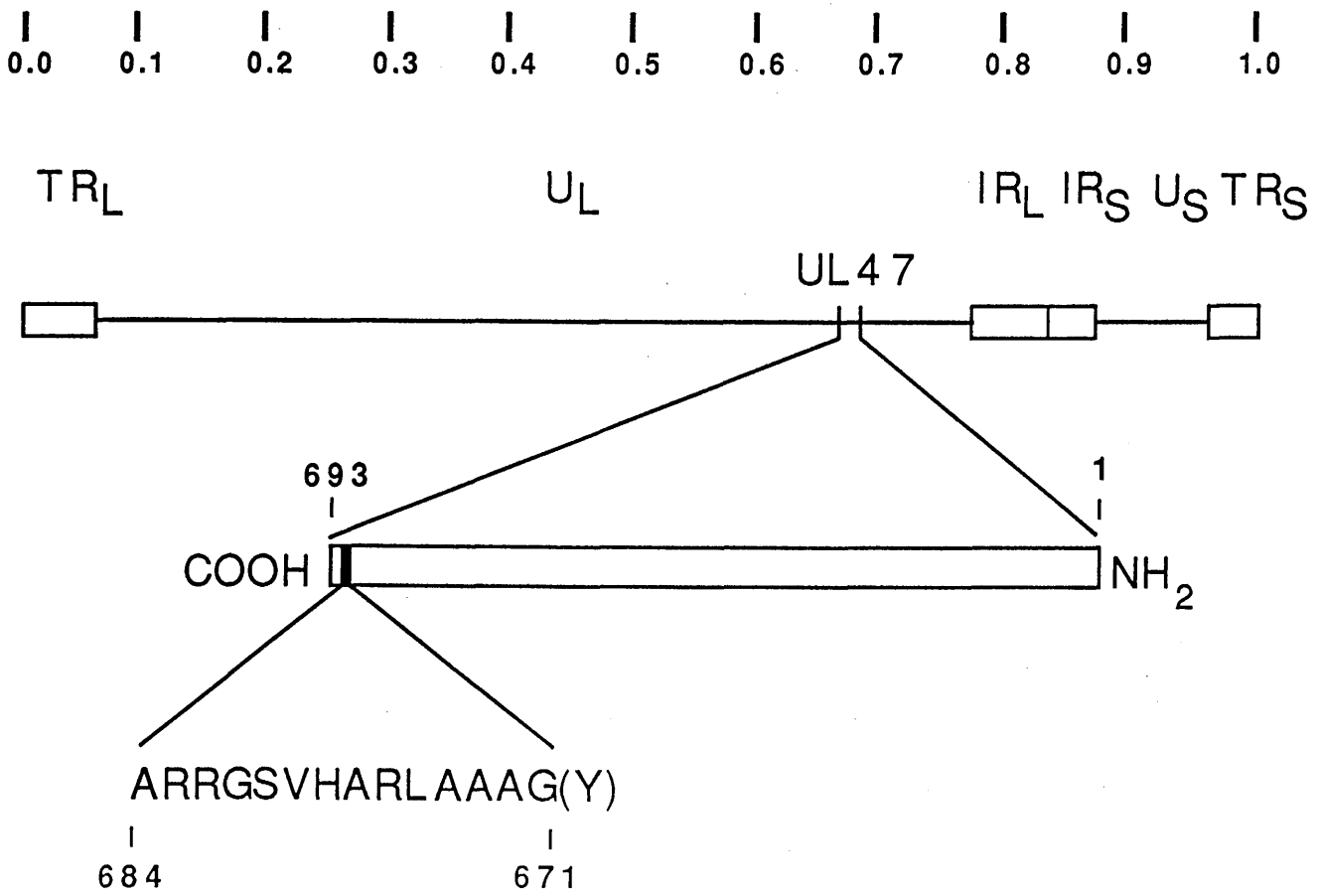
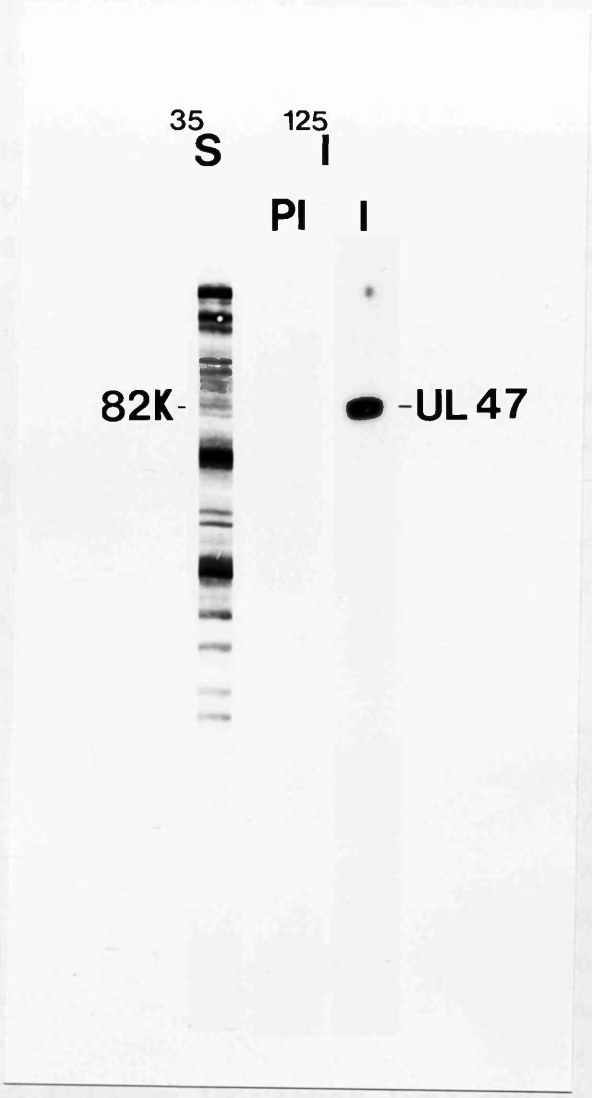

0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
TRL
UL
IRL IRS US TRS
UL47
693
1
COOH
NH2
ARRGSVHARLAAAG(Y)
684
671

FIGURE 16

Western blotting with the 94497 serum showing specific recognition of an 82K polypeptide in HSV-1 infected cells. The first lane (^{35}S) shows ^{35}S-labelled HSV-1 infected cell proteins transferred to nitrocellulose. The second and third lanes (^{125}I) shows polypeptides on the nitrocellulose that were detected by the pre-immune (PI) or immune (I) 94497 serum (1/100 diln.) respectively. In this and subsequent figures, unless otherwise stated, polypeptides were separated on 5-12.5% SDS polyacrylamide gels and alignment of the ^{35}S-labelled and ^{125}I-labelled lanes was as described by Haarr *et al.*, (1985).


35 S
125 I
PI
I
82K-
-UL 47

22. IDENTIFICATION OF HSV-1 GENE PRODUCTS

22.1 IDENTIFICATION OF THE PRODUCT OF GENE UL47

Identification of the product of gene UL47 using antiserum 94497: Figure 15, shows the position of the open reading frame of gene UL47 on the prototype arrangment of the HSV-1 genome (McGeoch *et al.*, 1988) together with the position and sequence of the peptide against which antiserum 94497 was raised. The reactivity on western blots of this antiserum with proteins from HSV-1 infected cells is shown in Figure 16. The immune serum specifically detects a strong polypeptide band of apparent M_r 82,000 in 5-12.5% gels which is not detected by a sample of the pre-immune serum (Fig. 16) and is also shown to be absent from a mock-infected cell extract (Fig. 21; Panel A, lane 1). This band was therefore considered to be the protein product of gene UL47.

To test whether the protein product of gene UL47 is a structural component of the HSV-1 virus particle, virions were first purified from the supernatant of ^{35}S-met labelled cells (Rixon *et al.*, 1988). Figure 17, is an electron micrograph of such a preparation and shows it to be substantially free of any visible contaminating cellular debris. Virion proteins were subjected to gel electrophoresis, transferred to a nitrocellulose membrane and probed with serum 94497. Both 5-12.5% gradient gels crosslinked with BIS (Figure 18, lanes 1-6) and 6% gels cross-linked with DATD (lanes 7-9) were used because in earlier experiments (Marsden *et al.*, 1976), a polypeptide doublet of apparent M_rs 82K and 81K had been observed in purified virions and it was anticipated that one of the gel systems might resolve the UL47 gene product as a doublet. Figure 18 (lanes 1 and 7), show the methionine-labelled virion polypeptides which were transfered to the membrane. The profile is similar to that reported earlier (Heine *et al.*, 1974; Marsden *et al.*, 1976; Dargan, 1986., Rixon *et al.*, 1988) in that the major proteins seen

FIGURE 17

Electron micrograph of the virion preparation used for the experiment shown in Fig 18. Virions were prepared as described in the text. 2ul of virion sample was applied to a parladion-coated copper grid, allowed to absorb for 5 min, blotted dry and and stained with 3% phosphotunstic acid (pH 7.0). Preparation of the virions used for this electron micrograph and the electronmicroscopy was kindly carried out by Dr.F.J. Rixon.

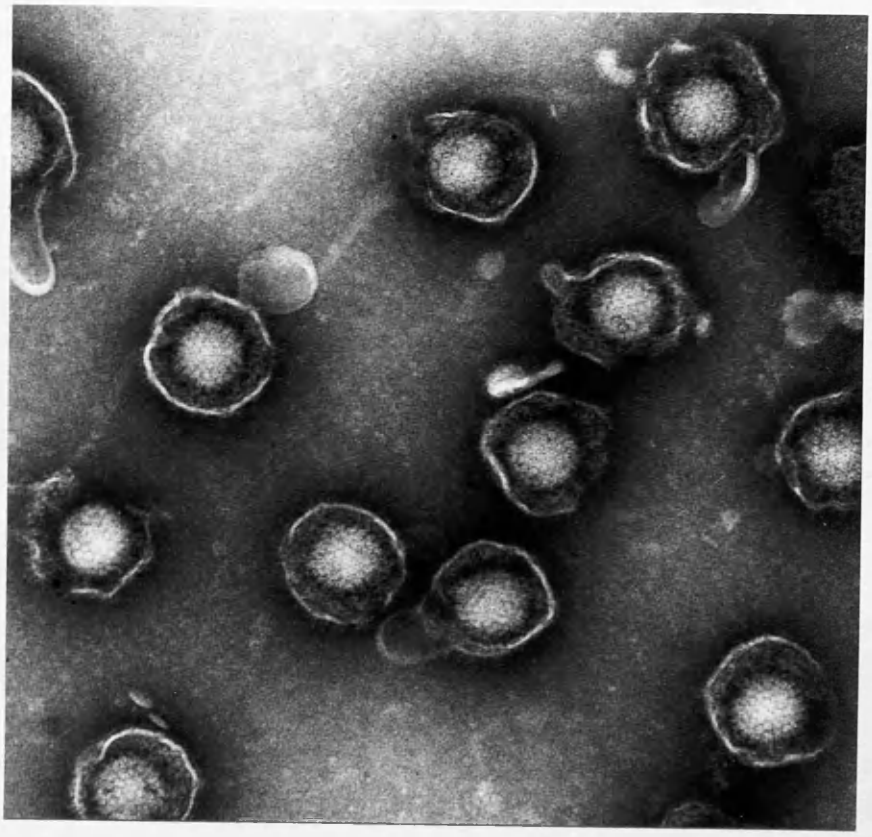


FIGURE 18

The 82/81K polypeptide products of gene UL47 are virion proteins. HSV-1 virions, labelled with ^{35}S-methionine, were separated by SDS-PAGE using either 5-12.5% gels crosslinked with BIS (lanes 1-6) or 6% gels cross-linked with DATD (lanes 7-10), then transferred to nitrocellulose membrane strips. Lanes 2-6, 8 and 9 were probed with serum 94497 [1/100 diln.] in the absence of polypeptide (lanes 2, 8 and 9) or in the presence of 1ug/ml (lane 3), 10ug/ml (lane 4) and 100ug/ml (lanes 5 and 10) of branched peptide $(YGAAALRAHVSGRRA)_8 K_7 A$ against which the antisera was raised or 100ug/ml of the unrelated branched peptide $(YLTPANLIRGDNA)_8 K_7 A$ (lane 6). Bound antibody was visualised with ^{125}I-protein-A (tracks 2-6, 8, 9 and 10). Lane 9 is a shorter exposure of lane 8. The major virion proteins transferred to nitrocellulose are indicated on the left.

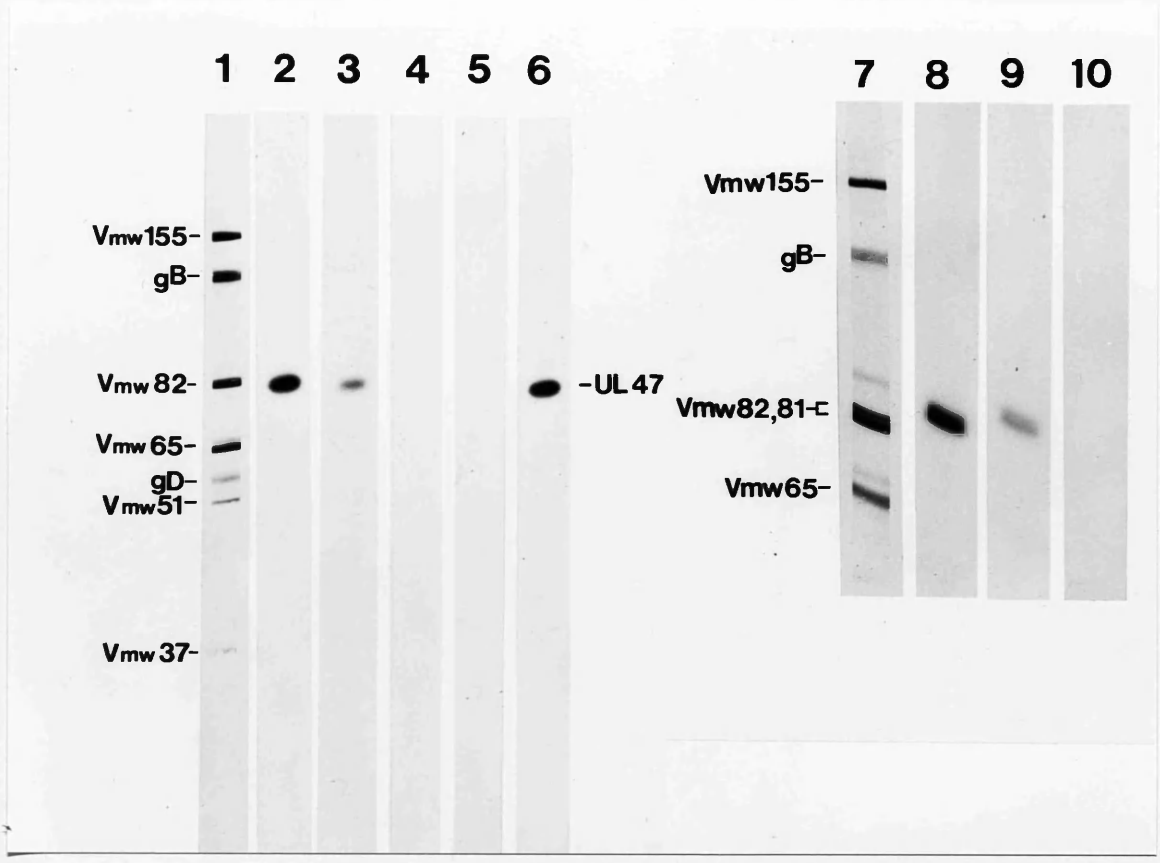
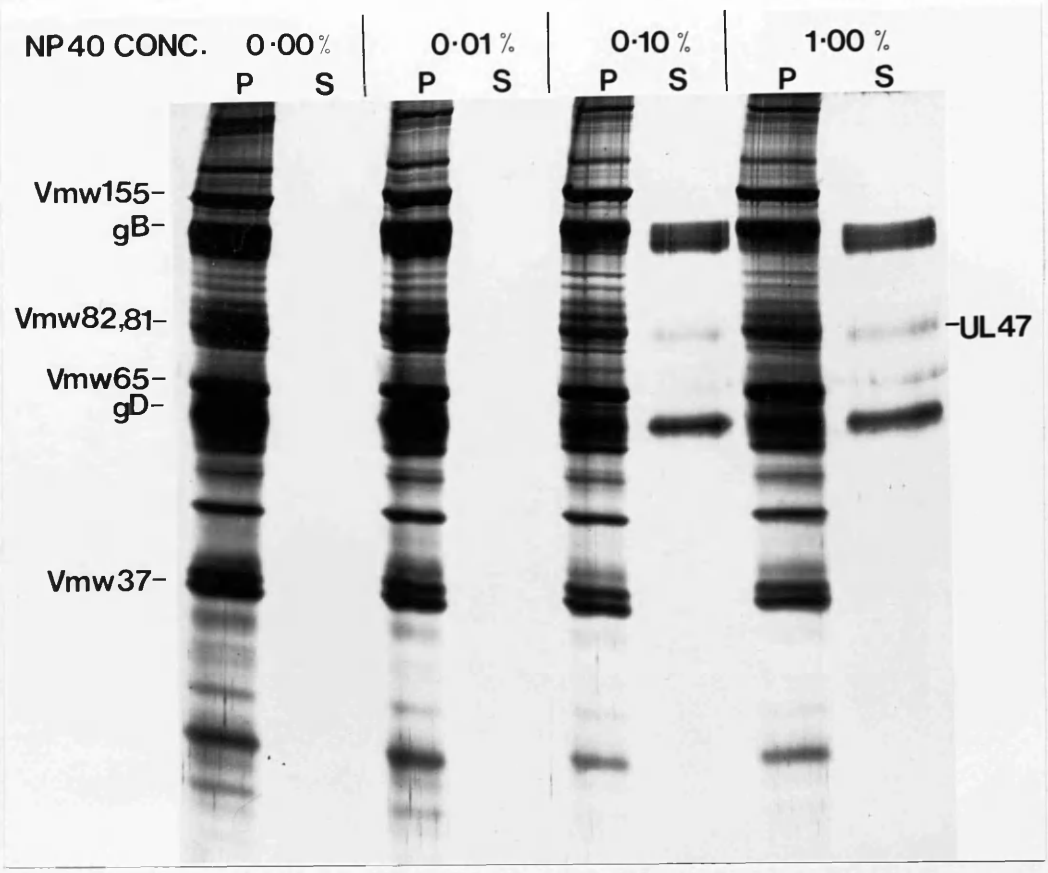

1
2
3
4
5
6
7
8
9
10
Vmw155-
gB-
Vmw82-
Vmw65-
gD-
Vmw51-
Vmw37-
-UL47
Vmw155-
gB-
Vmw82,81-
Vmw65-

FIGURE 19

Localisation of Vmw82/81 within the virion by treatment of virions with NP40. Following treatment of virions with various concentrations of NP40, solubilised proteins were identified as those remaining in the supernatant following ultracentrifugation as described. Proteins in the pellet (P) and supernatant (S) were separated by SDS-PAGE and silver stained.


NP40 CONC.
0·00%
0·01%
0·10%
1·00%
P
S
Vmw155–
gB–
Vmw82,81–
Vmw65–
gD–
Vmw37–
–UL47

include the major capsid protein (Vmw 155), gB, Vmw 82, the 65K transinducing factor (Vmw65 or $65K_{TIF}$), gD, Vmw51 (VP19c) and Vmw37. It lacks the large tegument protein Vmw273 because Vmw273 is too large to be electroeluted from the gel and transfered to the nitrocellulose membrane (Rixon *et al.*, 1988).

Serum 94497 reacts specifically with Vmw82 (lane 2) and Vmw82/81 (lanes 8 and 9) and this reaction is blocked by the peptide against which the antiserum was raised (lanes 3-5 and lane 10) but not by an unrelated peptide (lane 6). Lane 7 shows virion proteins blotted from a 6% gel cross-linked with DATD in which Vmw82/81 has been resolved as doublet. Two seperate exposures of a strip probed with antiserum 94497 show that both the 82K and the 81K polypeptides are recognised by the serum (Lanes 8 and 9) and that the reactions are blocked by the oligopeptide against which the antiserum was raised. This experiment identifies the UL47 82/81K gene product as the previously recognised Vmw82,81 major component of HSV-1 virions.

To investigate where in the virion the 82/81K protein is located, purified virions were treated with various concentrations of NP-40 and solubilised proteins separated from larger virion structures by centrifugation. Proteins in both pellet (P) and supernatant (S) were resolved by SDS-PAGE using a 5-12.5% gradient gel and proteins visualised by silver staining (Fig. 19). The data show that in the absence of NP-40 or with 0.01% NP-40 the virions appear intact as no proteins are found in the supernatant. With 0.1% and 1.0% NP-40 both gB (an envelope glycoprotein) and Vmw65 (a tegument protein) were solubilised as was Vmw82,81. In contrast, concentrations of NP-40 as high as 1% failed to solubilise Vmw155 (the major capsid protein) showing that the capsids remain intact. This data suggests that Vmw82,81 is located in the virion tegument or envelope.

To determine whether the product of gene UL47 was

FIGURE 20

Vmw82,81 is located in the tegument. Percoll-purified virions were incubated at 0-4° C either in the absence (lane 2) or the presence (lane 1) of 1% NP40. All samples were then iodinated: Acid insoluble radioactivity from ^{125}-iodine varied from 20,000 to 600,000 per ul. Polypeptides were then separated by SDS-PAGE slab gels of 9% acrylamide cross-linked with DATD. The heavily labelled band just above Vmw65 in lane 1 was also present after iodination of the BSA-containing Percoll solution and most likely represents BSA. This experiment was carried out by Drs. N. Langeland and L. Haarr.

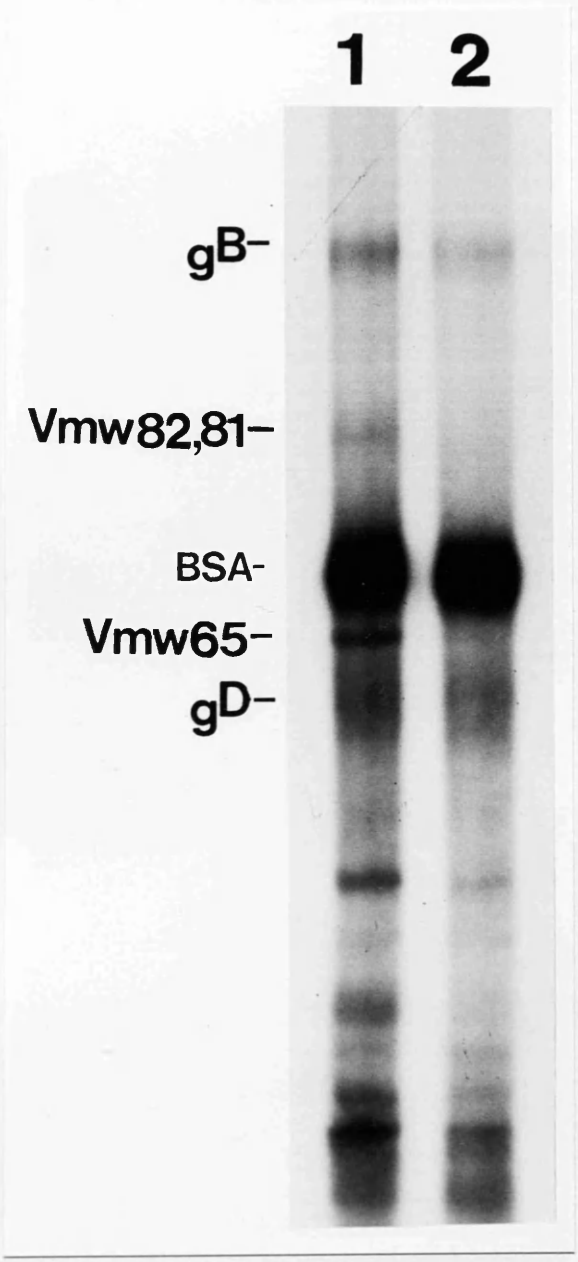
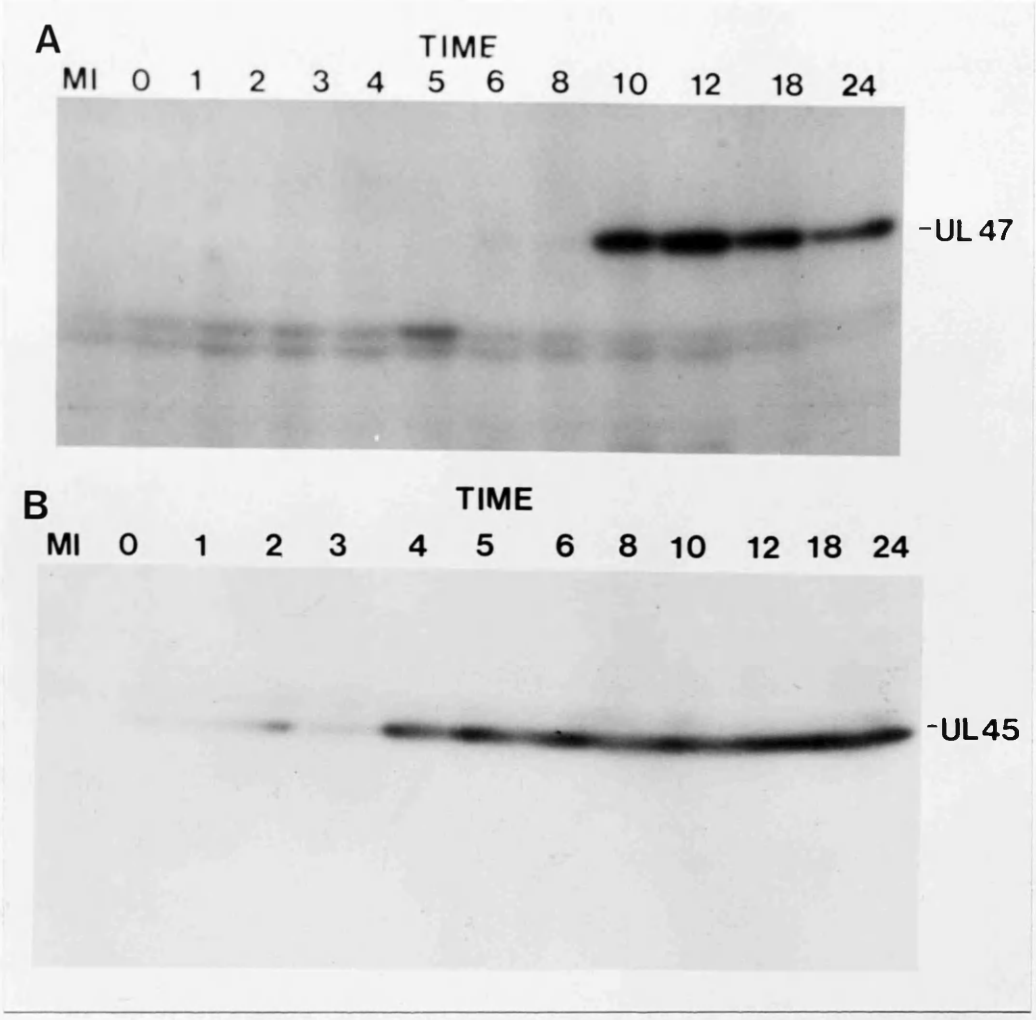

1
2
gB–
Vmw82,81–
BSA-
Vmw65–
gD–

FIGURE 21

The 82K product of UL47 is expressed late (Panel A) and the 38K product of gene UL45 is expressed early (Panel B) in HSV-1 infected cells. BHK cells were infected with HSV-1 strain 17, harvested at times shown (in h), separated by SDS page and probed with serum 94497 [1/100 diln.] (Panel A) and antiserum 23311 [1/50 diln.] (Panel B). Proteins from mock-infected (MI) cells were included for comparison. The figure shows autoradiographic images of the ^{125}I-protein A used to detect bound antibody; which have been trimmed to show only the relevant region.


A
TIME
MI 0 1 2 3 4 5 6 8 10 12 18 24
-UL47
B
TIME
MI 0 1 2 3 4 5 6 8 10 12 18 24
-UL45

located on the surface of the virion Percoll-purified virions were surface-labelled with ^{125}Iodine, after being subjected to treatment with 1% NP-40, (Fig. 20). Surface labelling with ^{125}I following NP-40 treatment shows the iodination of gB, Vmw82,81, gD, and Vmw65 (lane 1). Without pretreatment with NP-40, Vmw82,81 and Vmw65 were not iodinated (lane 2). This indicates that the UL47 protein is unlikely to be located on the surface of the virion particle. Together with the results of the previous experiments this suggests Vmw82, 81 are located in the tegument.

The regulation of expression of Vmw82,81 was then investigated. Cells were harvested at various times after infection and polypeptides were separated by SDS-PAGE, transferred to a nitrocellulose and probed with antiserum 94497 (Fig. 21, Panel A). The protein was first just detectable at low levels at 6 hours and 8 hours after infection. However it was then strongly upregulated at 10 hours and produced in higher amounts at 12 hours, its production then declined slightly after 24 hours (Fig. 21, Panel A). This dramatic up-regulation was reproducibly observed and suggests that UL47 is regulated as a late gene.

An operational definition of a true-late gene was suggested by Johnson *et al.*, (1986) to be those genes whose expression is most severely reduced, compared to all other groups of genes, under conditions of severely inhibited viral DNA replication. To determine whether gene UL47 was regulated as a true-late gene according to this definition the expression of the 82,81K protein was examined in the presence of 300ug/ml phosphonoacetic acid (PAA), as described in Methods section 12.2. This concentration reduces virus DNA replication to undetectable levels (<5% of the no drug control) in cells infected with HSV-1 strain 17 (Johnson *et al.*, 1986). The PAA-resistant mutant PAA^r-1 (Hay and Subak-Sharpe, 1976), which induces wild-type levels of viral DNA synthesis in the presence of 300ug/ml PAA, was included in the

FIGURE 22

The 82K product of gene UL47 is regulated as a true-late protein. Proteins were extracted from BHK cells infected with HSV-1 strain 17 in the absence (17^+ -PAA) or presence (17^+ +PAA) of 300ug/ml phosphonoacetic acid (PAA). A third set of cells were infected with a PAA-resistant mutant in the presence of 300ug/ml PAA (PAA^r-1 +PAA). Cells were harvested at 0, 6, 12, and 24 hours after infection, as indicated above the lanes and separated by SDS-PAGE. Three identical gels were run, polypeptides were transfered to nitrocellulose membranes and probed with serum 94497 [1/100 diln.] to detect the product of gene UL47 (panel A), serum 14327 [1/50 diln.] to detect the product of gene US11 (panel B), and serum 18826 [1/100 diln.] to detect the product of gene UL42 (panel C). Panel D shows an immunoblot with serum 94497 of doubling dilutions extracted from BHK cells infected with HSV-1 strain 17 in the absence of PAA and harvested 18 hours after infection. The autoradiographs have been trimmed to show only the relevant region.

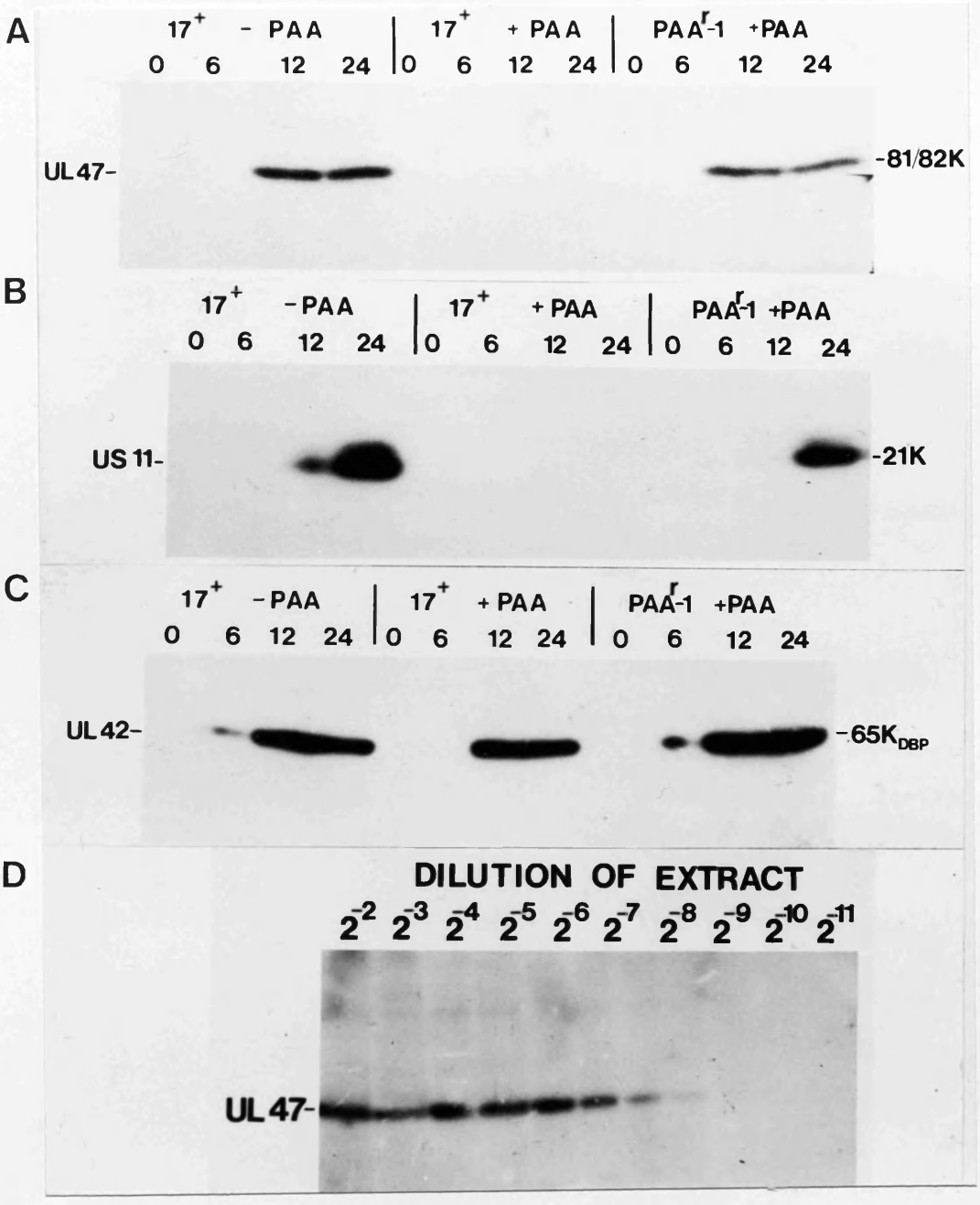
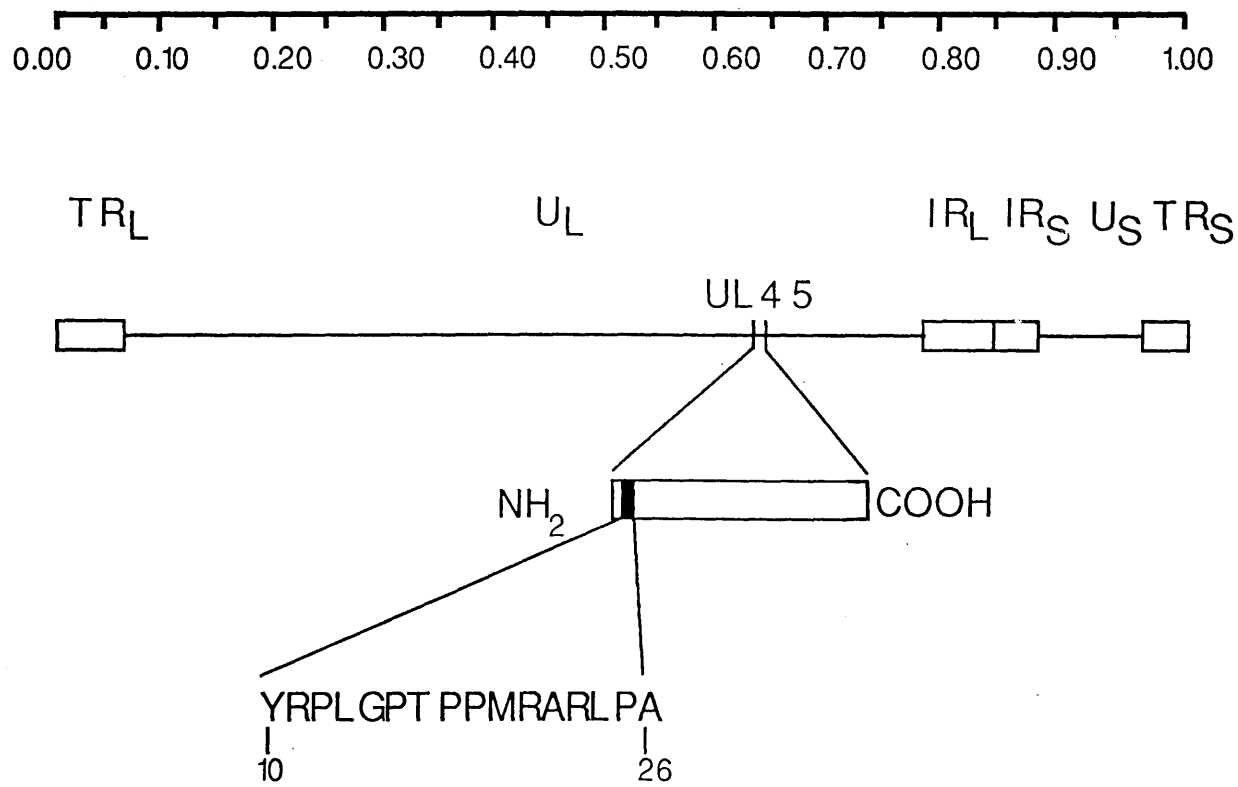

A
17+ - PAA
0 6 12 24
17+ + PAA
0 6 12 24
PAAr-1 +PAA
0 6 12 24
UL47-
-81/82K
B
17+ - PAA
0 6 12 24
17+ + PAA
0 6 12 24
PAAr-1 +PAA
0 6 12 24
US11-
-21K
C
17+ - PAA
0 6 12 24
17+ + PAA
0 6 12 24
PAAr-1 +PAA
0 6 12 24
UL42-
-65KDBP
D
DILUTION OF EXTRACT
2-2 2-3 2-4 2-5 2-6 2-7 2-8 2-9 2-10 2-11
UL47-

FIGURE 23

A schematic presentation of the predicted protein product of gene UL45 of HSV-1 strain 17 with the location and sequence of the peptide against which the antiserum 23311 was raised. The HSV-1 genome is represented as previously described for Fig. 15.


0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
TRL
UL
IRL IRS US TRS
UL45
NH2
COOH
YRPLGPT PPMRARLPA
10
26

experiment as a control.

For comparison the behaviour of two other proteins was also examined: 21K, a true-late protein (Johnson *et al.*, 1986) and 65K$_{DBP}$, an early protein (Schenk and Ludwig, 1988; Goodrich *et al.*, 1988) whose synthesis is reduced 3-4 fold in the presence of PAA (Goodrich *et al.*, 1988). The reactivity on Western blots of the rabbit antisera 18826 and 14327 used to detect the presence of 65K$_{DBP}$ and 21K respectively have previously been described (Parris *et al.*, 1988; MacLean *et al.*, 1987). Antiserum 18826 reacts with only a single protein 65K$_{DBP}$ (Parris *et al.*, 1988) whereas antiserum 14327 reacts with predominantly 21K and proteins of apparent M_r 22K, 17.5K, 14K and 11K which are related to 21K (MacLean *et al.*, 1987).

The results of this experiment are shown in Figure 22, in which infected cells were harvested at 0 hours, 6 hours, 12 hours and 24 hours after absorption. The 82,81K polypeptide was first detected at 12 hours after absorption both in the wild-type virus-infected cells in the absence of PAA and in PAA-resistant virus in the presence of PAA: In cells infected with wild-type virus in the presence of PAA no synthesis of 82,81K could be detected by antiserum 94497 at any time after infection (Panel A, Fig. 22). This finding is exactly what would be expected for a true-late protein and essentially parallels the result obtained with 21K (Panel B, Fig. 22), previously found to be a true-late (Johnson *et al.*, 1986). In contrast 65K$_{DBP}$ is clearly detectable by 6 hours post-infection and is only slightly inhibited by PAA in the wild-type virus-infected cells (Panel C, Fig 22).

The sensitivity of detection of 82,81K by antiserum 94497 was quantitated by Western-blotting two-fold serial dilutions of HSV-1 infected cells. The antiserum was able to detect 0.5% (i.e. 1 to 256 dilution) of the level of polypeptide induced in cells infected with wild-type virus in the absence of PAA (Panel D, Fig. 22). These

FIGURE 24

Western blotting with the 23311 serum showing specific recognition of a 38K polypeptide in HSV-1 infected cells. The first lane shows ^{35}S-labelled HSV-1 infected cell proteins transferred to nitrocellulose. The second and third lanes show polypeptides on the nitrocellose that were detected by pre-immune rabbit serum (PI) or immune (I) 23311 serum respectively.

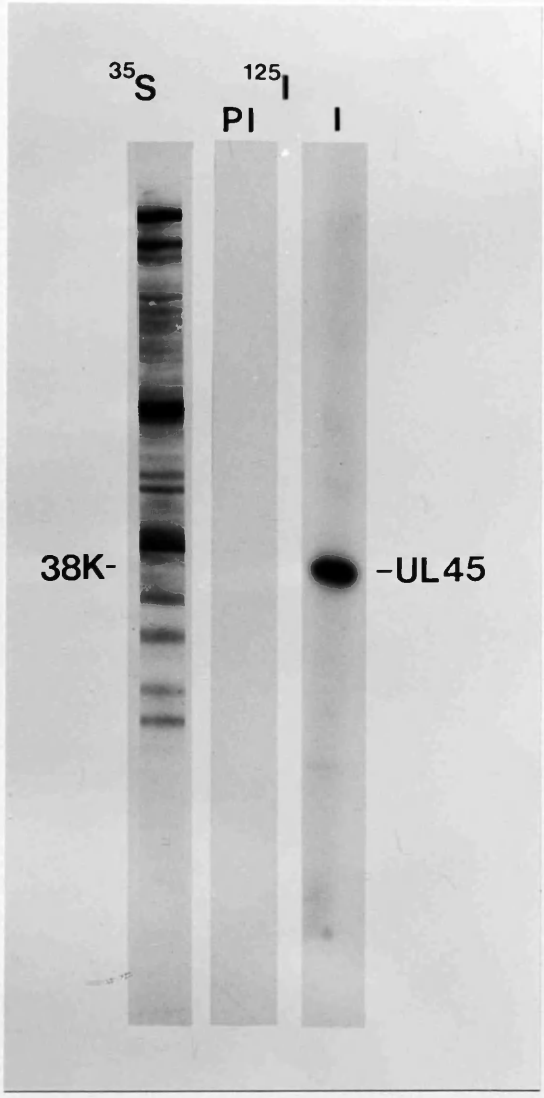
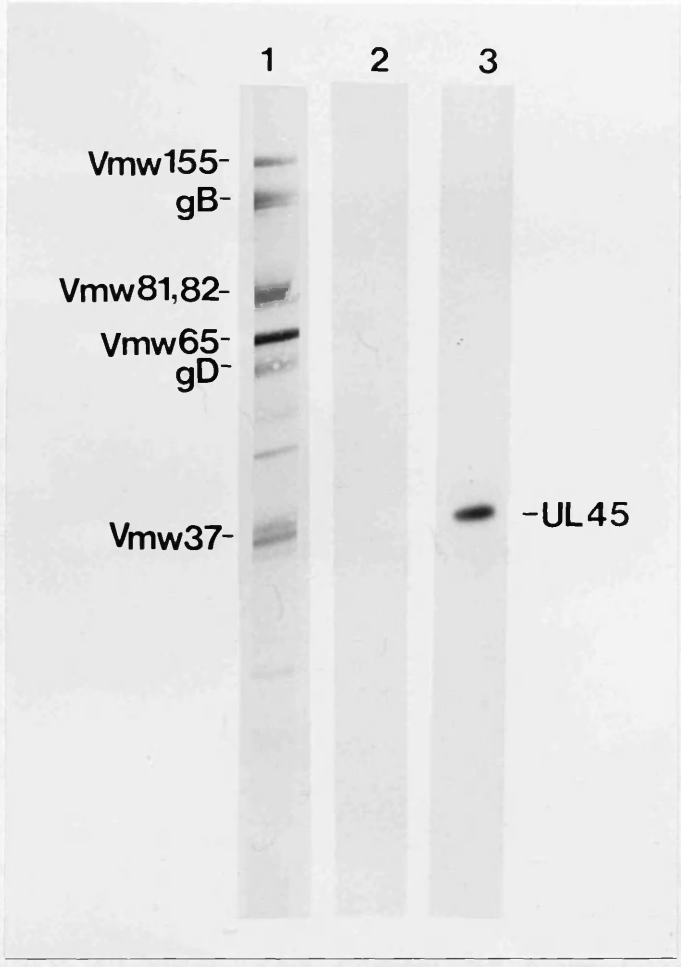

35S
125I
PI
I
38K-
-UL45

FIGURE 25

The 38K product of UL45 is a component of HSV-1 virions. Purified HSV-1 virions, labelled with ^{35}S-methionine, were separated by SDS-PAGE. Lane 1. shows the ^{35}S-labelled virion polypeptides transferred to nitrocellulose. Lanes 2 and 3 show polypeptides detected by pre-immune and immune 23311 serum [1/50 diln.] respectively.


1
2
3
Vmw155-
gB-
Vmw81,82-
Vmw65-
gD-
Vmw37-
-UL45

experiments show that in the absence of viral DNA synthesis the expression of gene UL47 is reduced to less than 0.5% of normal. The conclusion reached is that the UL47 gene, encoding the 82,81K polypeptides requires replication of viral DNA for expression.

21.2 IDENTIFICATION OF THE PRODUCT OF GENE UL45

Identification of the product of gene UL45 was achieved using antiserum 23311. Figure 23, shows the position of the open reading frame of gene UL45 on the prototype arrangment of the HSV-1 genome (McGeoch *et al.*, 1988) together with the position and sequence of the peptide against which antiserum 23311 was raised. The reactivity on western blots of this antiserum with proteins from HSV-1 infected cells is shown in Figure 24. The immune serum specifically detected a strong polypeptide band of apparent M_r 38,000 in 5-12.5% gels which was not detected by a sample of the pre-immune serum (Fig. 24) and which was also absent from a mock-infected cell extract (Fig. 21; Panel B, lane 1). This band was therefore considered to be the protein product of gene UL45.

Antiserum 23311 was also used to detect the presence of the UL45 gene product in purified HSV-1 virions. Figure 25 shows the result of a western blotting experiment in which purified HSV-1 virions were separated by SDS-PAGE and transferred to nitrocellulose, where they were probed with antiserum 23311. This antiserum detected a polypeptide band of M_r 38,000, which was the same M_r as the protein detected by this antiserum in HSV-infected cell extracts (Fig. 24). This polypeptide is a minor component of HSV-1 virions as the polypeptide is not visible in a normal profile of methionine labelled virions (Fig. 25, Lane 1).

A time course of protein production during viral infection was then carried out to investigate the regulation of expression of Vmw38. Cells were harvested

TABLE 8

Effect of pre-incubation of HSV-1 strain 17+ with antisera: 10555 (anti-HSV-1 serum, Column 1); 23074 (anti-carboxy-amino acids 156-168 of UL45, Column 2); antisera 23311, immune and pre-immune (columns 3 and 4 respectively). Antisera were diluted to the desired concentration shown, with PBS containing 4% guinea-pig complement and pre-incubated with virus for 1h at 31°C, followed by infection of cells with 200pfu virus (as described in Methods section 18.4).

TABLE 8

Neutralization of HSV-1 by serum 23311

ANTISERUM DILUTION	PLAQUES COUNTED			
	1.	2.	3.	4.
1	0	223	69	236
1/2	0	211	94	242
1/4	0	205	173	210
1/8	5	234	212	197
1/16	23	220	219	247

and probed with antiserum 23311 as previously described. The protein was first just detectable at low levels within 1 hour immediately after infection (Fig. 21, Panel B). However at 4 hours it then began to be produced in greater amounts reaching a peak around 12–24 hours. These kinetics are those of an early protein.

Due to the possibility that UL45 may be included in the virus membrane (for reasons discussed earlier, section 10); antiserum 23311 was also tested for its ability to interfere with HSV-1 infection of BHK cells. Virus was pre-incubated with 23311, in addition to a serum (23074) raised against a carboxy-terminal peptide (172, Table 1) from UL45 and a polyclonal anti-HSV-1 rabbit serum (10555, prepared by Dr.H.S. Marsden). Initial experiments were performed to determine the concentration of guinea-pig complement which produced maximum neutrilisation of virus by serum 10555 and 4% was chosen (data not presented). Antiserum 23311 was observed to give a slight (3-fold) reduction in the number of plaques produced when compared to pre-incubation with antisera 23074, 10555 and the 23311 pre-immune serum (Table 8).

To investigate this observation further, an oligopeptide was synthesised corressponding to the amino-terminal 35 amino acids of the predicted product of gene UL45: a sequence which includes the entire region predicted to be external to the potential membrane spanning domain. This peptide was synthesised as a monomer because peptides of this length would be expected to elicit a good immune response in the absence of a carrier protein and because uncertainty existed as to whether difficulties such as stearic hinderance might be encountered in synthesising a branched peptide of this size. Although this peptide (no. 258, Table 1) was successfully synthesised, had the correct Mr (Table 2), and was 94% pure (Fig. 7), it surprisingly failed to induce either an antipeptide or antiprotein response in rabbits (data not shown). Thus disappointingly, the sera

FIGURE 26

A schematic presentation of the predicted protein product of gene UL46 of HSV-1 strain 17 with the location and sequence of the peptide against which the antiserum 94490 was raised.The HSV-1 genome is represented as previously described for Fig. 15.

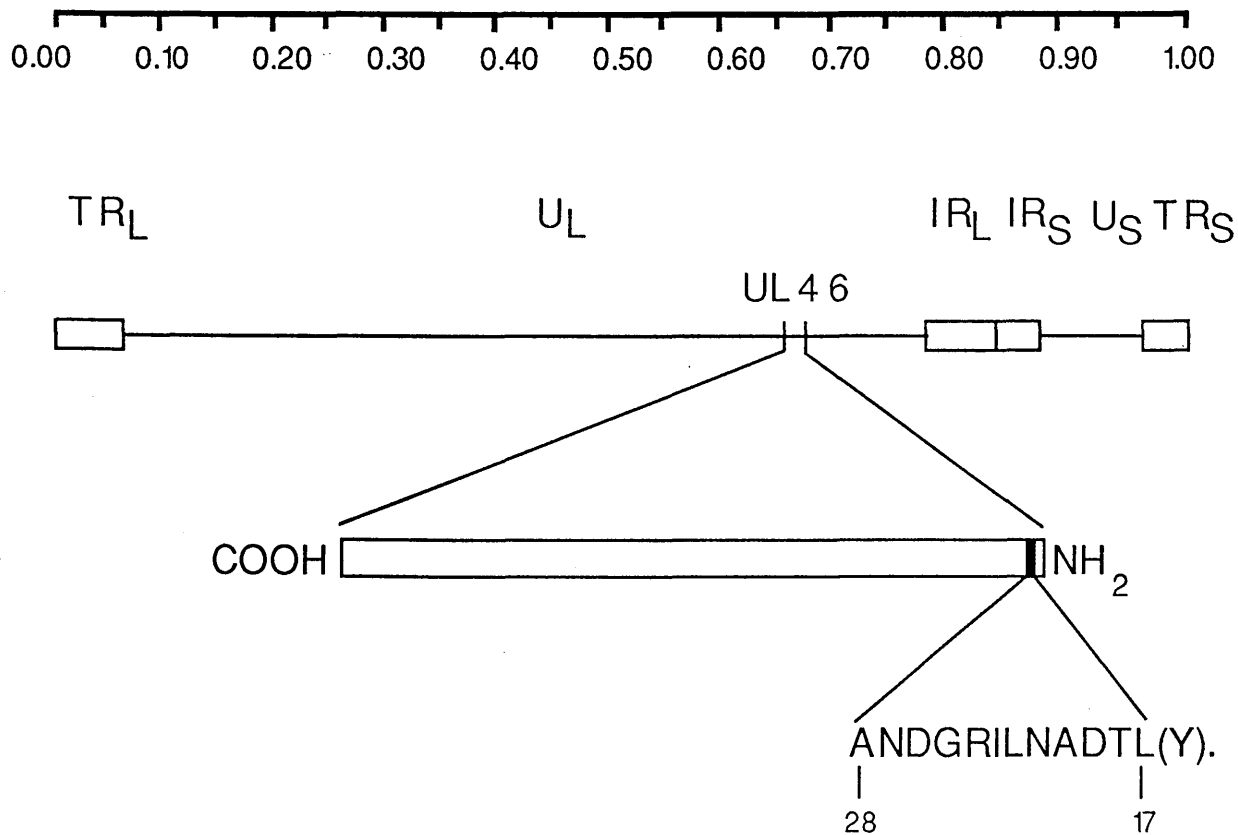

0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
TRL
UL
IRL IRS US TRS
UL 4 6
COOH
NH2
ANDGRILNADTL(Y).
28
17

FIGURE 27

Western blotting with the 94490 serum showing specific recognition of a 100K polypeptide in HSV-1 infected cells. The first lane shows ^{35}S-labelled HSV-1 infected cell proteins transferred to nitrocellulose. The second and third lanes show polypeptides on the nitrocellose that were detected by pre-immune (PI) or immune (I) 94490 serum respectively.

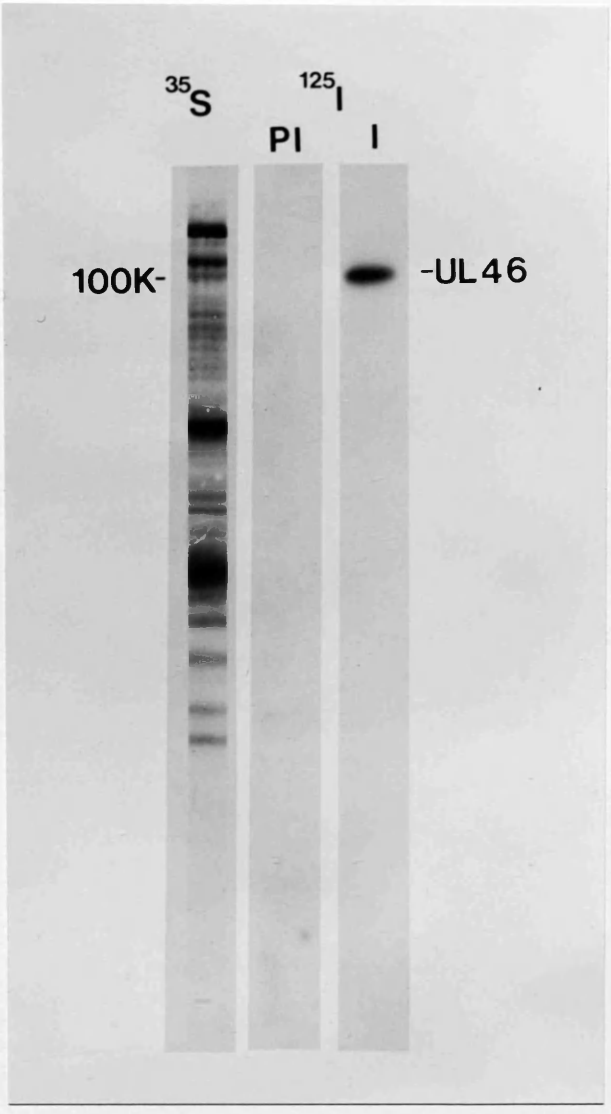
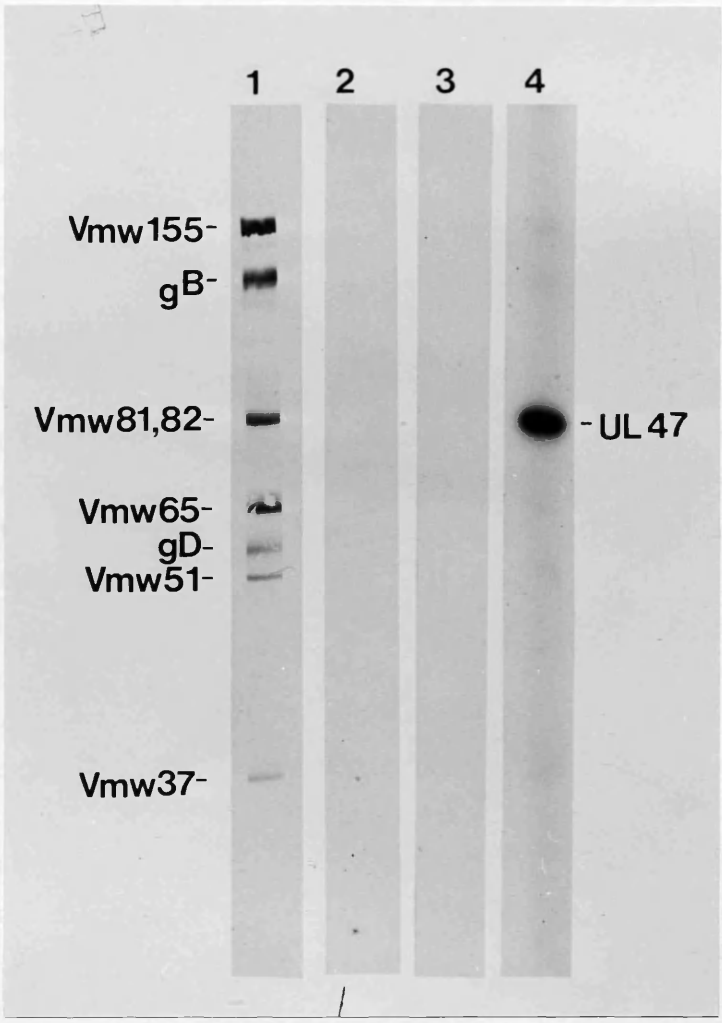

35S
125I
PI
I
100K-
-UL46

FIGURE 28

The products of genes UL46 and UL31 are not detected in HSV-1 virions. ^{35}S-methionine labelled virion polypeptides transferred to nitrocellulose are shown in Lane 1. Lanes 2 and 3 show polypeptides detected by antisera 94490 [1/100 diln.] and R85/1 [1/10 diln.] respectively. Lane 4 shows specific recognition of the 82,81K products of gene UL47 by antiserum 94497 as a control.


1
2
3
4
Vmw155-
gB-
Vmw81,82-
Vmw65-
gD-
Vmw51-
Vmw37-
-UL47

could not be used to further investigate whether antibodies directed against the amino-terminus of UL45 could neutr lise virus.

21.3 IDENTIFICATION OF THE PRODUCT OF GENE UL46

Identification of the product of gene UL46 was achieved using antiserum 94490. Figure 26 shows the position of the open reading frame of gene UL46 on the prototype arrangment of the HSV-1 genome (McGeoch *et al.*, 1988) together with the position and sequence of the peptide against which antiserum 94490 was raised. The reactivity on western blots of this antiserum with proteins from HSV-1 infected cells is shown in Figure 27. The immune serum specifically detected a polypeptide band of apparent M_r 100,000 in 5-12.5% gels which was not detected by a sample of the pre-immune serum (Fig. 27) and was also absent from a mock-infected cell extract (Fig. 29; Panel A, lane 1). This band was therefore considered to be the protein product of gene UL46.

Antiserum 94490 was also used to attempt to detect the presence of the UL46 gene product in HSV-1 virions. The result of a western blotting experiment are shown in Figure 28: Antiserum 94490 failed to detect any polypeptide band in purified HSV-1 virions (Fig. 28, lane 2).

A further time course of protein production during viral infection was then carried out to investigate the regulation of expression of Vmw100. Cells were harvested and probed with antiserum 94490 as previously described. The protein was first just detectable at low levels at 2 hours and 3 hours after infection (Fig. 29, Panel A). However it then began to be produced in larger amounts reaching a peak around 12-24 hours, again these kinetics are typical of an early protein.

FIGURE 29

The 100K product of UL46 is expressed at early times (Panel A) and the 30K product of UL31 is expressed late (Panel B) in HSV-1 infected cells. BHK cells were infected with HSV-1 strain 17, harvested at times shown (in h), separated by SDS page and probed with serum 94490 (Panel A) or antiserum R85/1 (Panel B). Proteins from mock-infected (MI) cells were included for comparison. The figure shows an autoradiographic image of the ^{125}I-protein A used to detect bound antibody.

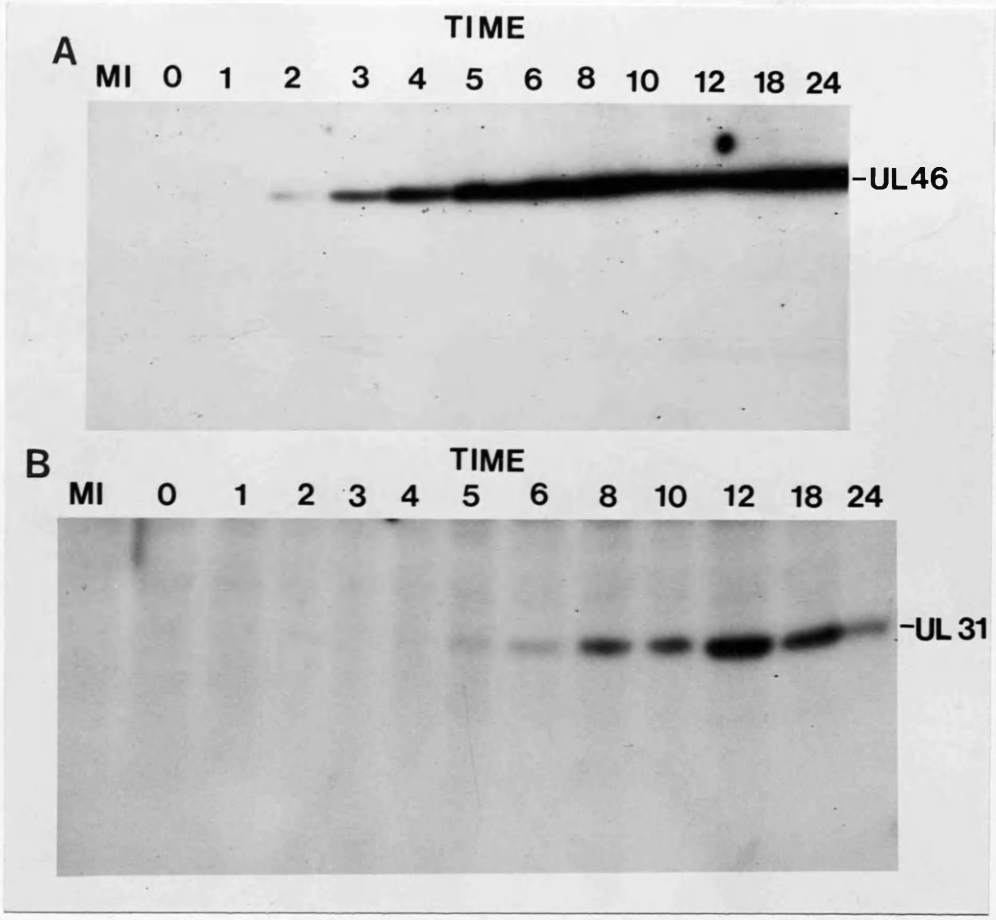
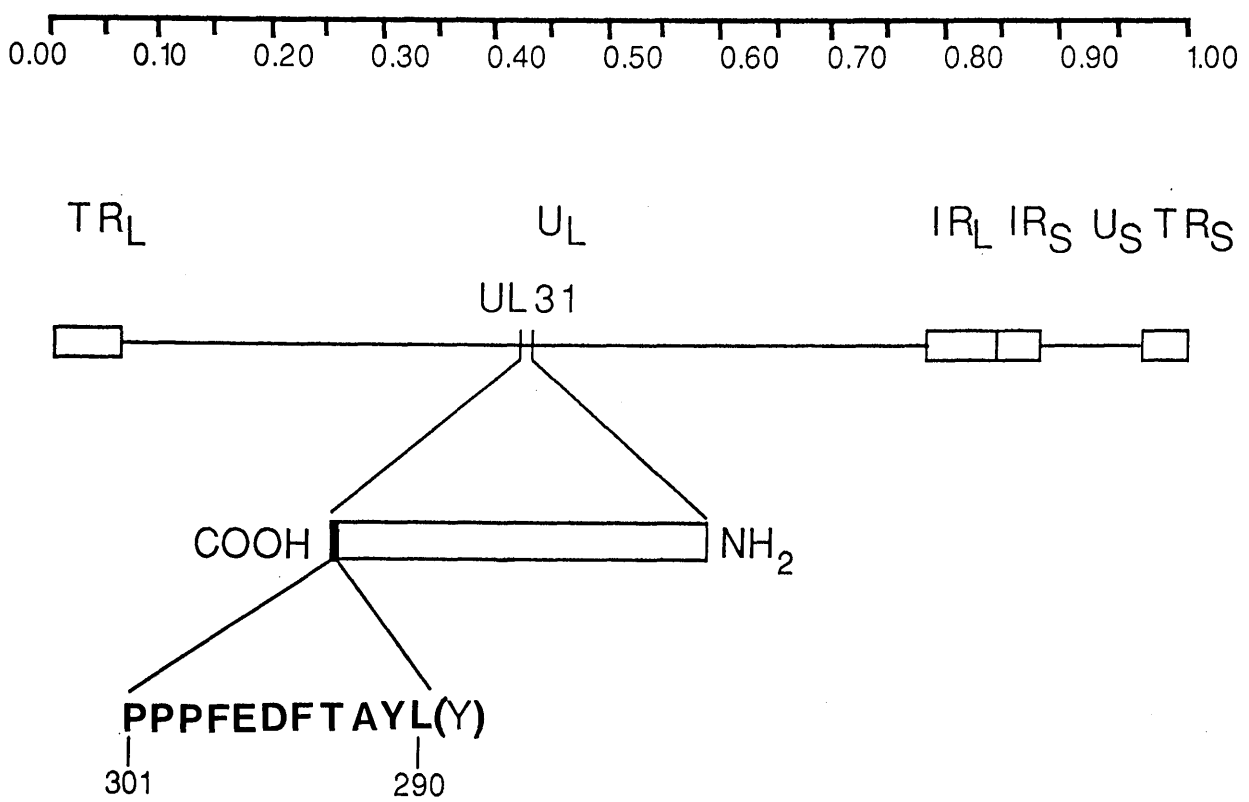

A
TIME
MI 0 1 2 3 4 5 6 8 10 12 18 24
-UL46
B
TIME
MI 0 1 2 3 4 5 6 8 10 12 18 24
-UL31

FIGURE 30

A schematic presentation of the predicted protein product of gene UL31 of HSV-1 strain 17 with the location and sequence of the peptide against which the antiserum R85/1 was raised. The HSV-1 genome is represented as previously described for Fig. 15.


0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
TRL
UL
IRL IRS US TRS
UL31
COOH
NH2
PPPFEDFTAYL(Y)
301
290

21.4 IDENTIFICATION OF THE PRODUCT OF GENE UL31

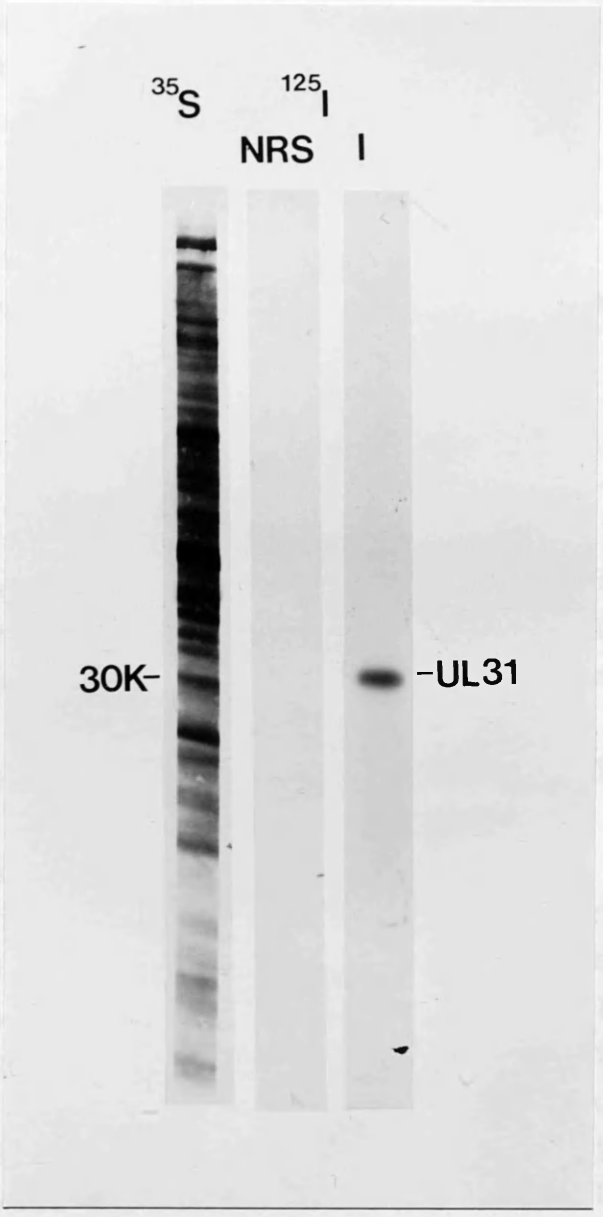
Identification of the product of gene UL31 was achieved using antiserum R85/1. This antiserum was prepared by Dr. John Palfreyman in rabbits using as immunogen the peptide coupled to BSA by procedures identical to those described in Methods section 17.1.2. It was used, rather than the antiserum prepared in this study, because experiments to identify and characterise the product of UL31 were initiated when R85/1 was the only antiserum available. Figure 30, shows the position of the open reading frame of gene UL31 on the prototype arrangment of the HSV-1 genome (McGeoch *et al.*, 1988) together with the position and sequence of the peptide against which antiserum R85/1 was raised. The reactivity on western blots of this antiserum with proteins from HSV-1 infected cells is shown in Figure 31. The immune serum specifically detected a strong polypeptide band of apparent M_r 30,000 in 5-12.5% gels which was not detected by a sample of normal rabbit serum (NRS) (Fig. 31) and was also absent from a mock-infected cell extract (Fig. 29; Panel B, lane 1). This band was therefore considered to be the protein product of gene UL31.

Antiseum R85/1 was also used to attempt to detect the presence of the UL31 gene product in HSV-1 virions. The result of a western blotting experiment are shown in Figure 28. Antiserum R85/1 failed to detect the presence of the UL31 polypeptide in HSV-1 virions (Fig. 28, Lane 3.)

The regulation of expression of Vmw30 was then investigated. Cells were harvested at various times after infection and polypeptides were separated by SDS-PAGE, transferred to a nitrocellulose and probed with antiserum R85/1 (Fig. 29, Panel B). The protein was first just detectable at low levels at 5 hours and 6 hours after infection. However it then began to be produced in larger amounts reaching a peak around 12 hours. The late appearance of this polypeptide indicated that it was

FIGURE 31

Western blotting with the R85/1 serum showing specific recognition of a 30K polypeptide in HSV-1 infected cells. The first lane shows ^{35}S-labelled HSV-1 infected cell proteins transferred to nitrocellulose. The second and third lanes (^{125}I) show polypeptides on the nitrocellulose that were detected by normal rabbit serum (NRS) or immune (I) R85/1 serum respectively.


35S
125I
NRS
I
30K-
-UL31

regulated as a late gene.

To determine whether gene UL31 is regulated as a true-late gene according to the operational definition suggested by Johnson *et al.*, (1986), described earlier (see Section 22.1), the expression of the 30K protein was examined in the presence of 300ug/ml phosphonoacetic acid (PAA), as described previously for UL47. The PAA-resistant mutant PAA^r-1 (Hay and Subak-Sharpe, 1976), which induces wild-type levels of viral DNA synthesis in the presence of 300ug/ml PAA was also included in the experiment. Antisera 14423 and 18812 (previously described) were again used as previously described as controls.

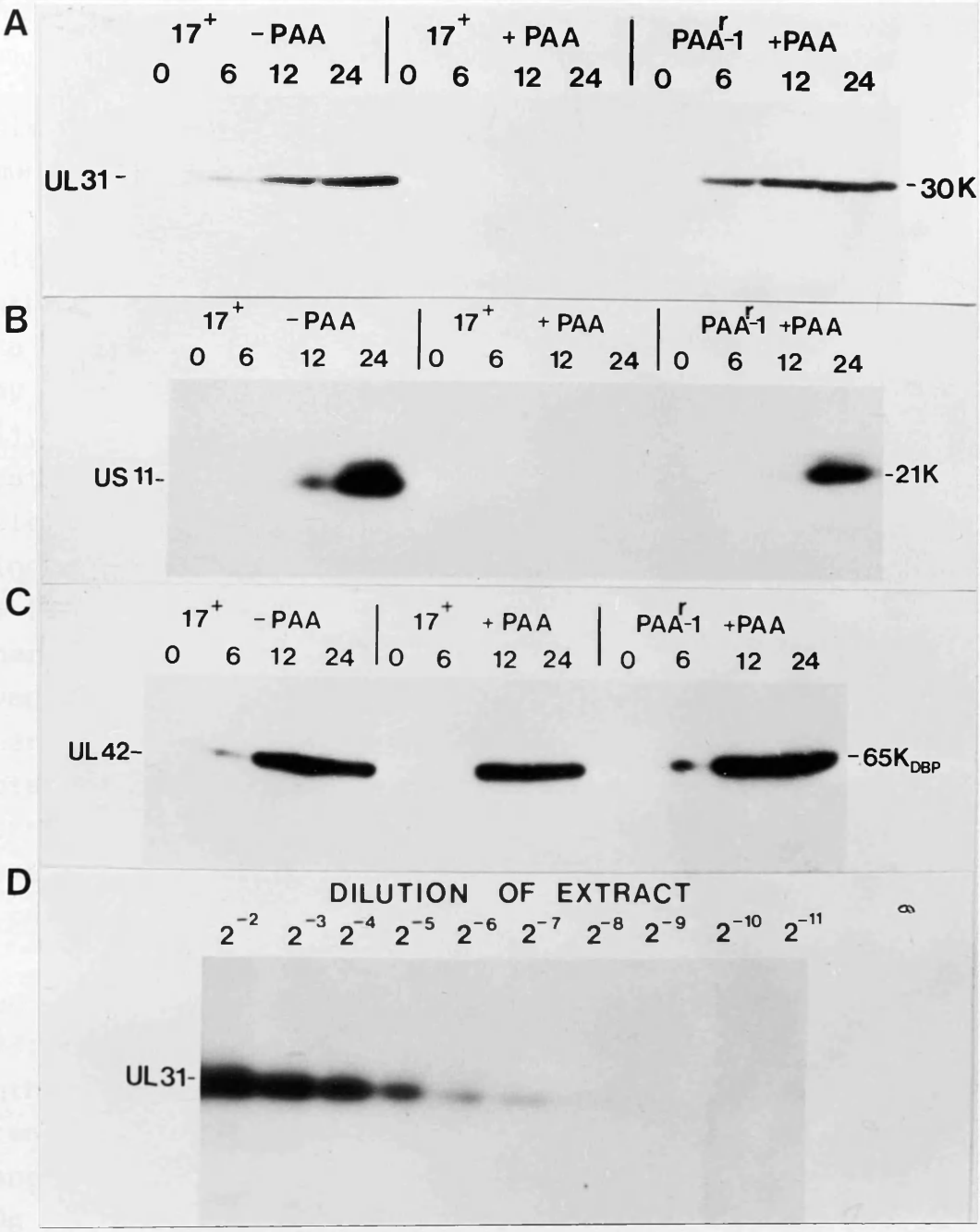
The results of this experiment are shown in Figure 32, in which infected cells were harvested as previously described. Polypeptide 30K was first detected in minor amounts at 6 hours after absorption both in the wild-type virus-infected cells in the absence of PAA and in PAA-resistant virus in the presence of PAA: In cells infected with wild-type virus in the presence of PAA no synthesis of 30K could be detected by antiserum R85/1 at any time after infection (Fig. 32, Panel A). This finding is again exactly what would be expected for a true-late protein and essentially parallels that obtained with 21K (Fig. 32, Panel B), previously found to be a true-late (Johnson *et al.*, 1986). In contrast 65K$_{DBP}$ was clearly detectable by 6 hours post-infection and was only slightly inhibited by PAA in the wild-type virus-infected cells (Fig. 32, Panel C).

The sensitivity of detection of 30K by antiserum R85/1 was examined by Western-blotting two-fold serial dilutions of HSV-1 infected cells. The antiserum was able to detect 1.0% (i.e. 1 to 128 dilution) of the level of polypeptide induced in cells infected with wild-type virus in the absence of PAA (Fig. 32, Panel D). These experiments show that in the absence of viral DNA synthesis the expression of gene UL31 was reduced to less than 1.0% of normal. The conclusion reached is that the

UL31 gene, encoding the 30K polypeptide requires replication of viral DNA for its expression.

FIGURE 32

The 30K product of gene UL31 is regulated as a true-late protein. Proteins were extracted from BHK cells infected with HSV-1 strain 17 in the absence (17+ -PAA) or presence (17+ +PAA) of 300ug/ml phosphonoacetic acid (PAA). A third set of cells were infected with a PAA-resistant mutant in the presence of 300ug/ml PAA (PAA^r-1 +PAA). Cells were harvested at 0, 6, 12, and 24 hours after infection, as indicated above the lanes and separated by SDS-PAGE. Three identical gels were run, polypeptides were transfered to nitrocellulose membranes and probed with serum R85/1 [1/10 diln.] to detect the product of gene UL31 (panel A), serum 14327 [1/50 diln.] to detect the product of gene US11 (panel B), and serum 18826 [1/100 diln.] to detect the product of gene UL42 (panel C). Panel D shows an immunoblot with serum R85/1 of doubling dilutions extracted from BHK cells infected with HSV-1 strain 17 in the absence of PAA and harvested 18 hours after infection. The autoradiographs have been trimmed to show only the relevant region.


A
17+ -PAA
0 6 12 24
17+ +PAA
0 6 12 24
PAAr-1 +PAA
0 6 12 24
UL31 -
-30K
B
17+ -PAA
0 6 12 24
17+ +PAA
0 6 12 24
PAAr-1 +PAA
0 6 12 24
US11-
-21K
C
17+ -PAA
0 6 12 24
17+ +PAA
0 6 12 24
PAAr-1 +PAA
0 6 12 24
UL42-
-65KDBP
D
DILUTION OF EXTRACT
2-2 2-3 2-4 2-5 2-6 2-7 2-8 2-9 2-10 2-11
UL31-

DISCUSSION.

22. GENERATION OF ANTISERA TO SYNTHETIC PEPTIDES

The experiments carried out to produce antipeptide antisera were designed to compare quantitatively the immunogenicity of branched peptides (Tam, 1988; Posnett *et al.*, 1988) with that of peptides conjugated to carrier proteins or linked to the resin on which they were synthesised. This topic is important because antipeptide sera have already proved extremely useful reagents, for many types of experiments (described earlier, Section 9.1). Their main use to date has been in identifying the putative polypeptide products, inferred by analysis of nucleic acid sequences. However, antipeptide sera produced using peptides conjugated to carrier proteins are often of low titre, so that any method of presenting peptides which generates sera of higher titres would be extremely advantageous. In fact, even if branched peptides were to generate sera with only equal titres to those elicited by protein-conjugated peptides they would still be a more attractive form of immunogen because the method avoids all the steps and difficulties involved in coupling peptides to carrier proteins.

The method of Merryfield chemistry (1963) originally used for the synthesis of the branched lysine core (Tam, 1988; Posnett *et al.*, 1988) was successfully modified for synthesis using Fmoc chemistry so avoiding the use of the potentially hazardous chemical hydrogen fluoride. The branched core was conveniently synthesised in batches of 1.0g (of a 0.1 mM equivalent Pepsin KA resin) then stored at -20° C, in 0.125g aliquots for subsequent synthesis of the branched peptides. Yields from 0.125g of resin-liked branched lysine core were typically 50-100mg of branched peptides: about 100 times that required for a series of immunisations. Batches of branched lysine core produced have all been successful as judged by mass spectrometric analysis and subsequent synthesis of branched peptides. The branched peptides produced by this method were

homogeneous as judged by SDS-polyacryamide gel electrophoresis demonstrating coupling of the carboxy-terminal amino acid of the unique peptide sequence to all eight amino groups of the branched lysine core.

22.1 PRODUCTION OF ANTISERA IN RABBITS

A comparison was made of the immunogenicity of six different peptide sequences (from five different HSV-1 genes) when presented to two different species of rabbits in each of three different forms. The data obtained convincingly demonstrate that rabbits immunised with branched peptides yield sera with higher antipeptide and antiprotein titres than rabbits immunised with either protein-conjugated or resin-linked peptides.

The most usual criterion by which antipeptide sera are judged is their ability to recognise the protein of which the peptide forms an integral part. Table 4. shows that the titres of sera from rabbits immunised with branched peptides were more than four-fold higher than those of rabbits immunised with protein conjugated peptides. Initially, a four-fold difference may not seem very significant, however, in terms of the usefulness of the antisera for experimentation it is, because, sera with anti-protein titres of 10 or less give higher backgrounds on Western blots and are consumed very rapidly by even a modest series of experiments.

As judged by ability to recognise proteins, 7 out of 13 (54%) rabbits immunised with branched peptides produced useful sera, while the comparable figure for rabbits immunised with protein conjugated peptides was only 3 out of 12 (25%). An alternative way to view these results is that four of the five HSV-1 gene products could be investigated using branched-peptide-induced antisera compared with two out of five for the protein-conjugated sera. This result also means that many fewer animals (about half) would have to be immunised in order to have a good probability of producing a useful antiserum. Any

increase in efficiency which leads to the use of a lower number of experimental animals is of course highly desirable.

Resin-linked peptides initially seemed an attractive approach to generate anti-protein sera being potentially the easiest method, as it is necessary to remove the side-chain protecting groups. The matrix can be ground up to a fine powder for immunisations. Disappointingly, none of the animals immunised with peptides presented in this form produced sera reactive in Western blots with protein. The reason why this approach should have proved so unsuccesful is not clear. However there are various possibilities: perhaps the particles produced were simply too large to be processed in a normal way by the immune system; it is also possible that the presence of the synthetic resin in some way hampered the ability of the immune system to respond to the attached peptides, which may be inaccessible to cells of the immune system by their position within the matrix. Unfortunately it must be concluded that this approach is not useful.

Two species of rabbits were used in this study: New Zealand White for peptides 161, 172 and 173 (Table 1); and Sandy Half-Lop for peptides 177, 175 and 202 (Table 1). The data presented in Figures 8 and 9 (panels a, c and f show data from New Zealand White rabbits) demonstrate that the observed relative immunogenicity of the three different forms of peptide presentation was similar for both rabbit strains. The effectiveness of branched peptides in eliciting high titre sera was therefore independent of the rabbit strain used.

One peptide, 177, elicited no anti-protein sera, regardless of the method of presentation. There are several possible reasons for this: it is possible that the region on the protein from which the 177 sequence originated is inaccessible to antibody even after the denaturation that is involved with Western blotting. Alternatively, there may be something inherent in the amino acid sequence that results in low immunogenicity.

This seems to be confirmed by the antipeptide titres obtained for this sera (Figure 8. Panel b), with the average titre elicited by this peptide being lower than that elicited by the other five peptides. Interestingly this peptide is extremely basic (net charge +5) although this is not neccesarily responsible for the results obtained.

These experiments also demonstrated, that rabbits immunised with branched peptides responded to give sera of high titre after one single immunisation, whereas it required at least two or more immunisations with monomeric peptides to achieve a satisfactory response. The reason for this observation has not been investigated, although it may be due to the multivalent nature of the branched peptide molecules.

This result is particularly interesting, with the potential development of synthetic vaccines based around peptide immunogens. Especially with respect to production of future veterninary vaccines, where economic considerations dictate that a single dose of vaccine, should be sufficient to confer long lasting protective immunity. Whether branched peptides are capable of generating such a response requires further investigation.

The successful adaptation of Fmoc chemistry to the synthesis of branched peptides facilitated the use of this chemistry to explore the effects of adding synthetic T-cell epitopes to branched peptides as originally suggested by Tam (1988) [see later]. The observation that the branched peptides produced high antipeptide titres, after even a single injection of antigen, provides strong support for the suggestion (Tam, 1988) that these structures may provide potent immunogens for the development of peptide-based vaccines.

22.2 PRODUCTION OF ANTISERA IN MICE

Although polyclonal antipeptide sera raised in rabbits are very useful (for reasons described earlier), they are however restricted in their potential, as there is always a limited volume of a particular serum available for experimentation. Therefore the logical progression of the use of branched peptides would be to use them as immunogens to produce monoclonal antibodies in mice. Such antibodies would have the advantage of providing a potentially limitless supply of reagent, which especially in the case of ascites fluid would be of much greater titre than any polyclonal sera generated in rabbits.

Initial attempts to immunise Balb/c mice with branched peptides in an identical manner to that used for rabbits, unfortunately proved unsuccessful with no antipeptide response detectable (results not presented). One possible reason for this lack of success could be that the inbred, Balb/c mice are genetically identical. This might place a genetic restriction on the ability of the immune system to respond to a short peptide sequence, through lack of availability of a large repertoire of potential T_H-cell receptor (T_HCR) sequences. A severe restriction is therefore placed on the number of potential epitopes that can be recognised by their T_HCRs. This restriction may be playing an inhibitory role on the antigenic potential of branched peptides, because, as discussed earlier, it is believed that an immunogenic peptide must contain antibody recognition sites (B-cell epitopes) and sites capable of eliciting T-cell help for antibody production (T_H-cell epitopes) (Mitichson, 1971).

Recent experiments had shown that conjugation of B-cell epitopes with either a "natural" or "forgein" T_H-cell epitope sequence could confer immunogenicity on previously poorly immunogenic peptides (Good *et al.*, 1987; Leclerc *et al.*, 1987; Section 9.3). Of particular interest in this respect, were the experiments of Francis *et al.*, (1988), who demonstrated the successful use of SWM-1, a T-

cell epitope from Sperm Whale myoglobin, shown to be active in Balb/c mice (Berkower *et al.*, 1985). This sequence was used successfully to considerably enhance immunogenicty on monomeric peptides from FMDV.

The experiments described here demonstrate that by the use of the same sequence in combination with branched peptides, a very strong immunogenic potential could be conferred on these molecules. In these experiments the SWM-1 T_HCE was attached to the amino-terminus of the branched peptides. This was in contrast to the work of Francis *et al.*, (1988), whose work with a FMDV peptide used the T_HCE at the carboxy-teminus of the B-cell epitope. In this case at least, addition of the T_HCE to the amino-terminus seems to have been as successful. However to demonstrate fully if this lack of constraint on orientation holds true, it would be neseccary to attach the T_HCE to both the carboxy and amino termini of the same B-cell epitope. It would not be surprising, due to the high degree of degradation undergone by antigens taken-up by antigen presenting cells like macrophages, to find that the T_HCE could be added to either end.

Of the 30 mice immunised with branched peptides without T_HCEs only 10 produced an antipeptide reponse. With only one exception these sera were all of very low titre. Whereas in combination with SWM-1, the branched peptides almost uniformally produced antipeptide sera of high titre. Interestingly, the only peptide sequence that gave low titre sera and failed to give five out of five positive mice in the presence of the T_HCE, was 177. This sequence had previously been shown to display poor immunogenicity in the experiments carried out in rabbits (Figure 8, panel b). Reference to Table 5 indicates that immunisation with peptide 177E failed to generate any antibodies to the T_HCE, in contrast to all other sequences tested. It therefore seems likely that the T_HCE failed to attach successfully to the 177 branched peptide. It is not known, however, whether the poor response to this immunogen is due to the lack of the T_HCE or some inherent

low immunogenicity of this peptide, as already observed in rabbits. It is most likely a combination of both.

This method of producing the T_HCE-branched peptide conjugates, whereby the T_HCE portion of the immunogen was added as a "cassette" in a one step addition process, obviously provides advantages over a continuous step-by-step addition of each amino acid residue. By adding the complete T_HCE to any available branched peptide, it considerably reduces the synthesis time required to produce the peptide, as addition of subsequent amino acids to peptides longer than 20 amino acids require twice as much time as adding amino acids to short peptides since double-coupling and long recirculation times are routinely used. In practice one large "batch" of protected T_HCE/ needs only to be produced aliquots of which can then be added to each branched peptide as and when required. Also since the amount of branched peptide needed for immunisations is usually only a small fraction of what is synthesised most can be left uncoupled and available for other uses e.g. ELISA assays, instead of having to initiate a further synthesis.

The ability to confer strong immunogenicity on branched peptides, in inbred animals, obviously makes them attractive canditates for the subsequent generation of monoclonal antibodies and could provide the means to generate monoclonal antibodies, directed against the product of any gene for which nucleic acid sequence data is available. The subsequent recognition of proteins by these sera cannot of course be guaranteed.

A method which employs branched peptides to produce monoclonal antibodies has a number of potential advantages over conventional techniques: Firstly, the majority of monoclonal antibodies produced to date have used either purified or partially purified proteins as immunogens. Although frequently successful, this approach often involves considerable time to produce the immunogen. Secondly, if a non-homogeneous, partially purified protein extract is used, then initial screening of any clones

produced has to be carried out by immune precipitation or a similar method which is also very time consuming. The use of branched peptides in combination with T_HCEs could help surrmount these problems by providing a homogeneous well defined immunogen.

Branched peptides also facilitate very rapid screening of the supernatant of hybridoma clones generated during a fusion by ELISA assays. Such early screening for peptide reactive clones can remove the need to propagate negative cell lines at an early stage. This avoids the time consuming process of freezing all cell-lines for storage until it has been determined by immune precipitation whether they are of interest. Of course, many clones will be selected having anti-peptide antibodies which do not then react with the protein of which the peptide forms a part. Such clones can only be eliminated by subsequently screening them against protein in some other immunoassay.

It may be possible to reduce the proportion of peptide-reactive, protein non-reactive antibodies by initial immunisation with branched peptide in combination with T_HCE, followed by "booster" immunisation with a partially purified extract containing the protein of interest. In this way there is the possibility that only those memory cells capable of secreting antipeptide antibody which is reactive with the native protein would be stimulated to proliferate and secrete immunoglobulin. This would further increase the likelihood of generating a useful cell-line.

An added advantage of monoclonal antibodies raised against synthetic peptides, is that the epitope which they recognise within the target protein is already known, which may prove useful in subsequent experimentation for example immunoaffinity purification.

23. SENSITIVITY OF BRANCHED AND MONOMERIC PEPTIDES IN ELISA ASSAYS

The results presented here demonstrated that the amount of branched peptide needed to detect rabbit antibodies specific for peptide was 100 to 10,000-fold lower than the amount of the corresponding linear, monomeric peptide, a result that was observed for all six peptides tested (Table 7).

The basis for the finding that the amounts of branched peptide needed to detect antibodies in rabbit sera is lower than for monomeric peptides has not been investigated. However it is most likely that the difference reflects the multivalent nature of the branched peptides compared with the monovalent nature of the linear peptides. Multivalent binding between antibody and branched peptide (avidity) would result in a considerable increase in stability, compared to the simple monovalent binding of linear peptide. Thus there may be a 10^3-fold increase in the binding energy of IgG when both valencies (binding sites) are occupied (Roitt *et al.*, 1989). Similarily if IgM were the antibody under test and a multivalent antigen was used, binding energy could increase by up to 10^7-fold. Multivalency might therefore account for the finding that the amount of branched peptide needed to detect antipeptide sera produced in rabbits is as much as 10,000 (10^4-fold) less than the amount of monomeric peptide needed.

An alternative explanation for apparent increased sensitivity of branched peptides might be that branched peptides bind more readily to the microtitre wells than do linear peptides so that the amount absorbed to each well is greater. Although further experiments are neseccary to exclude this possibility it seems unlikely, as other experiments have shown that the ratio of branched peptide to monomeric peptide needed to detect antibodies in rabbit and human sera is different (Marsden, Owsianka, Graham, McLean and Subak-Sharpe, manuscript in preparation).

Synthesis of branched peptides is no more difficult or expensive (weight for weight) than that of monomeric peptides, suggesting that significant savings are potentially available by using branched peptides for the development of peptide-based serodiagnostic kits. If such kits were designed to detect IgM antibodies (perhaps for diagnosis early in infection) then for reasons discussed above this saving could potentially be greater than those already observed, with perhaps as much a 10^7-fold reduction in the amount of peptide required. Although this remains to be experimentally tested. This makes the use of branched peptides in serodiagnosis worthy of further investigation.

24. IDENTIFICATION OF THE HSV-1 GENE PRODUCTS

Following the publication of the complete DNA sequence of HSV-1 strain 17 (McGeoch *et al.*, 1988) it is clear that much work remains to be done in the characterisation of HSV-1 genes. The products of many of the genes remain to be identified and their ability to encode proteins confirmed. Antisera generated in the previously described studies on peptide immunogenicity, was used to attempt to identify the putative protein products of five HSV-1 genes, predicted by the DNA sequence of McGeoch *et al.*, (1988); namely, UL31, UL41, UL45, UL46 and UL47. (Antiserum R85/1, which was used to identify the protein product of the UL31 gene product was generated by Dr. John Palfreyman, and was used because it was available before the sera described in this thesis were made).

These genes were of initial interest because no protein products had been assigned to them. Identification and characterisation of the products of genes with antipeptide sera is important not only as an insight into their possible role in the virus life cycle but also as a useful confirmation of the accuracy of the DNA sequence.

Within the DNA sequence of strain 17 there are 11 proposed overlaps of coding sequences within UL, these occur in two major groups of genes: UL5 to UL14 and UL30 to UL33 (McGeoch *et al.*, 1988). The majority of these overlaps are at present proposed soley on the interpretation of the DNA sequence. Because of uncertainty at mapping translational start sites most of these remain tent tive (McGeoch *et al.*, 1988). The predicted DNA sequence for gene UL31 proposes an overlap in "tail-to-tail" fashion of 19 codons with the predicted sequence of gene UL30, the HSV-1 encoded DNA polymerase (Quinn and McGeo h, 1985).

The oligopeptide (Y)LYATFDEFPPP which was used to generate antiserum R85/1 represents amino acids 291-301 of the predicted sequence of the UL31 gene product, which lies completely within the region of preposed overlap with gene UL30 (Quinn and McGeoch, 1985; McGeoch *et al.*, 1988). The data presented here showing that antiserum R85/1 specifically identifies the product of gene UL31 as a product in HSV-1 infected cells of apparent M_r 30,000 confirms the accuracy of the UL31 DNA sequence obtained for strain 17 (McGeoch *et al.*, 1988). The DNA sequence for gene UL30, the HSV-1 DNA polymerase has now been published by three independent groups (Quinn and McGeoch, 1985; Gibbs *et al.*, 1985; Knopf, 1986; McGeoch *et al.*, 1988), in 3 different virus strains. All of these authors identify the same carboxy-terminus as that of McGeoch *et al.*, (1988). This information together with the result presented here which identifies the UL31 gene product using an antiserum raised against a peptide sequence predicted to be wholly encoded from within this overlap, provides strong evidence to support the proposed overlap of the carboxy-termini of genes UL30 and UL31.

Attempts at the identification of the UL41 gene product, the virion component involved in shut-off of host cell macromolecular synthesis (Read and Frenkel, 1983; Kwong, *et al.* 1988; Simbert and Smiley, 1990) were unfortunately unsuccessful. This lack of success was almost certainly

due to the failure to produce a high titre antiserum in either rabbits or mice with peptide 177 (representing amino acid numbers 13-23 of the predicted product of UL41). The sequence of this peptide seems to have a particular lack of antigenicity (discussed earlier).

The predicted M_r of the product of gene UL45 from its DNA sequence is 18,178 (McGeoch *et al.*, 1988). This is considerably lower than the apparent M_r of 38,000 observed on polyacrylamide gels, western blotted and probed with antiserum 23311. The reason for this large increase in apparent M_r is not known, however sequence data indicates that UL45 is potentially membrane associated, containing an extremely hydrophobic amino terminus. Although there is a lack of any apparent N-glycosylation sites within the UL45 sequence (McGeoch *et al.*, 1988). Further experiments will have be carried out to determine the nature of any modifications made to the UL45 primary translation product.

Significant differences exist in the published DNA sequences obtained for gene UL47 from HSV-1 strain F (McKnight *et al.*, 1987) and HSV-1 strain 17 (McGeoch *et al.*, 1988). In particular there is an extra G residue at base 101163 of strain 17 which is not present in the published sequence for strain F. As a consequence the predicted amino acid sequences differ from the aligned proline residues at position 650 of strain F and 651 of strain 17: The former continues for a further 14 residues whilst the latter continues for a further 42 residues. The sequence (Y)GAAALRAHVSGRRA in the branched peptide against which antiserum 94497 was raised corresponds to amino acids 671-684 of the UL47 gene product predicted for strain 17 (McGeoch *et al.*, 1988), plus an additional N-terminal tyrosine, but is entirely absent from the UL47 product predicted for strain F (McKnight *et al.*, 1987).

The data obtained here with antiserum 94497 specifically identifies the products of gene UL47 of HSV-1 strain 17 as abundant virion proteins of apparent M_rs 82,000 and 81,000 confirms the correctness of the UL47 DNA

sequence obtained for strain 17. However it should be stated that this does not exclude the possibility that there are strain-specific sequence differences between strain 17 and strain F.

24.1 KINETICS OF SYNTHESIS OF UL31, UL45, UL46 AND UL47

The results presented have established that regulation of expression of the UL47 and UL31 genes is in a true-late manner. These findings support and extend those described in two earlier studies which reported two true-late transcripts, most likely originating from what we now call UL47 and UL31. The first was a 4.7Kb true-late mRNA transcribed in a leftward direction from the region of the genome predicted to encode gene UL47 (Hall *et al.*, 1982) and the second was a 1.4 Kb true-late mRNA transcribed in a leftward direction around map unit 0.44 corresponding to the position of gene UL31 (Holland *et al.*, 1984). The quantitative data reported here (Figures 22 and 32) shows that in the absence of viral DNA synthesis the UL47 protein product levels are less than 0.5% of those in a normal infection, while the amounts of UL31 product present are reduced to less than 1.0%.

Regulation of HSV-1 gene expression has been most frequently examined at the mRNA level. Evidence exists for 9 true-late mRNAs (Holland *et al.*, 1980; Frink *et al.*, 1981; Hall *et al.*, 1982; Holland *et al.*, 1984; Silver and Roizman, 1985; Johnson *et al.*, 1986). Comparison of the positions and sizes of these transcripts with the DNA sequence obtained by McGeoch *et al.* (1988) suggests that these true-late mRNAs may originate from genes UL22, UL31, UL32, UL38, UL44, UL45, UL47, UL49 and US11, but quantitative data for mRNA regulation has been obtained only for gene UL49 (Silver and Roizman, 1985) and US11 (Johnson *et al.*, 1986). Here UL31 and UL47 have been shown to be true-late Quantitative data for either mRNA or protein will have to be obtained for the others to identify these genes unambiguously as true-late.

At present the designation of HSV-1 gene UL38 as a

true-late gene seems doubtful: First, assembly of capsids is not dependent on virus DNA replication (Reviewed by Dargan, 1986) but is dependent on expression of HSV-1 UL38 (Pertuiset *et al.*, 1989), the product of which is an abundant capsid protein (Rixon *et al.*, 1990). Second , qualitative data has been obtained by Yei *et al.* (1990) suggesting that the HSV-1 gene is not regulated as a true-late gene while the HSV-2 gene is.

The results presented in this thesis (Figure 21, Panel B) place some considerable doubt on the designation of gene UL45 as "true-late" as experiments with antiserum 23311 have shown that the 38K product is present in infected cells early in infection (1 hour p.a.) at a time before initiation of viral DNA synthesis.

At the protein level only two genes, US11 and UL36 have been shown to be regulated in a true-late manner (Johnson *et al.*, 1986; McNabb and Courtney, 1990, Abstracts: 15th International Herpesvirus workshop), US11 encodes a 21K non-structural protein (Rixon and McGeogh, 1984) which localises to the nucleoli of infected cells (MacLean *et al.*, 1987) and binds directly or indirectly to DNA, most likely to the *a* sequence (Dalziel and Marsden, 1984; MacLean *et al.*, 1987). UL36 encodes the large 273K tegument protein (Batterson *et al.*, 1983).

Analysis of the kenetics of synthesis of gene UL46 have shown the 100K product is present in infected cells early in infection at 2-3 hours p.a., reaching peak amounts at 18-24 hours p.a. (Fig. 29, Panel A). This supports and extends the findings of Hall *et al.*, (1982) who mapped a 2.5Kb beta-gamma mRNA transcribed in a leftward direction in this region of the genome. This transcript was translated *in vitro* into a 85K polypeptide.

24.2 FURTHER CHARACTERISATION OF PROTEINS

Both antisera R85/1 and 94490 failed to detect the 30K and 100K products respectively of genes UL31 and UL46 in purified HSV-1 virions, while in parallel experiments

the UL45 and UL47 gene products were readily identified as virion proteins. The finding that the product of gene UL31 is likely to be a non-structural protein might initially seem surprising: from its true-late expression it might have been expected to play a role in the virion structure. However the product of gene US11, has also previously been shown to be regulated as a "true-late" protein (Johnson *et al.*, 1986), and also plays a non-structural role in the virus life cycle with its 21K protein localising to the nucleoli of infected cells (MacLean *et al.*, 1987).

Identification of the product of gene UL46 as non-structural is also an interesting result as the results of McKnight *et al.*, (1987), indicated a role of UL46 with UL47 in modulation of the activity of Vmw65. Results presented here identifiying UL47 as an abundant tegument protein, supported the possibility that it may act with the UL48 gene product.

Antiserum 23311, against UL45, detected a polypeptide band of similar M_r in both infected cells and in purified HSV-1 virions. It seems to be present in very low amounts in virions, as it is not visible as a ^{35}S-methionine labelled band (Figure 25), and is only detectable with the ^{125}I-probe in Western blotting experiments. Experiments to determine the position of this polypeptide within the virion particle have not as yet been carried out. Although the presence of a highly hydrophobic N-terminus and a potential membrane spanning domain make it a candidate for an envelope-associated polypeptide.

The preliminary experiments presented here, indicated that antiserum 23311, had a slight (3-fold) inhibitory effect on absorption of HSV-1 to BHK cells. Although not a highly significant reduction, it was worthy of further investigation in view of the possible membrane location of UL45. Unfortunately, attempts to investigate this further by synthesis of the entire predicted outermembrane domain of UL45- and subsequent antisera generation, failed to produce any neutralizing antisera.

Unexpectedly the "free" 35-mer lacked immunogenicity in rabbits. Further experiments using the 35 amino acid peptide in a branched form should hopefully produce a useful antiserum with which to investigate this effect further (work in progress, Dr.H.S.Marsden).

Results presented here have shown that the 82/81K products of gene UL47 are abundant structural proteins and since Vmw82,81 are readily released from virions by NP40 they must be components of the tegument or envelope. Iodination in the presence but not the absence of NP40 indicates that Vmw82,81 is located in the tegument (McLean *et al.*, 1990). Examination of the amino acid sequence predicted for UL47 (McGeoch *et al.*, 1988) reveals no features characteristic of a membrane protein, which lends support to this conclusion.

By examination of earlier studies (Roizman and Furlong, 1974; Heine *et al.*, 1974; Honess *et al.*, 1973) and comparison of the apparent relative intensity and virion location of the proteins described there, it could be speculated that Vmw82,81 corresponds to VPs 13,14. However this speculation remains to be experimentally tested.

The tegument is a poorly defined structure which lies between the capsid and the envelope and comprises approximately 65% of the virion by volume (Schrag *et al.*, 1989). Many tegument proteins are poorly characterised but some appear to function at the early stages of infection. Predominant among these is Vmw65 (VP16, $65K_{TIF}$) a protein of apparent M_r 65,000 which stimulates transcription from immediate-early genes (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell *et al.*, 1984). It has been reported (McKnight *et al.*, 1987) that the products of genes UL46 and UL47 act to modulate (positively and negatively respectively) the activity of Vmw65. It is therefore of interest that the 82/81K products of gene UL47 should be present in virions in amounts comparable or greater than Vmw65.

Vmw65 is also of importance as a structural

component of the virion, since a *ts* mutation in the gene encoding this protein confers decreased thermostability on virions (Moss, 1989). Whether Vmw82,81 will also have an equivalent structural role is unknown, although this possibility seems unlikely as recent experiments have shown that this gene along with the product of the UL46 gene is dispensible for virus growth in tissue culture cells (Barker and Roizman, 1990).

It is of interest that two proteins originate from UL47. In a previous analysis using 2D gel electrophoresis (Marsden *et al.*, 1983) two polypeptides were identified (spots 24 and 25) of apparent M_r 81,000 and one polypeptide (spot 402) of apparent M_r 82,000. It is not possible to unambiguously equate spots 24, 25 and 402 with either of 82K or 81K polypeptides. However, since no other spots having these apparent M_rs were detected on 2D gels and since all three spot are labelled following iodination of NP40 treated virions but not untreated virions (McLean *et al.*, 1990), it seems likely that spot 402 corresponds to Vmw82 and spots 24 and 25 correspond to Vmw81. Earlier studies (Marsden *et al.*, 1983) demonstrated that spot 402 was a processed product while spots 24 and 25 were synthesised *in vitro*. It could therefore be speculated that spots 24 and 25 both arise from UL47 by initiation of translation at the first and second AUGs (codons 1 and 34 respectively) of the mRNA. There is a precedent for this suggestion from studies on the translation of HSV thymidine kinase, in which it was shown that translation is initiated at the first three AUGs of the mRNA (Haarr *et al.*, 1985).

The results presented in this thesis support the continued use of antipeptide antisera for the identification and characterisation of viral gene products and demonstrate that methods are now available which are both more convenient and more successful than those at present widely employed. In addition the availability of antisera reactive with the products of genes UL31, UL45, UL46 and UL47 should enable the further characterisation

of these proteins, with a view to investigate their role in the virus life cycle.

25. FUTURE WORK

The experiments presented in this thesis give a number of interesting findings worthy of further investigation. It has been demonstrated that branched peptides are highly successful at eliciting a response against a target peptide sequence. These have been shown to be effective both in outbred strains of rabbits and in inbred Balb/c mice, when presented in combinaton with a T_H CE. Future work would involve further development of the generation of a stong response in mice to subsequently produce monoclonal antibodies. It should be possible to develop a quick and efficient method for initial screening of clones produced from a fusion, allowing work to be concentrated only on clones secreting antibodies of interest. The probability of further increasing the number of clones secreting antipeptide antibodies which were reactive with the target protein, could also be investigated by giving a second immunisation which contained the protein (as discussed earlier, section 22.2).

The availability of antisera reactive against four HSV-1 gene products will allow further characterisation of gene products UL31, UL45, UL46 and UL47. In particular the identification of the UL47 gene product as an 82/81K virion protein and the availability of antiserum directed against this protein, should facilitate its purification and allow *in vitro* experiments to test directly its speculated role in modulating the activity of Vmw65. It should be possible to ascertain whether UL47 is invloved in direct interaction with Vmw65. In addition, experiments could be carried out to determine the form of post-translational modification that is undergone by the UL45 gene product. This would involve labelling experiments to look for modification such as glycosylation and

myristylation. It should also be possible to determine whether UL31, UL45, UL46 and UL47 possess any DNA-binding properties.

The preliminary data presented here demonstrating a possible effect of an anti-UL45 antiserum on HSV-1 infection of BHK cells should be investigated further. The production of the 35 amino acid peptide from the amino terminus of gene UL45 in a branched form should produce a higher titre antiserum with which to better investigate this observation.

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