Interactions of Herpes Simplex Virus Type-1 with the Cell Surface

By

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A Thesis Presented for the
Degree of Doctor of Philosophy
in
The Faculty of Science
at the University of Glasgow

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October 1993

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Acknowledgements

I am grateful to Professor John H. Subak-Sharpe for providing research facilities in the Institute of Virology, and his overall supervision and support during my studies in Glasgow.

I would like to thank Valerie G. Preston for her supervision and for extensively proof-reading this thesis. Thanks to Iris McDougall for providing excellent technical advice throughout my studies and listening to my constant moaning.

I would also like to thank the many members of staff in the Institute who have helped with the writing of this thesis; in particular Fraser Rixon, Ben Luisi, and John McLauchlan for helpful discussions, and Joan Macnab and Nigel Stow for providing antisera. My thanks also go to Jim Aitken for not laughing at me too much during my attempts to learn electron microscopy.

Thanks are also due to the Clydesdale Bank for giving me the opportunity to be indebted to them for the next 20 years.

The continued support of some great friends made the completion of this thesis possible, especially Ann, Aileen, Iain, Cathy, Julia and Ruby, whose friendships have been greatly appreciated. My sincere thanks also go to Liz, a true friend, who deserves far more than whatever thanks I can give her here.

Finally, I would like to dedicate this thesis to my family, in particular my parents, Margaret and Dave, for their constant moral and financial support throughout my life.

The author was a recipient of a medical Research Council studentship. Except where specified, all of the results described in this thesis were obtained by the authors own efforts.

Summary

The present study was concerned with the characterisation of the herpes simplex virus type 1 (HSV-1) UL25 gene product and analysis of the phenotype of the HSV-1 mutant *ts*1204 which has a temperature sensitive (ts) mutation in the UL25 gene. Previous work by Addison *et al.* (1984) suggested that *ts*1204 was unable to penetrate the cell membrane at the non-permissive temperature (NPT). This early defect could be overcome by brief incubation of mutant infected cells at the permissive temperature (PT). Upon further incubation of mutant-infected cells at the NPT, low numbers of intermediate capsids lacking DNA assembled in the nucleus. No full capsids were observed, indicating that *ts*1204 also had a defect in the production of virus particles. Although the work presented here confirmed that *ts*1204 has a defect in a step prior to the onset of viral protein synthesis, the data indicated that *ts*1204 had an uncoating defect at the NPT.

A rabbit polyclonal antiserum raised against an oligopeptide representing an amino acid sequence present in the UL25 polypeptide was used to study the synthesis, processing and location of the UL25 gene product. The antiserum recognized a 67,000MW protein in virus-infected cell extracts and in purified virions, suggesting that the UL25 protein was a structural component of the virion. Using immunoprecipitation assays the UL25 gene product was detected at 1hr post-infection in virus-infected cells at 37^0 and reached a peak of synthesis at 4-5 hr post-infection. Analysis of the synthesis of the UL25 protein in the presence of inhibitors of viral DNA replication suggested that the UL25 protein was regulated as a leaky late viral gene product. Pulse chase analysis of infected cell extracts showed that the UL25 protein did not undergo any processing during synthesis. The location of the UL25 protein within the virion was determined by detergent extraction of purified radiolabelled virions. Although treatment of purified virions with 1% NP-40 was sufficient in removing a significant proportion of the envelope and Vmw65, the UL25 protein remained predominantly associated with the capsid. This finding suggests that the UL25 protein forms a tight association with the capsid structure as might be expected if it were to function in capsid stability. Suprisingly, the UL25 protein was detected in L-particles, which lack capsids.

In general, HSV infection leads to a decrease in host cell protein synthesis (Fenwick and Walker, 1978). However, the converse is true for a minority of cellular proteins, whose expression is upregulated in HSV-infected cells (Kemp *et al.*, 1986; Lathangue *et al.*, 1988). Although the expression of many HSV-induced cellular genes is dependent on viral protein synthesis, a minority of cell proteins appear to accumulate under conditions which restrict the induction of HSV polypeptides. Thus, it is assumed that these cellular proteins are induced by events occuring prior to the onset of viral protein synthesis, such as attachment of the virion to the cell membrane or entry of the nucleocapsid into the cytoplasm.

The abundant HSV-induced host cell protein p56 is an example of a cellular protein that is induced by events occuring prior to the onset of viral protein synthesis (Addison *et al.*, 1984; Preston , 1990). The p56 polypeptide accumulates in human foetal lung (HFL) cells infected with the HSV-1 mutant *ts*1204 at the NPT. Further evidence indicated that the accumulation of p56 in infected cells occurs by the transcriptional activation of the p56 gene. The significance of p56 induction in HFL cells during HSV-infection is unclear. Accumulation of p56 may assist in the establishment of the cellular antiviral state, or alternatively, p56 may facilitate viral infection in HFL cells.

The aim of the present study was to isolate cDNAs representing polyA$^+$ RNAs that accumulate in *ts*1204-infected HFL cells at the NPT.

In order to isolate cDNA clones derived from cellular genes that were induced in *ts*1204-infected HFL cells at the NPT, a cDNA library was prepared from polyA$^+$ RNA prepared from *ts*1204-infected cells.

The differential screening technique was used to identify cDNA clones of genes encoding cellular proteins induced in *ts*1204-infected cells at the NPT. Some 3000 bacterial clones were screened in this manner, of which 23 clones appeared to hybridize preferentially to the probe synthesized from *ts*1204-infected cell polyA$^+$ RNA. These selected clones represented approximately 0.8% of the members of the library that were initially screened. Confirmation that these clones represented polyA$^+$ RNAs that are induced during *ts*1204-infection at the NPT was obtained by RNA slot blot analysis of each cDNA clone.

Two groups of clones were identified on the basis of DNA-cross hybridization analysis. The first group designated C6, comprised six members, each containing a cDNA insert of approximately 1.5kbp. The relatedness of these clones was

confirmed by sequence analysis and restriction enzyme mapping of each clone. Since all of the clones identified within this group were 1.5kbp in size, it was assumed that the cDNA inserts represented full length polyA$^+$ RNA molecules. Transcription and translation of these clones *in vitro* revealed a protein of approximately 40,000 MW (p40). Southern blot analysis and immunoprecipitation studies suggest that p40 was distinct from the 42,000MW mitochondrial aspartate aminotransferase found to be increased in HSV-2 infected cells (Lucasson, 1992). Partial sequence analysis of the C6 cDNA followed by subsequent data base searching, failed to identify the protein product encoded by the C6 cDNA insert.

Four cDNA clones appeared to share similar DNA sequences on the basis of DNA cross-hybridization studies. This second group of clones termed C4 contained variable sized inserts, of which the largest was approximately 2kbp. An *alu* rich repetitive sequence was identified by sequence analysis of the 5' terminus of one member of C4. Further studies revealed that the members of C4 shared DNA sequences distinct from the *alu* repetitive sequence. The identification of the protein product encoded by this group of cDNA clones remains unknown.

The remaining 13 clones have yet to be analyzed.

Abbreviations

A	adenine
AIDS	acquired immune deficiency syndrome
Amp	Ampicillin
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney
bFGF	basic fibroblast growth factor
bp	base pair
BSA	bovine serum albumin
C	cytosine
CAV	cell associated virus
cDNA	complementary DNA
CHO	chinese hamster ovary
Ci	Curies
cm	centimetre
CPE	cytopathic effect
cpm	counts per minute
CS	calf serum
CTP	cytidine-5'-triphosphate
Da	Daltons
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxynucleoside-5'-triphosphate
dUTP	2'-deoxyuridine-5'-triphosphate
DBP	DNA binding protein
DEPC	diethylpirocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
E	early

EBV	Epstein Barr Virus
E. coli	*Escherichia coli*
EDTA	sodium ethylendiamine tetra-acetic acid
EHV	equine herpes virus
EM	electron microscopy
FCS	foetal calf serum
FITC	fluorescein isothiocyanate conjugated
G	guanine
g	gram
gD	glycoprotein D
HCMV	human cytomegalovirus
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HFL	human foetal lung
HHV	human herpes virus
HIV	human immunodeficiency virus
hr(s)	hour(s)
HS	heparan sulphate
hsp	heat shock protein
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVS	herpesvirus samiri
IE	immediate early
IFN	interferon
Ig	immunoglobulin
IR	internal repeat
k	kilo
l	litre
L	late
LAT	latency associated transcripts
LHS	left hand side
LFP	large (klenow) fragment DNA polymerase
M	molar
mA	milliampere
MasPAT	rat aspartate amino-transferase
MCMV	murine cytomegalovirus
mi	mock-infected
min	minute
ml	millilitre
mm	millimetre

mM	millimolar
moi	multiplicity of infection
MW	molecular weight
mRNA	messenger RNA
N	unspecified nucleotide or amino acid
ng	nanogram
nm	nanometre
NPT	non-permissive temperature
NP40	Nonident P40
OD	optical density
ORF	open reading frame
ORI	origin of DNA replication
^{32}P	phosphorous-32 radioisotope
P	pellet
PAA	phosphonoacetic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming unit
polyA	polyadenylic acid
PRV	pseudorabies virus
PT	permissive temperature
R	purine moiety
RHS	right hand side
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second(s)
SN	supernatant
SV40	simian virus 40
syn	syncytial plaque morphology (*syn*+= non syncytial *syn*-= syncytial)
T	thymidine
TBS	tumour bearing serum
TCA	trichloracetic acid
TEMED	n,n,n',n'-tetramethylethylene diamine

TK	thymidine kinase
TR	terminal repeat
Tris	tris(hydroxymethyl)aminomethane
ts	temperature sensistive
UV	ultra violet
V	volt
vhs	virion host shut-off
vol	volume
Vmw	apparent molecular weight of virus-induced protein
v/v	volume/volume
VP	virion protein
VZV	varicella zoster virus
w/v	weight/volume
wt	wild type
Y	pyrimidine moiety

Greek Symbols

α	
β	
γ	
μ	
δ	

Amino Acid Symbols

A alanine	G glycine	M methionine	S serine
C cysteine	H histidine	N asparagine	T threonine
D aspartate	I isoleucine	P proline	V valine
E glutamate	K lysine	Q glutamine	W tryptophan
F phenylalanine	L leucine	R arginine	Y tyrosine

1. Introduction

Section 1.A The Herpesviruses

1. Description and Classification

Herpesviruses are found in a wide range of animal species (reviewed by Roizman, 1990). Members of the *Herpesviridae* family share a similar morphology. A typical herpesvirus particle (120-200nm in diameter) has a double stranded DNA genome enclosed within an icosadeltahedral capsid (approximately 100-110nm in diameter). The capsid is surrounded by the tegument, a proteinaceous layer comprising 65% of the total virion volume, which is enveloped in a trilaminar membrane containing the viral glycoprotein spikes.

An interesting property of many herpesviruses is the ability to exist in a latent state within the infected host. Following a primary infection, the viral DNA remains in an inactive form in certain host cells, from which it can be reactivated to produce a secondary lytic infection. In addition to the ability of herpesviruses to form latent infections, they share three other significant biological properties. All of the herpesviruses encode virus-specific enzymes, many of which are involved in the synthesis of viral DNA. Furthermore, viral DNA synthesis and capsid assembly occur within the infected-cell nucleus. Finally, the production of infectious progeny virus is accompanied by the death of the host cell. Despite these similarities, the herpesviruses vary greatly in their biological properties, and on this basis have been divided into 3 sub-families (Alpha-, Beta- and Gamma-herpesvirinae)

Alphaherpesvirinae have a variable host range in tissue culture, and exhibit a short reproductive cycle followed by destruction of infected cells and rapid spread of the virus in tissue culture. Latent infections are frequently but not exclusively established in sensory and autonomic ganglia. Members of this group

include herpes simplex virus types 1 and 2 (HSV-1, HSV-2), Equine herpes virus type 1 (EHV-1), Pseudorabies virus (PRV) and varicella zoster virus (VZV).

Betaherpesvirinae have a narrow host range both *in vivo* and *in vitro* and a slowly spreading growth in tissue culture. Enlargement of infected cells (*cytomegalia*) occurs both *in vitro* and *in vivo.* Latent infections are generally established in secretory glands, lymphoreticular cells and the kidneys. Members of this sub-family include human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV).

Gammaherpesvirinae infect and establish latency in B or T lymphocytes. The experimental host range is very narrow. Members of this sub-family include Epstein Barr virus (EBV) and herpesvirus samiri (HVS).

Human herpesvirus 6 (HHV-6) was originally assigned to the *Gammaherpesvirinae* on the basis of its tropism for lymphocytes. However, on the basis of sequence homology and gene organisation, HHV-6 was later shown to be related to HCMV and was subsequently placed within the *Betaherpesvirinae.*

2. Pathogenicity and Epidemiology of Human Herpesviruses

Seven different human herpesviruses have been identified: HSV-1, HSV-2, VZV, HCMV, EBV, HHV-6 and human herpes virus 7 (HHV7).

HSV-1 and 2 normally cause cold sores and genital sores respectively. The virus can establish a latent infection reactivating spontaneously. Reactivation of latent HSV can be triggered by nerve damage, UV-light exposure, fever or immune suppression. Occasionally, infection with HSV can result in herpes simplex encephalitis. Although HSV-2 is the primary cause of genital herpetic lesions, changing patterns of sexual behaviour have resulted in an increase of genital herpes associated with HSV-1 infection. HSV-2 infection has long been implicated in the development of cervical carcinoma, but its role in transformation is unclear.

VZV causes two clinically distinct diseases (reviewed by Gelb, 1990). Varicella or chicken-pox, as it is more commonly called, is the primary infection. Following the initial infection, VZV normally establishes latency in the dorsal root ganglia

and remains so until an appropriate stimulus leads to its reactivation. Reactivation of the virus from sensory ganglia, leads to the eruption of painful vesicles and this clinical manifestation has been designated herpes zoster or shingles.

EBV infects B-lymphocytes in the peripheral blood and is the causative agent of infectious mononucleosis (reviewed by Miller, 1990). EBV is also strongly associated with Burkitt's lymphoma and nasopharyngeal carcinoma.

HCMV infections are normally asymptomatic. However, HCMV can cause opportunistic infections in patients with lower than normal resistance to infection, for example, AIDS sufferers, neonates and organ transplant patients. In addition, *in utero* infection can lead to a severe generalized infection, called cytomegalia inclusion disease, which can result in birth defects.

HHV-6 (reviewed by Lopez and Honess, 1990), previously called human B lymphotropic virus, was discovered in 1986 during the search for a causative agent of lymphomas associated with AIDS. The virus can infect T cells *in vitro* and is the primary aetiological agent of *exanthum subitium*, characterized by high fever and the appearance of a rash.

HHV-7 was recently isolated from CD4+ T cells (Frenkel *et al.*, 1990). The virus has yet to be connected with any specific disease in humans.

3. Structure And Genetic Content Of The HSV-1 Genome

The HSV genome is a linear, double stranded DNA molecule approximately 152kbp in length and comprises a long (L) and a short (S) component which are covalently linked. A schematic representation of the arrangement of the HSV-1 genome is represented in Figure 1 . The L component consists of the long unique region, U_L flanked by a terminal repeat (TR_L) and an internal repeat (IR_L) (Sheldrick and Berthelot, 1974). Likewise, the S component consists of a short unique region (U_S) flanked by inverted repeats, designated TR_S and IR_S. The L and S repeat elements are unrelated apart from a 400bp region termed the *a* sequence (Roizman, 1979). A single copy of the *a* sequence is normally found at the S terminus whilst multiple copies are detected at the L terminus and a variable number in inverted orientation at the L-S junction. During viral DNA

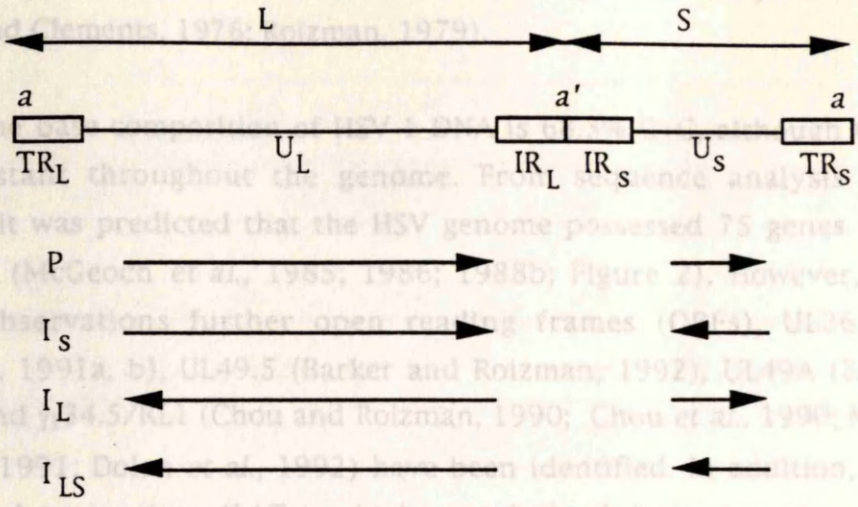


Figure 1. Structure Of The HSV-1 Genome

A representation of the HSV-1 genome as two covalently joined components, L and S. The unique sequences, U_L and U_S, are shown as solid lines. The major repeat elements, TR_L, IR_L, TR_S and IR_S, are shown as open boxes. The terminal *a* sequences and the internal inverted *a* sequence (*a'*) are indicated. The relative orientations of the L and S components in the four isomers, designated P, I_L, I_S and I_{LS} are indicated by the four arrowed lines

replication, the L and S components can invert relative to each other, such that a *wt* virus DNA preparation usually contains 4 isomers in equimolar amounts differing in the orientation of the L and S components (Hayward *et al.*, 1975; Delius and Clements, 1976; Roizman, 1979).

The base composition of HSV-1 DNA is 68.3% G+C, although this figure is not constant throughout the genome. From sequence analysis of the HSV genome it was predicted that the HSV genome possessed 75 genes encoding 72 proteins (McGeoch *et al.*, 1985; 1986; 1988b; Figure 2). However, since these initial observations further open reading frames (ORFs), UL26.5 (Liu and Roizman, 1991a, b), UL49.5 (Barker and Roizman, 1992), UL49A (Barnett *et al.*, 1992), and $\gamma_1$34.5/RL1 (Chou and Roizman, 1990; Chou *et al.*, 1990; McGeoch and Barnett, 1991; Dolan *et al.*, 1992) have been identified. In addition, the latency-associated transcripts (LATs) which are derived from a region in the long repeat, and are the only viral transcripts to accumulate in latently infected tissues, have been characterized (Rock *et al.*, 1987; Stevens *et al.*, 1987; Fraser *et al.*, 1992). However, there is little evidence to suggest that the LATs encode viral proteins. Table 1 indicates the HSV-1 genes, whether the gene is essential for growth of the virus in tissue culture, and what is known of the encoded protein and its function.

4. HSV Latency

An interesting feature of HSV is the ability to establish a latent infection in neuronal ganglia. The viral genome exists as a circularised molecule in latently infected cells (Mellerick and Fraser, 1987; Rock and Fraser, 1983; 1985). Although the majority of viral genes remain dormant during latency, a few are expressed. The latency associated transcripts (LATs) were shown to accumulate in the nuclei of neurons of latently infected cells (Stevens *et al.*, 1987). The LATs are transcribed in the opposite direction to the IE-1 mRNA, and are in part complementary to the 3' terminus of the IE-1 mRNA. On this basis, it was postulated that the function of the LATs is to preclude the expression of Vmw110 (Stevens *et al.*, 1987). Deletion mutants, lacking the LATs gene of HSV-1, were shown to be fully capable of establishing a latent infection (Javier *et al.*, 1988), although reactivation of latent virus *in vitro* and in experimental animals was

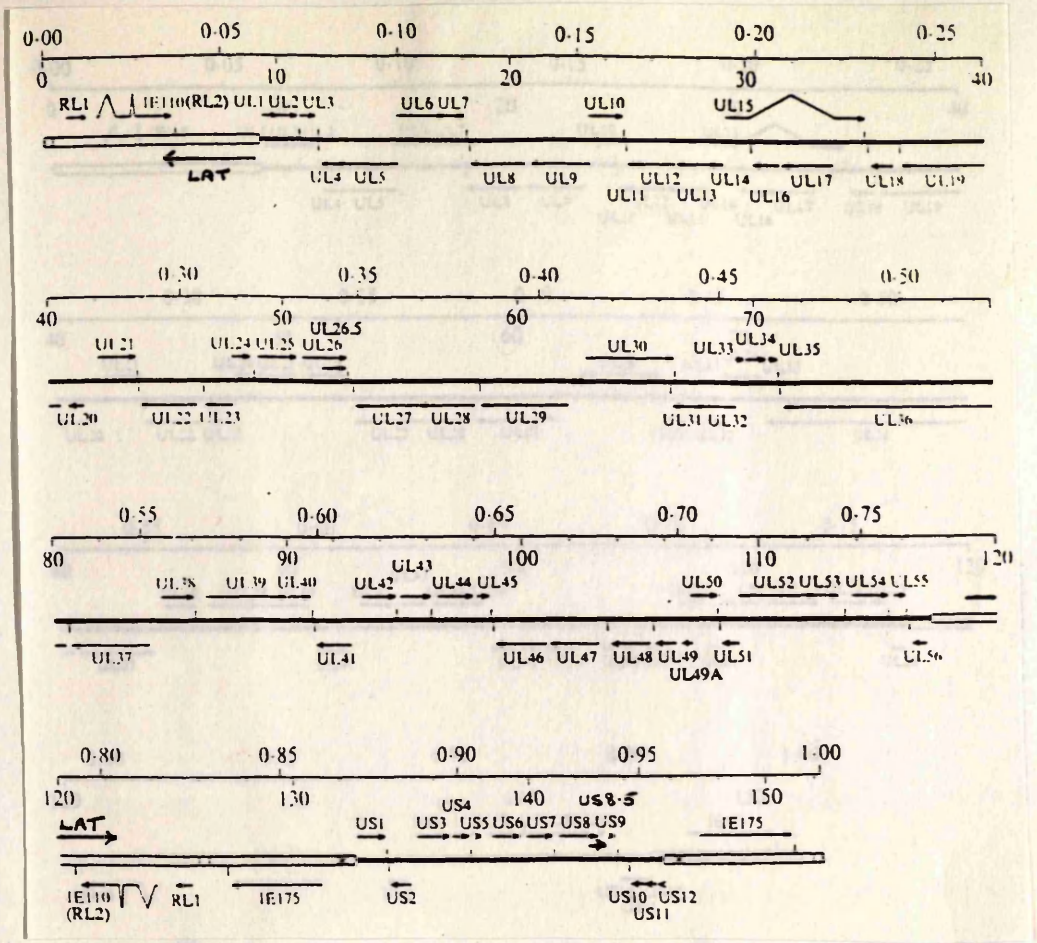


Figure 2. Layout of the HSV-1 genes.

The genome in the P orientation is represented by four successive lines with unique regions shown as 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 size and orientation of the proposed functional open reading frames are shown by the arrowed lines, with splicing within coding regions indicated. Overlaps of adjacent, similarly orientated ORFs are not shown explicitly. Location of proposed transcription polyadenylation sites are indicated as short vertical bars. The location of the origins of DNA replication are shown as X. Genes UL1-UL56 indicated on the top three lines. The genes US1-US12 are shown on the bottom line. The position of the genes encoding the IE polypeptides Vmw175 and Vmw110 are indicated. This figure is reproduced from McGeoch *et al.* (1988b), with the addition of UL26.5 (Liu and Roizman, 1991a,b), UL49.5/49A (Barker and Roizman, 1992; Barnett *et al.*, 1992), $\gamma_1$34.5/RL1 (Chou and Roizman, 1990; Chou *et al.*, 1990; McGeoch and Barnett 1991; Dolan *et al.*, 1992), and the major stable LAT (Rock *et al.*, 1987; Stevens *et al.*, 1987; Fraser *et al.*, 1992) and US8.5 (Georgopoulou *et al.*, 1993).

Table 1 HSV-1 Gene Products This figure is reproduced from A. Davison (1993).

HSV-1 Gene	*Status*	*Protein/Function (names)*	*References*
a sequence	e	400 bp terminal redundancy, with oppositely orientated copy a' internally at the IR_L/IR_S junction. Contains the signals required for the processing/packaging of viral DNA	Stow *et al.*, 1983
RL1	ne	Neurovirulence factor (ICP34.5, γ134.5)	Chou and Roizman, (1990); Chou *et al.*, (1990); Dolan *et al.*, (1992); McGeoch and Barnett, (1991)
RL2	ne	IE Protein; transcriptional activator (ICP0, Vmw110, IE110, α0, IE1).	Cai and Schaffer, (1989); Everett, (1989); Sacks and Schaffer, (1987); Stow and Stow, (1986)
LAT	ne	Family of transcripts expressed during latency. The function of the LATs and the proteins they encode are uncertain	Perry and McGeoch, (1988); Javier *et al.*,(1988) Wagner *et al.*, (1988a); Block *et al.*, (1990);
UL1	e?	Glycoprotein L; complexes with glycoprotein H	Hutchison *et al.*, (1992a); Little *et al.*, (1981)
UL2	ne	Uracil-DNA glycosylase	Mullaney *etal.*, (1989); Worrad *et al.*, (1988)
UL3	ne	Unknown	Baines and Roizman, (1991)
UL4	ne	Unknown	Baines and Roizman, (1991)
UL5	e	Posesses helicase motifs and is a component of the DNA helicase primase complex which is essential for DNA replication.	Weller *et al.*, (1985); Crute *et al.*, (1989); Wu *et al.*, (1988); Zhu and Weller, (1988); Zhu and Weller, (1992a,b)

UL6	e	Role in virion morphogenesis (VP11-12?)	Sherman and Bachenheimer, (1987); Sherman and Bachenheimer, (1988); Weller *et al.*, (1983a)
UL7	-	Unknown	
UL8	e	Component of the helicase-primase complex essential for DNA replication	Weller *et al.*, (1985); Carmichael and Weller, (1989); Crute *et al.*, (1989); Zhu and weller, (1988)
UL9	e	Ori-binding protein essential for DNA replication; DNA helicase	Carmichael *et al.*, (1988); Fierer and Challberg, (1992); Olivo *et al.*, (1988); Wu *et al.*, (1988)
UL10	ne	Function unknown; probably an integral membrane protein	MacLean *et al.*, (1991); Baines and Roizman, (1991)
UL11	ne	Myristylated tegument protein; role in envelopment and transport of nascent virions	Baines and Roizman, (1992); MacLean *et al.*, (1989); MacLean *et al.*, (1992)
UL12	e	Deoxyribonuclease: role in the maturation and packaging of viral DNA	Weller *et al.*, (1990)
UL13	ne	Tegument protein with a protein kinase activity	Cunningham *et al.*, (1992); Overton *et al.*, (1992).
UL14	-	Unknown	
UL15 exon 1	e?	Function unknown; possible NTP-binding motifs	Baines and Roizman, (1991); Baines and Roizman, (1992); Davison, (1992); Dolan *et al.*, (1991)
exon 2	e?	Function unknown; possible NTP-binding motifs	
UL16	ne	Unknown	Baines and Roizman, (1991)

Table 1 (continued)

UL17	e?	Unknown	Baines and Roizman, (1991)
UL18	-	Capsid protein (VP23)	Rixon *et al.*, (1990)
UL19	e	Major capsid protein (VP5)	
UL20	e/ne	Integral membrane protein; function in virion egress	Baines *et al.*, (1991); MacLean *et al.*, (1991)
UL21	-	Unknown	
UL22	e	Virion envelope glycoprotein H involved in cell entry; forms a complex with glycoprotein L	Desai *et al.*, (1988); Forrester *et al.*, (1992) Gompels *et al.*, (1991); Weller *et al.*, (1983)
UL23	ne	Thymidine kinase	Coen and Schaffer, (1980); Darby *et al.*, (1981); Larder *et al.*, (1983); Sanders *et al.*, (1982); Summers *et al.*, (1975)
UL24	ne	Function unknown	Jacobsen *et al.*, (1989); Sanders *et al.*, (1982)
UL25	e	Virion protein; function unknown	Addison *et al.*, (1984)
UL26	e	Capsid protein (VP24); Protease, active in virion maturation	Braun *et al.*, (1984); Davison *et al.*, (1992) Liu and Roizman, (1991a,b); Preston *et al.*, (1983); Preston *et al.*, (1992); Rixon *et al.*, (1988)
UL26.5	e?	Internal protein of B capsids (VP22a) processed by UL26 protease	Liu and Roizman, (1991a,b); Preston *et al.*, (1992)
UL27	e	Virion envelope glycoprotein B; involved in cell entry	Cai *et al.*, (1987); Cai *et al.*, (1988); Holland *et al.*, (1983); Kousoulas *et al.*, (1984); Little and Schaffer, (1981)

Table 1 (continued)

UL28	e	Role in capsid maturation/DNA packaging	Addison *et al.*, (1990); Pancake *et al.*, (1983)
UL29	e	ssDNA binding protein essential for DNA replication (ICP8)	Conley *et al.*, (1981); Gao *et al.*, (1988); Orberg and Schaffer, (1987); Weller *et al.*, (1983b)
OriL	ne	Origin of DNA replication	Polvino-Bodnar *et al.*, (1987); Weller *et al.*, (1985)
UL30	e	Catalytic subunit of replicative DNA polymerase; complexes with UL42 protein	Chartrand *et al.*, (1979); Chartrand *et al.*, (1980); Coen *et al.*, (1982); Coen *et al.*, (1984); Gottlieb *et al.*,(1990); Honess *et al.*, (1984)
UL31	e?	Possible nuclear phosphoprotein	Chang and Roizman, (1993)
UL32	-	Function unknown	Coen *et al.*, (1984); Weller *et al.*, (1983a)
UL33	e	Role in capsid maturation/DNA packaging	Al-Kobaisi *et al.*, (1991)
UL34	-	Membrane associated phosphoprotein; substrate for the UL13 protein kinase	Purves *et al.*, (1991); Purves *et al.*, (1992)
UL35	-	Capsid protein (VP26)	McNabb and Courtney, (1992a); Davison *et al.*, (1992)
UL36	e	Large tegument protein (VP1-3, Vmw273)	Batterson *et al.*, (1983)
UL37	-	Function unknown; forms a complex with ICP8; may have a role in DNA-binding	Anderson *et al.*, (1980); Shelton *et al.*, (1990)
UL38	e	Capsid protein (VP19C)	Pertuiset *et al.*, (1989); Rixon *et al.*, (1990); Yei *et al.*, (1990)
UL39	e/ne	Ribonucleotide reductase large subunit (ICP6, Vmw136, R1)	Goldstein and Weller (1988a,b)

Table 1 (continued)

UL40	e/ne	Ribonucleotide reductase small subunit (Vmw38, R2)	Preston *et al.*, (1988)
UL41	ne	Virion protein; involved in shut-off of host cell protein synthesis.	Fenwick and Everett, (1990a,b); Kwong *et al.*, (1988)
UL42	e	Subunit of replicative DNA polymerase; increases processivity; complexes with UL30 protein	Gallo *et al.*, (1989); Gottlieb *et al.*, (1990); Johnson *et al.*, (1991); Marchetti *et al.*, (1988)
UL43	ne	Function unknown; probably integral membrane protein	MacLean *et al.*, (1991)
UL44	ne	Virion envelope glycoprotein C; role in cell entry	Draper *et al.*, (1984); Holland *et al.*, (1983); Homa *et al.*, (1986)
UL45	ne	Virion protein	Visalli and Brandt, (1991)
UL46	ne	Modulates IE gene transactivation by the UL48 protein	Barker and Roizman, (1990); McKnight *et al.*, (1987)
UL47	ne	Tegument protein (VP13-14?); modulates IE gene transactivation by the UL48 protein	McLean *et al.*, (1990); Barker and Roizman, (1990); McKnight *et al.*, (1987); Zhang *et al.*, (1991)
UL48	e	Major tegument protein; transactivates IE genes (VP16, Vmw65, a-TIF)	Ace *et al.*, (1989); Campbell *et al.*, (1984); Weinheimer *et al.*, (1992)
UL49	-	Tegument protein (VP22)	Elliot and Meredith, (1992)
UL49A	e?	Possible membrane protein	Barker and Roizman, (1992); Barnett *et al.*, (1992)
UL50	ne	Deoxyuridine triphosphatase	Fisher and Preston, (1986); McGeoch, (1990)

Table 1 (continued)

UL51	e/ne	Function unknown	Barker and Roizman, (1990)
UL52	e	Component of the DNA helicase/primase complex essential for DNA replication	Crute *et al.*, (1989); Goldstein and Weller, (1988c)
UL53	e?	Glycoprotein K	Debroy *et al.*, (1985); Hutchinson *et al.*, (1992b); MacLean *et al.*, (1991); Pancake *et al.*, (1983)
UL54	e	IE protein; post-translational regulator of gene expression (ICP27, Vmw63, IE2)	McCarthy *et al.*, (1989); McMahan and Schaffer, (1990); Pancake *et al.*, (1983); Sacks *et al.*, (1985); Smith *et al.*, (1992)
UL55	ne	Unknown	MacLean and Brown, (1987)
UL56	ne	Unknown	MacLean and Brown, (1987); McGeoch *et al.*, (1991)
RS1	e	IE protein; transcriptional regulator (ICP4, Vmw175, IE175, IE3)	DeLuca *et al.*, (1985); DeLuca *et al.*, (1988); Dixon *et al.*, (1980); Paterson and Everett, (1990) Paterson *et al.*, (1990); Preston *et al.*, (1981); Russell *et al.*, (1987); Schroder *et al.*, (1985)
OriS	e?	Origin of DNA replication	Stow, (1985); Stow and McMonagle, (1983)
US1	e/ne	IE protein; regulatory protein (ICP22, Vmw68, IE4)	Post and Roizman, (1981)
US2	ne	Function unknown	Longnecker and Roizman, (1987); Weber *et al.*, (1987)

Table 1 (continued)

US3	ne	Protein kinase; phosphorylates the UL34 protein	Frame *et al.*, (1987)Longnecker and Roizman, (1987); Purves *et al.*, (1987); Purves *et al.*, (1991); Purves *et al.*, (1992)
US4	ne	Virion surface glycoprotein G	Longnecker and Roizman, (1987); Weber *et al.*, (1987)
US5	ne	Virion surface glycoprotein J	Weber *et al.*, (1987)
US6	e	Virion surface glycoprotein D; role in cell entry	Ligas and Johnson, (1988); Minson *et al.*, (1986)
US7	ne	Virion surface glycoprotein I	Johnson *et al.*, (1988); Longnecker *et al.*, (1987)
US8	ne	Virion surface glycoprotein E	Johnson *et al.*, (1988); Longnecker and Roizman, (1986); Neidhart *et al.*, (1987)
US9	ne	Virion protein	Umene, (1987); Frame et al., (1986b).
US10	ne	Virion protein	Umene, (1987); Longnecker and Roizman, (1987)
US11	ne	Virion protein; associated with ribosomes in the infected cell.	MacLean *et al.*, (1987); Roller and Roizman, (1992); Umene, (1987)
US12	ne	IE protein; function unknown (ICP47, Vmw12, IE5)	Umene, (1987); Longnecker and Roizman, (1987)

(e) Genes known to be essential for growth of virus in tissue culture
(e/ne) Status depends on culture conditions, ie temperature etc.
(ne) Non-essential genes

Table 1 (continued)

greatly reduced (Hill *et al.*, 1990). Thus it is likely that the LATs are not required for the establishment of latency.

No gene products have been shown to be specifically required for the establishment and maintenance of latency. However, Vmw110 (Russell *et al.*, 1987; Clements and Stow, 1989), the HSV-encoded thymidine kinase (TK) (Coen *et al.*, 1989; Efsthathiou *et al.*, 1989) and the HSV ribonucleotide reductase (RR) (Jacobsen *et al.*, 1989) appear to be important for the reactivation of latent virus in various assays.

Section 1.B The HSV-1 Lytic Cycle

1. Adsorption And Penetration

The initial interaction between HSV and cells is the adsorption of the virus to the cell surface and the subsequent fusion of the virion envelope with the plasma membrane, either at the cell surface or in endocytic vesicles (Fan and Sefton, 1978; Marsh, 1984). Early electron microscopy (EM) studies have suggested that both mechanisms of virus entry occur (Morgan *et al.*, 1968; Dales and Silverberg, 1969). However, recent work has shown that compounds known to inhibit the endocytic process do not inhibit entry of HSV (Wittels and Spear, 1990). Moreover, virus internalised in an endocytic vesicle does not establish a lytic infection of the host cell probably because it is degraded in the vesicle (Campadelli- Fiume *et al.*, 1988a).

At present, the majority of opinion favours the idea of entry by direct fusion of the virion envelope with the cell membrane, resulting in the release of nucleocapsids into the cytoplasm (Morgan *et al.*, 1968). Immunological data supporting this theory showed that neutralising monoclonal antibodies inhibited virus entry but not attachment of virus to the cell surface (Fuller and Spear, 1987; Fuller *et al.*, 1989). Virus infectivity was restored when the neutralized virus bound to the cells was treated with polyethylene-glycol (PEG), a membrane fusing compound. Other evidence in support of the fusion model has come from the work of Para *et al.*, (1980) who showed that the virion

glycoprotein gE was transferred from the virion envelope to the cell membrane in infected cells prior to DNA synthesis.

HSV-1 specifies at least 11 glycoproteins; gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM (Spear, 1976; Marsden *et al.*, 1978; 1984; Bauke and Spear, 1979; Buckmaster *et al.*, 1984; Roizman *et al.*, 1984; Spear, 1985; Frame *et al.*, 1986; Gompels and Minson, 1986; Longnecker *et al.*, 1987; Johnson and Feenstra, 1987; Hutchison *et al.*, 1992a,b; Ramaswamy and Holland, 1992). Five of the envelope glycoproteins, gB gD, gH, gK and gL are required for virus entry into cultured cells (Cai *et al.*, 1988; Forrester *et al.*, 1992; Hutchison *et al.*, 1992ab; Ligas and Johnson, 1988; Roop *et al.*, 1993). It is likely that all of the HSV glycoproteins are present in the virion envelope, although direct evidence is lacking for gL, gK and gJ. It is possible that the product of UL34, which is a membrane-associated phosphoprotein (Purves *et al.*, 1991; 1992), and the UL20 and UL43 gene products, which have multiple hydrophobic domains (Baines *et al.*, 1991; MacLean *et al.*, 1991) , may also be envelope proteins.

Immune electron microscopic analysis of negatively stained virus preparations revealed that gB, gC and gD each form distinct morphological structures projecting from the virion envelope (Stannard *et al.*, 1987). Although the organisation of the envelope proteins is unknown, their structure and location suggest that they may play important roles in both adsorption and entry of HSV-1 into the host cell.

The initial interaction between HSV and the cell is via cell surface heparan sulphate proteoglycans (HS) (WuDunn and Spear, 1989). However, binding of HSV-1 to HS is believed to be only the first in a series of interactions between HSV-1 and the cell surface, culminating in the fusion of the virion envelope with the cell membrane. Observations that virions bind to heparin, a related glycosaminoglycan, and that heparin blocks virus adsorption support this conclusion. Furthermore, virus binding to cells which lack HS is greatly impaired. Interestingly, virus adsorbed to cells at 4^0 can be removed by brief exposure to low pH or soluble heparin, suggesting that HSV-1 binds to HS with low affinity (Huang and Wagner, 1964). In addition, virus bound to the low affinity receptor can be neutralized by HSV-specific monoclonal antibodies (Highlander *et al.*, 1987).

Glycoprotein gC, a non-essential protein for virion infectivity, is principally responsible for the initial adsorption of *wt* HSV virions to cell

surface HS (Herold *et al.*, 1991). The conclusion that gC binds to HS was based on the finding that gC bound to heparin-sepharose under physiological conditions in affinity chromatography experiments. This idea was supported by the observation that mutant HSV virions devoid of gC bound less efficiently to cells compared to *wt* virus (Langeland *et al.*, 1990; Herold *et al.*, 1991). Glycoprotein III, the gC homologue of PRV is also important for the adsorption of virus to cells and, like HSV-1, is not absolutely essential for virus infectivity (Robbins *et al.*, 1986; Schreurs *et al.*, 1988). The idea that gC makes the initial contact with the cell surface is supported by the work of Stannard *et al.* (1987) who showed that gC formed long slender structures (up to 24nm in length) projecting from the virion envelope.

Neomycin and polylysine aminoglycoside antibiotics have been shown to inhibit HSV-1 to a much greater extent than HSV-2 binding to cells (Langeland *et al.*, 1987; 1988). These polycationic drugs are thought to block the attachment of virus to the cell surface by binding to HS. This idea is supported by the work of Oyan *et al.*, (1993) who showed that the region on the HSV genome responsible for the resistance to polycationic drugs maps to the amino-terminal of gC. Since gC of both HSV-1 and HSV-2 bind to heparin, it is possible that the two serotypes may interact differently with cell surface HS, either having different affinities for, or binding to separate epitopes on the HS molecule. An alternative explanation is that, in the presence of drugs binding to HS, HSV-2 can interact with another cellular component via gC or another viral glycoprotein. The idea that HSV-1 and HSV-2 interact differently with cell surface HS was also suggested by WuDunn and Spear (1989) who showed that HSV-1 bound to soluble heparin with a much higher affinity than HSV-2.

Although gC greatly facilitates adsorption of virus to cells it is not the only glycoprotein which can mediate this process. Kuhn *et al.*, (1990) showed that gB, gC and gD formed complexes with cell membrane components during the adsorption process, confirming the results obtained by earlier workers who suggested roles for these glycoproteins in viral attachment (Fuller and Spear, 1985; Johnson *et al.*, 1984). HSV mutants that lack gC, gB or gD can still attach to cells, indicating that this function of a missing glycoprotein can be replaced by other glycoproteins. Evidence has been obtained suggesting that the attachment of gC negative virus to the cell surface HS is mediated by gB, and that this mechanism is resistant to neomycin and polylysine (Herold *et al.*, 1991). The contribution, however, made by gB to the adsorption process must be small, since gB minus virions adsorb to cells as efficiently as *wt* virus (Cai and

Person, 1988; Herold *et al.*, 1991). Whether the cell component described by Kuhn *et al.* (1990) that binds to gB is HS or another cellular component is unknown. It is likely, however, that gB interacts with another cellular component as well as HS, since cells which lack HS are susceptible to *wt* virus infection (Tufaro *et al.*, 1993).

Following the low affinity attachment of HSV to cell surface HS, tighter binding, presumably via a high affinity receptor then occurs, after which bound virus is resistant to acid treatment and neutralisation by monoclonal antibodies. Fuller and Lee (1992) speculated that the stable attachment of virus to the cell surface was necessary for the fusion of the virion envelope with the cell membrane. Kohn (1979) proposed that high affinity binding involved interactions between a number of viral and cellular components. Evidence supporting multivalent attachment of virus to cells comes from Rosenthal (1984) who demonstrated that HSV-1 binding to cells rapidly reduced cell surface protein mobility, similar to that caused by the multivalent attachment of other ligands such as antibodies or other cells. The hypothesis that more than one type of HSV attachment can occur is consistent with the ability of herpesviruses to bind to a wide range of cell types from diverse species.

In a recent report, Kaner *et al.* (1990) claimed that the basic fibroblastic growth factor (bFGF) receptor was a "portal of cellular entry" for HSV-1. Evidence supporting this idea came from two observations. Firstly, the attachment and infectivity of HSV-1 was reduced when cells were incubated with bFGF prior to the addition of virus. Secondly, the uptake of HSV-1 in Chinese hamster ovary (CHO) cells, which have very few bFGF receptors, was increased after transfection of the bFGF receptor cDNA. Moreover, the uptake of virus in CHO cells which express the bFGF receptor was blocked by polyclonal antibodies specific for bFGF (Baird *et al.*, 1990). From these results it was concluded that the interaction between HSV and the bFGF receptor is mediated by the association of bFGF with the virion, rather than a direct interaction between a HSV glycoprotein with the receptor itself. The proposal that HSV-1 entry occurs through the bFGF receptor was attractive because bFGF binds to cell surface HS prior to binding to the bFGF receptor (Vlodavsky *et al.*, 1987). However, subsequent studies have since disproved that the basic FGF receptor is involved in HSV-1 entry (Shieh and Spear, 1991; Mirda *et al.*, 1992; Muggeridge *et al.*, 1992). The observed effects were probably a result of the interaction of basic FGF with cell surface HS (Shieh and Spear, 1991; Muggeridge *et al.*, 1992).

HSV-1 gD is a major component of both the virion envelope and infected cell membranes. The idea that a high affinity attachment occurs between gD and a cell surface receptor came from the work of Ligas and Johnson, (1988) who showed that virus which lacked gD adsorbed to cells as efficiently as *wt* virus, but the bound virus could not block infection by superinfecting *wt* virus. This defect could be overcome by treating the virus infected cells with PEG, a compound which promotes membrane fusion. Similarly, cell lines expressing gD were shown to be resistant to infection by both HSV serotypes (Campadelli-Fiume *et al.*, 1988ab; Johnson and Spear, 1989), which suggests that gD present in the plasma membrane of transfected cells binds to and sequesters a cell surface receptor and thereby blocks the entry of HSV. Finally, further studies demonstrated that the target of the gD mediated restriction to superinfection, was the gD present in the superinfecting virion (Campadelli-Fiume *et al.*, 1990). Ligas and Johnson (1988) recently obtained evidence for a cell surface receptor required for the entry of HSV-1 and 2 into cells. The receptor sites are more limited in number than HS and appear to bind to gD in the virion envelope (Johnson *et al.*, 1990). The identity of this cellular receptor is presently being investigated (D. Johnson, personal comunication).

Cell lines expressing gB or gC are not resistant to HSV infection and there is no evidence to date for saturatable cell surface receptors for these viral proteins.

Cell fusion is the next stage in virus entry and follows the stable attachment of virus to the cell surface. Studies with *ts* mutants, monoclonal antibody resistant mutants, syncytial mutants and null mutants have suggested that gB functions directly in virus entry and virus induced cell fusion (Sarmiento *et al.*, 1979; Bzik *et al.*, 1984b; Cai *et al.*, 1988; Highlander *et al.*, 1988).

To date, four viral genes UL53 (gK) (Debroy *et al.*, 1985; Pogue-Geile and Spear, 1987), UL27 (gB) (Kousoulas *et al.*, 1984; Bzik *et al.*, 1984a), UL24 (Sanders *et al.*, 1982; Jacobsen *et al.*, 1989), and UL20 (Baines *et al.*, 1991) have been associated with the syncytial phenotype. A fifth syncytial locus has been suggested to map within the inverted repeats flanking U_L although the gene product involved has not yet been identified (Romanelli *et al.*, 1991).

While both gD and gB have been implicated in virion cell induced fusion, it remains to be seen whether the attachment of these proteins to cellular membrane components is a prerequisite to their role in viral penetration.

Suggestions that gD and gB form a complex inducing membrane fusion are unfounded, since gB and gD are spatially separated in the virion envelope (Stannard *et al.*, 1987). Furthermore, gB forms homodimers which are not found to be associated with any other glycoproteins (Claesson-Welsh and Spear, 1986). Nonetheless, it is possible that gB and gD may interact with other viral components prior to the initiation of cell fusion.

Using biochemical and elecron microscopy approaches, the entry process of HSV-1 was examined in detail by Fuller and Lee (1992) who observed discernible changes of the virion envelope and tegument after contact of infectious virus with the cell plasma membrane. Virions rendered non-infectious by anti-gD or anti-gH neutralising antibodies bound to cells but failed to form a visible fusion bridge. On the basis of these investigations, together with earlier findings, a model of HSV entry into cells was proposed, and this is illustrated in Figure 3.

According to the proposed model, virion attachment to cell surface HS is mediated by gC and to a lesser extent gB, after which, a more stable attachment of the virus to the cell occurs via an unidentified cellular receptor. The viral glycoprotein thought to be involved in the stable attachment of virus to cells is gD, since bound virus lacking gD are more sensitive to removal by heparin or high ionic strength washes than *wt* virus adsorbed to cells. Moreover, soluble gD can reduce virus attachment when added to cells prior to or during virus adsorption (Fuller and Lee, 1992). The stable attachment of the virion is believed to prime the virus for fusion with the cell membrane, on the basis that gD-minus and gD-neutralized virus bind to cells but are unable to penetrate the cell membrane (Fuller and Spear, 1987; Highlander *et al.*, 1987; Ligas and Johnson, 1988). The model proposes that gD has two functions in viral entry. Firstly, gD interacts with a cellular receptor, bringing virus and cell closer together. Secondly, gD is believed to interact with another viral component that is directly involved in the fusion initiation event. The idea that gD is bi-functional comes from the observation that antibodies specific to gD, which prevent attachment, do not bind in the same regions as those blocking penetration (Eisenberg *et al.*, 1985). Furthermore, the finding that cell surface gD interferes with superinfection, possibly by interacting with gD or another viral glycoprotein on the virion, is consistent with the hypothesis that gD interaction with another virion component triggers the fusion initiation event. However, it can also be argued that gD mediated inhibition of superinfection results from the sequestering of a cellular receptor by cell surface gD. This idea

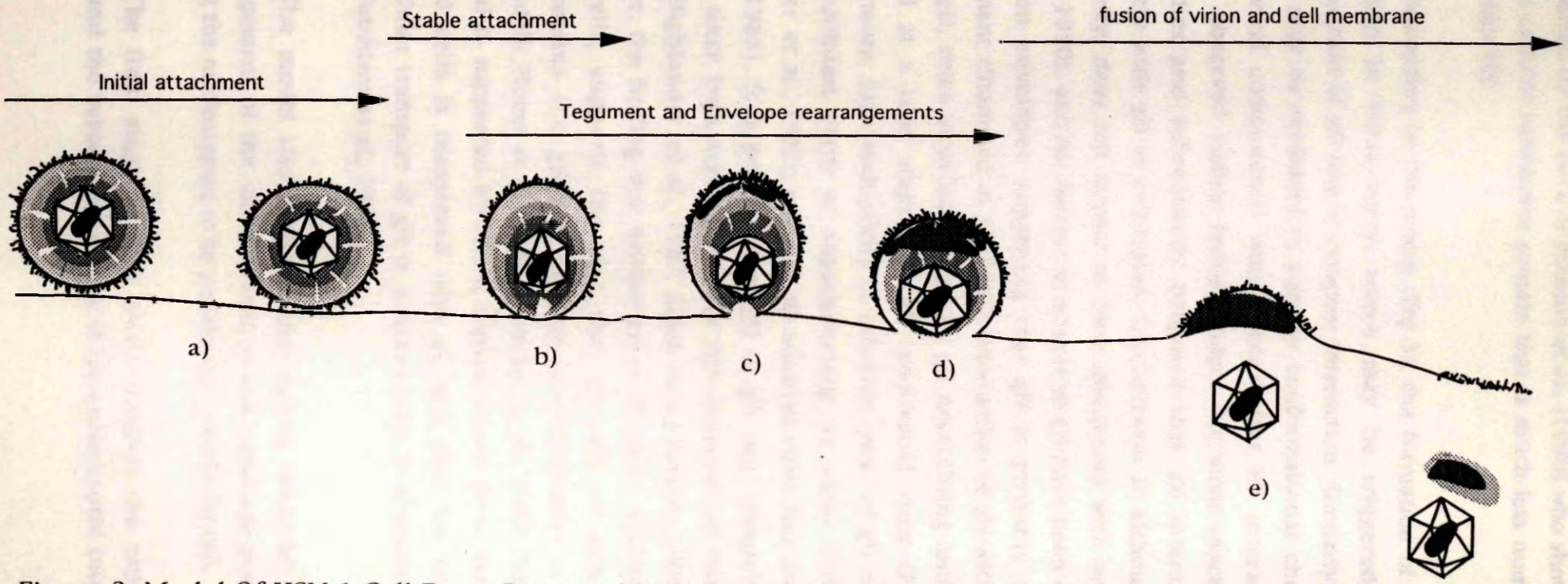


Figure 3. Model Of HSV-1 Cell Entry Proposed By Fuller and Lee (1992)

a) Initial attachment of virus to the cell surface; b) stable attachment; c) initiation of a fusion bridge ; d) expansion of the fusion bridge; e) release of nucleocapsid into the cytoplasm. The proposed rearrangements of the virion envelope, tegument, and nucleocapsid in relationship to the cell plasma membrane are diagrammed. This diagram was reproduced from Fuller and Lee (1992).

is supported by the work of Johnson *et al.*, (1990) who showed that gD bound to a 170kDa cellular membrane protein that is much less numerous in number than cell surface HS.

According to the model (Fig 3), the formation of a fusion bridge is the next step in virus entry, which may be triggered by changes in the conformation of gD due to receptor interaction. Similarly, formation of a fusion bridge may be mediated in part by conformational changes in gC or gB (or other viral components) which occur after HS interaction. Fuller and Lee (1992) observed visible fusion bridges for virus inactivated by gH specific antibodies and subsequently proposed that gH interacts either directly or indirectly with gD in cell fusion. Glycoprotein H, although essential for virion infectivity, does not appear to form complexes with any cellular component (Kuhn, 1990), and no distinct structures of gH have been detected on the surface of virion envelopes, suggesting that gH is probably not involved in virus attachment (Stannard *et al.*, 1987). Interaction of gH with the cell is thought to follow gD, since virus inactivated by gH neutralising antibodies appeared to be blocked at a later stage than gD inactivated virus (Fuller and Lee, 1992). Furthermore, high multiplicity of infection (moi) of gH negative virus bound to cells inhibited entry of superinfecting *wt* virus (Fuller and Spear, 1987; Forrester *et al.*, 1992), whereas gD negative virus had little effect (Johnson and Ligas, 1988). It is possible that gD or gH may independently mediate virion fusion, since both are required for HSV-induced cell fusion (Fuller and Spear, 1987; Highlander *et al.*, 1987; Ligas and Johnson, 1988; Fuller *et al.*, 1989). However, the finding that neither gD or gH alone mediate substantial fusion of most cells, supports the idea that gH and gD and perhaps other viral glycoproteins (i.e gB and gK) act synergistically to mediate HSV fusion (Campadelli Fiume *et al.*, 1988b; Butcher *et al.*, 1990; Peeters *et al.*, 1992). This proposal is supported by recent reports which show that most or all of gH in infected cells is complexed with gL and that the correct processing and intracellular transport of gH in infected cells, is dependent on the coexpression of gL (Hutchison *et al.*, 1992a).

The model also proposes that during initiation of the fusion bridge, rearrangement of the virion envelope and tegument proteins occur (Fig 3 d), allowing the nucleocapsid to be positioned towards the cell.

The final stage of viral entry, involves the expansion of the fusion bridge and the subsequent release of the nucleocapsid into the cytoplasm (Fig 3

e and f). It is likely that gB has an essential role in virus induced cell fusion, since gB-null syncytial virus can cause complete fusion of gB-transformed cells, but no fusion on untransformed cells (Cai *et al.*, 1988). Furthermore the recently identified glycoproteins gK and gL, in addition to other viral components, may play important roles in mediating and controlling the changes observed in the virion particle or cell membrane and subsequent release of the nucleocapsid into the cytoplasm (Hutchison *et al.*, 1992b; Ramaswam y and Holland, 1992)

The role of non-glycosylated proteins in viral attachment and fusion have not been thoroughly addressed. Previous investigations showed that virus attachment can occur when glycosylation is inhibited, suggesting that other viral proteins are important in virus binding (Svennerholm *et al.*, 1982; Kuhn *et al.*, 1988). An alternative explanation is that glycosylation is not required for the interaction of viral attachment proteins to the cell receptors. Furthermore, the non-glycosylated UL25 gene product has been implicated in viral entry but not adsorption (Addison *et al.*, 1984).

2. Uncoating Of The Virus Genome

Once HSV nucleocapsids have been released into the cytoplasm, they are transported across the cytoplasm to the nucleus (Batterson *et al.*, 1983) by a mechanism probably involving host cell microfilaments (Lycke *et al.*, 1984). At the nuclear membrane disassociation of the capsid occurs and viral DNA is released. Dissasociation of capsid pentamers may be prerequisite to the release of viral DNA, since these are frequently absent from preparations of flattened capsid sheets visualized in infected cells (Vernon *et al.*, 1984).

Viral DNA enters the nucleus via nuclear pores (Hummeler *et al.*, 1969). Since a lytic infection can occur with transfected viral DNA, translocation does not appear to be dependent on the presence of viral proteins.

The uncoating process can take place in the presence of nucleic acid and protein synthesis inhibitors, indicating that *de novo* RNA and protein synthesis are not required for this process. In light of these observations it was proposed that a structural component of the virus or a cellular enzyme mediates the uncoating step (Hochberg and Becker, 1968). A function essential for the

uncoating event has been mapped to the UL36 gene (Batterson *et al.*, 1983), which encodes the tegument protein Vmw273 (Macnabb and Courtney, 1992b). The possibility exists that other tegument proteins may also be required for uncoating.

3. Effects Of HSV-Infection On Host Cell Macromolecular Synthesis

3.1 Shut-Off Of Host Cell Protein Synthesis

One of the first changes to occur in HSV-1 infected cells is the margination of host cell chromatin (Nii *et al.*,1968). Margination may be a function of a virion component or an IE gene or the cellular stress response, since mutant virus limited to the IE stage of replication are still able to induce this effect (Dargan and Subak-Sharpe, 1983). Other changes such as distortion of the nucleus and disintegration of the nucleolus occur early in HSV-1 infected cells and are possibly caused by HSV-1 IE proteins (reviewed by Dargan, 1986). Alterations in cell membrane permeability have also been observed (Kohn, 1979).

Infection of cells with HSV-1 results in the rapid shut-off of host protein synthesis by disruption of polysomes and degradation of host mRNA (reviewed by Fenwick, 1984). Virus entry is thought to be required for the induction of host cell shut-off, since protein synthesis is not inhibited in cells infected with virus, inactivated either by heat treatment at 56^0 or with human γ-globulin (Fenwick and Walker, 1978; Nishioka and Silverstein, 1978).

Two stages of shut-off of host cell protein synthesis have been defined (Nishioka and Silverstein, 1978). The first, termed virion associated shut-off, is mediated by a virion component. It occurs in the presence of actinomycin D (inhibitor of gene expression) and in cells infected with UV-inactivated virus (Fenwick and Walker, 1978; Fenwick *et al.*, 1979; Kwong and Frenkel, 1987). A further reduction in host cell protein synthesis, termed secondary shut-off, occurs later in infection, and requires viral gene expression. Virion induced degradation of mRNA is not selective, resulting in both the shortening of the half lives of both host and viral mRNA (Kwong and Frenkel, 1987). Read and

Frenkel,(1983) isolated a series of virion host shut off (*vhs*) mutants that were unable to mediate virion-associated shut-off of host protein synthesis. The *vhs* mutation was later mapped to a region on the HSV genome containing the UL41 ORF, which is predicted to encode a 58 kDa protein (Kwong *et al.*, 1988; McGeoch *et al.*, 1988b). Subsequent work by Smibert *et al.*, (1992), using rabbit antipeptide antisera specific for the UL41 protein, confirmed that the UL41 gene product was a 58 kDa protein found in virions. Finally, the UL41 protein was shown to be a component of the virion tegument (McLauchlan *et al.*, 1992a).

Although the UL41 protein has a role in host shut-off of protein synthesis, the protein is expendable for growth of virus in tissue culture, since mutants defective in host cell shut-off are able to produce lytic infection in cells (Fenwick and Clark, 1982; Read and Frenkel, 1983; Fenwick and Everett, 1990b). Nonetheless, the *vhs* protein does confer a growth advantage to the *wt* virus, shown by the rapid selection of *wt* virus during serial virus propagation (Kwong *et al.*, 1988). Since viral gene expression involves the sequential activation of groups of genes comprising the IE, E and L genes, nonselective degradation of mRNA may facilitate translation of the most recently transcribed mRNA (Kwong and Frenkel, 1987; Oroskar and Read, 1989).

3.2 Induction Of Cellular Proteins

Challenge of a susceptible host with HSV can result in abortive, latent and productive infections. HSV-2 has also been implicated in transformation and oncogenesis. This variability in the response to HSV-infection suggests that host cell factors may determine the outcome of the virus-cell interactions. The expression of a number of cellular proteins has been frequently observed during viral infection.

a) Interferons

The interferons are a family of cellular proteins, not normally expressed, that are produced in response to viral infection. They are released from virus-infected cells and bind to interferon receptors on neighbouring cell membranes. Two classes of human interferon, IFN-α and IFN-β have been

shown to bind to the same cellular receptor, whereas IFN-γ binds to a separate cell receptor (Branca and Baglioni, 1981; Sarkar and Gupta, 1984). Binding of interferon to the cellular receptor promptly activates the transcription of a defined set of cellular genes, many of which are involved in establishing an antiviral state whereby viral replication is inhibited. In this respect they are believed to be the body's first line of defence against viral infection. Development of the antiviral state by interferon involves the induction of two enzymatic activities, a protein kinase and a 2'-5'- oligoadenylate synthetase (Farell, 1979), both of which are involved in the degradation of mRNA and inhibition of viral protein synthesis.

In addition to the establishment of an antiviral state, the effects of interferons are accompanied by changes in surface membrane proteins (Lengyel, 1982), and in the morphology of the cytoskeleton (Fellous *et al.*, 1982).

A number of other cellular proteins, several of which are in the same size range (Colonno, 1981), are known to accumulate in IFN-treated cells. Ball (1979) showed that interferon treatment induced the synthesis of a 56 kDa protein that appeared to be related to the 2'-5'-oligoadenylate synthetase. Since then, four other 56 kDa cellular proteins have been shown to accumulate in interferon-treated cells. Although these proteins are the same size, they appear to be distinct. For instance, the 56,000 Mr protein (C56) identified by Chebath *et al.* (1983) does not have any detectable synthetase activity or share common sequences with the 2'-5'-oligoadenylate synthetase cDNA, suggesting that the C56 is distinct from the protein described by Ball (1979). The synthesis of the 56kDa protein identified by Kusari and Sen (1987) occurs in cells treated with IFN-α or IFN-β but not in cells treated with IFN-γ. This protein is probably distinct from the 56,000Mr IFN-induced protein described by Rubin *et al.*, (1988), which is induced in both IFN-α, IFN-β and IFN-γ treated cells. The functions of the majority of IFN-induced proteins in mediating the effects seen in IFN-treated cells have yet to be determined.

b) Heat shock proteins

The cellular stress response occurs in cells subjected to environmental stresses such as increases in temperature. During the stress response, the synthesis of cell proteins that are currently being synthesized is down-regulated, and a new set of proteins is synthesized. These proteins are known as the heat shock or stress proteins. The high degree of conservation of the amino acid sequences among the heat shock proteins in all organisms together with the observation that many of these proteins are present when the organism is not subjected to stress, suggests that they have functions essential to normal cellular operations but are required to a higher degree under stress conditions. Recent studies of the hsp70 and hsp90 classes of heat shock proteins support the notion that these proteins act as molecular chaperones (Ellis, 1990). Molecular chaperones are defined as a family of unrelated proteins that mediate the correct assembly of other polypeptides. Thus it was proposed by Pelham (1986) that the heat shock proteins not only mediate protein assembly but also promote the disassembly of proteins that have been damaged as a result of stress.

As well as heat shock, the heat shock proteins are synthesized in response to other conditions, such as viral infection (Nevins, 1982; Collins and Hightower, 1982; Wakakura *et al.*, 1987). There have been numerous reports of the induction of heat shock proteins during HSV-infection. For instance, Notarianni and Preston (1982) showed that heat shock proteins accumulated in cells infected with the HSV-1 strain 17 *tsk* but not in *wt* virus-infected cells. The *tsk* mutant carries a lesion in the IE protein Vmw175 and it was proposed that accumulation of heat shock proteins was important in eliminating non-functional forms of Vmw175 or neutralizing their damaging effects, rather than merely resulting from viral IE protein accumulation (Russell *et al.*, 1987). This effect may be important *in vivo*, since natural isolates frequently show *ts* mutations in the gene encoding Vmw175 (Knipe *et al.*, 1981)

LaThangue *et al.* (1984) identified a 57,000 Mr cellular protein (p57) that was transcriptionally induced in heat-shocked cells and in HSV-2 infected cells. An accumulation of this protein was also shown to occur in HSV-1 infected cells, albeit to a lower level (Patel *et al.*, 1986). The fact that p57 accumulated both in HSV-infected cells and during heat shock suggests that these stimuli activate the stress response and it is likely therefore that p57 has a similar function in both cases. Thus induction of heat shock proteins in HSV-infection may represent an attempt by the cell to deal with the presence of foreign viral

proteins. Clearly in the majority of cases the viral lytic cycle is not inhibited by the stress response. Nonetheless, it is possible that induction of the stress response in HSV-infection of some cell types *in vivo* may cause the lytic cycle to be aborted, resulting in the establishment of cellular transformation (Minson, 1984).

c) Cellular Proteins and HSV-induced Transformation.

The involvement of herpesviruses with tumour induction has been observed for some time (reviewed by Macnab, 1987). Although there are no direct links between HSV-infection and subsequent cancer development, there is nonetheless a tenuous association between previous infection with HSV-2 and the development of cervical neoplasia, inasmuch as a higher prevalence of HSV-2 seropositivity has been demonstrated in cervical cancer patients than in normal control patients (Rawls, 1983).

The mechanism of HSV-2 induced cell transformation is unclear but may involve in part the amplification of some cellular genes (reviewed by Macnab, 1987).

A number of heat shock proteins have been shown to accumulate in HSV-transformed cells (Kelley and Schlesinger, 1982). In immunoprecipitations with the monoclonal antibody TG7A, which was raised against the DNA-binding proteins of HSV-2 infected cells (LaThangue and Chan, 1984), LaThangue and Latchman (1988) identified two cell encoded polypeptides, p90 and p40, that accumulated during HSV-infection. Previous studies indicated that similar sized polypeptides were overexpressed in transformed and immortalized cells (Macnab *et al.*, 1985). The expression of p90 was also induced by heat shock and it was proposed that p90 was related to the previously defined heat shock protein of this molecular weight, hsp90 (Kelley and Schlesinger, 1982; LaThangue and Latchman, 1988). Interestingly, p40 accumulated primarily in HSV-1 infected cells, whilst p90 was overexpressed during HSV-2 infection. In addition to p40, TG7A reacted with a 32kDa cell protein in HSV-1 infected cells, albeit at a lower concentration relative to p40. The differential accumulation of p40 and p90 suggests that the infecting virus possesses the factors which determine whether p40 or p90 accumulates. It is intriguing to speculate that the accumulation of these proteins in HSV-infection and in HSV-transformed cells

* Mitochondrial aspartate amino-transferase is the target of anti-tumour drugs (Thomasset *et al.*, 1992; Vila *et al.*, 1990).

may have important implications for the mechanism of HSV-induced cellular transformation. However, the difficulty with this suggestion is that p40 and p90 are present in many different transformed cell lines, not just those transformed by HSV, suggesting that the overexpression of p40 and p90 is a general feature of all transformed cells (Macnab *et al.*, 1985).

Work recently carried out by Hewitt *et al.* (1991) led to the identification of a transformation-specific polypeptide U90 that accumulated in HSV-2 infected cells. U90 represents the upper polypeptide band of a 90kDa doublet. Both U90 and the polypeptide L90 from the lower 90 kDa band can be immunoprecipitated from transformed cells by antisera from tumour bearing animals (TBS) or by the mouse monoclonal antibody TG7A (Macnab et al., 1985). In contrast to p90 that was identified using TG7A in western blot analysis by LaThangue and Latchman (1988), neither U90 or L90 were induced by heat shock. Indeed, Hewitt *et al.*, (1991) failed to identify any heat shock proteins in HSV-infected and transformed cells using the TG7A antibody in immunoprecipitation assays. Thus it was concluded that while TG7A and TBS recognize similar U90 and L90 polypeptides in immunoprecipitations, in western blots TG7A recognizes a different epitope to that recognized by TBS. Therefore, U90, immunoprecipitated from rat cells with TG7A, is distinct from p90 that was recognized by TG7A in western blots. On the basis of this data, it was proposed that the heat shock proteins recognized in HSV-infected cells by western blot analysis using TG7A (Lathangue and Latchman, 1988) do not express the epitopes characteristic of U90 or L90 that were recognized by the immunoprecipitation assays using the same antibody. In support of this idea is the observation that TG7A recognizes an epitope on the *E. coli lon* protease (Latchman *et al.*, 1987a), whereas TBS fails to react with any *E. coli* proteins (D. McNab and J.C.M. Macnab, unpublished results).

Recently, it was shown that patients with cervical cancer produced an antibody response to a tumour specific cell polypeptide that is overexpressed in HSV-2 infected cells (Macnab *et al.*, 1992). The 40kDa protein was immunoprecipitated from tumour cells using TBS and TG7A, and limited amino acid sequence analysis suggested that it was homologous to the mitochondrial aspartate amino-transferase (Huynh *et al.*, 1981; Lucasson, 1992). There has been evidence to suggest an involvement of this enzyme in cancer, and disturbances in mitochondrial function have been implicated in the development of malignancy (reviewed by Shay and Werbin, 1987).

To date, the role of the HSV-inducible cell polypeptides in the lytic cycle is unknown. The fact that HSV-infection can result in the overexpression of some cellular proteins which also accumulate in transformed cells may have important implications for the mechanism of HSV-induced transformation. However, it remains to be seen whether the overexpression of certain cellular proteins is directly involved in the establishment of the transformed phenotype, or is simply a consequence of it.

d) Mechanisms of Induction

The accumulation of the majority of cellular proteins during HSV-infection appears to be mediated by the transcriptional induction of the corresponding cellular gene (Kemp *et al.*, 1986a; Patel *et al.*, 1986). Many of these gene products require functional viral IE proteins, in particular Vmw175, for their induction (Patel *et al.*, 1986; Latchman *et al.*, 1987b; Kemp and Latchman, 1988; Esteridge *et al.*, 1989; reviewed by Everett, 1987). In the case of adenovirus, the induction of a 70kDa heat shock protein is dependent on the expression of the early gene product E1a (Nevins, 1982; 1989). The accumulation of the cell polypeptide p40 in HSV-infected cells (LaThangue and Latchman, 1988) was shown to be dependent on the expression of the HSV IE protein Vmw63 (Esteridge *et al.*, 1989). However, in the absence of viral replication, transfection with a plasmid containing the gene encoding Vmw63 either alone or in concert with the other IE genes or that encoding Vmw65, was not sufficient to induce significant p40 accumulation. This finding suggested that Vmw63 alone was not sufficient for maximal p40 accumulation and that other factors in infected cells are required. Since p40 accumulation occurs in *tsk*-infected cells, where lytic infection is limited to the IE stage, it is likely that these factors must be a component of the virion other than Vmw65 or a relatively non-specific stimulus such as viral attachment or entry of the capsid into the cytoplasm. Since the activation of the majority of cellular genes during viral infection is dependent upon the expression of the IE proteins, it is intriguing to speculate that the cellular promoters have sequence homology to viral promoters and as a result are transcriptionally activated by the IE proteins during lytic infection. However, evidence has suggested that the activation of cellular genes requires no specific *cis*-acting sequences within the cellular promoter. In support of this idea was the observation that active endogenous β-globin genes were shut off by HSV infection in a manner that

was characteristic of the majority of cellular genes, whilst transfected globin genes were activated in apparently the same manner as HSV genes, (Everett, 1984a; 1984b; Everett and Dunlop, 1984; Everett, 1985). These observations suggest that an open chromatin structure is an important factor in the non-specific activation of cellular genes during viral infection. Thus cellular genes which are transcribed during lytic infection may simply have easily accessible promoters (Everett, 1985). This idea is supported by the fact that the heat shock genes, which accumulate in HSV-infections, have open promoters in normal cells (Wu, 1980).

A few cellular genes can be induced in herpesvirus infected cells in the absence of viral protein synthesis (Kemp *et al.*, 1986b; Preston, 1990). During HCMV infection, Boldogh *et al.*, (1990) detected a rapid increase in the RNA levels of three proto-oncogenes, in conditions which severely inhibited the synthesis of the viral IE proteins. These workers concluded that the expression of these oncogenes was induced by events occuring before the onset of viral protein synthesis, perhaps by the interaction of the virus particle with the cell surface. Although the cellular receptor to which HSV binds remains to be determined, it is likely that it is a normal cell surface component. The cellular receptors of many viruses have been determined and all perform normal functions in cells. For instance, EBV infects T-lymphocytes by means of the C3d complement receptor (Fingeroth, 1984), human immunodeficiency virus (HIV) primarily uses the CD4 glycoprotein receptor (Dalgleish *et al.*, 1984), and vaccinia virus can enter the cell by first interacting with the epidermal growth factor receptor (Eppstein *et al.*, 1985). It is conceivable that in HSV binding to its cellular receptor, the virus may mimic the normal ligand for this receptor and subsequently cause the induction of cellular genes normally induced by this binding.

There is some evidence to suggest that regulation of cellular genes may also occur in the absence of viral protein synthesis, at stages subsequent to receptor binding (Kemp *et al.*, 1986b). In this case the induction of cellular genes may be regulated in a similar manner to the viral IE proteins. Induction of the HSV IE proteins is mediated by the viral *trans*-activator, Vmw65 and is dependent on the presence of certain cellular factors and the TAATGARAT motif within the promoter region of the induced gene (see section 1.2). However, there is no evidence yet to suggest that TAATGARAT-like motifs exist within the HSV-inducible cell promoters.

Although the exact mechanisms of induction of cellular proteins during viral infection are unknown, it is likely that this effect is of functional significance to the infected cell. For instance, the induction of interferon during viral infection is important in establishing the antiviral state, which serves to restrict the spread of infection to other cells. Furthermore, the activation of the stress response by infecting virus would have a similar effect. However, the possibility remains that the virus may induce some cellular proteins that may facilitate or affect the outcome of a viral infection. In the case of HSV, viral infection can result in both lytic and latent infections as well as transformation of the infected cell. Indeed, the observation that HSV can induce a number of cellular proteins, which appear to accumulate in transformed cells, suggests that HSV-transformation may occur in part by the activation of cellular genes.

4. Temporal Regulation Of HSV-1 Gene Expression

HSV genes were initially classified into three broad groups, immediate early (IE or α), early (E or β) and late (L or γ) (Honess and Roizman, 1974; Clements *et al.*, 1977). The IE proteins are the first viral proteins to be synthesized in the virus growth cycle and in general are involved in the regulation of gene expression. Studies using metabolic inhibitors revealed that IE gene expression does not require prior viral protein synthesis. Early proteins are synthesized after the expression of the IE proteins, prior to DNA replication. Indeed, many of the early proteins are involved in viral DNA metabolism. The late genes are defined as genes whose expression requires prior synthesis of IE and E proteins in addition to viral DNA synthesis and, in general, encode structural proteins (reviewed by Wagner, 1985; 1991). The identification of several late viral genes expressed prior to viral DNA synthesis lead to the classification of two groups of late genes: the leaky late viral genes (βγ or γ_1) and the true late genes (γ_2). The maximal expression of both of these groups of genes is dependent on viral DNA replication. However, in contrast, to the true late viral proteins, the leaky late proteins can be detected prior to the synthesis of viral DNA (Wagner, 1985). More recently, Johnson *et al.*, (1986), reported the detection of very low levels of the protein encoded by the true late gene US11, under conditions of severely restricted viral DNA synthesis. These

workers subsequently proposed that a true late gene is a gene whose expression under conditions which severely restrict viral DNA synthesisis is reduced to less than 95% of normal levels.

4.1 The IE Proteins

Five IE proteins , Vmw110, Vmw63, Vmw175, Vmw68 and Vmw12, which are encoded by the HSV IE 1, 2, 3, 4 and 5 genes respectively have been described.

a) Vmw12 and Vmw68

In contrast to the other IE proteins, Vmw12 is non-phosphorylated and is located within the cytoplasm (Marsden *et al.*, 1982). Since viruses with deletions of the Vmw12 gene are viable, Vmw12 is not essential for virus growth in tissue culture (Longnecker and Roizman, 1986). The function of Vmw12 is poorly understood.

Vmw68 appears to have a role in the regulation of late gene expression. Analysis of a deletion mutant indicated that Vmw68 was only essential in some cell types, suggesting that a cellular function may replace its activity (Post and Roizman, 1981)

b) Vmw63

The observation that *ts* mutant viruses carrying lesions in the Vmw63 gene over-expressed early gene products and did not synthesize detectable amounts of late genes (Sacks *et al.*, 1985), suggested that Vmw63 was essential for virus growth in tissue culture. The requirement of Vmw63 for late gene expression and possibly the down regulation of IE genes was confirmed by a subsequent study of Vmw63 deletion mutants (McCarthy *et al.*, 1989). Recent studies have showed that Vmw63 plays an important role in the regulation of late gene expression through a post transcriptional mechanism (Sandri-Goldin and Mendoza, 1992; Smith *et al.*, 1992), probably by increasing the processing at the 3' poly(A) signal of late transcripts (McLauchlan *et al.*, 1992b). Recently, it

was reported that purified Vmw63 could bind to zinc ions and interact with single-stranded DNA (Vaughn *et al.*, 1992). Furthermore, the aminoterminal acidic region was shown to be important for the regulatory activities of Vmw63 in both transfected and infected cells (Rice *et al.*, 1993).

c) Vmw110

Vmw110 is a transactivator of a variety of viral promoters (O'Hare and Hayward, 1985a, 1985b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986; Everett, 1986; Everett *et al.*, 1991b). Although, Vmw110 is not essential for virus growth in tissue culture, it does, however, confer a strong growth advantage on the virus (Stow and Stow, 1986; Sacks and Schaffer, 1987). Transactivation by Vmw110 was shown to be increased in the presence of Vmw175, and vice versa (Everett, 1984b), however, this synergism was shown to depend greatly on the experimental conditions used (Everett *et al.*, 1988). Nonetheless, further studies showed that Vmw175 affected the intranuclear location of Vmw110, which suggests that the two proteins interact (Knipe and Smith, 1986).

d) Vmw175

Vmw175 is the major viral transcriptional regulatory protein and is essential for virus growth (Preston, 1979), and the transcriptional activation of the E and L genes (Preston, 1979; Everett, 1984a, 1984b; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; DeLuca and Schaffer, 1985; Quinlan and Knipe, 1985). In addition, Vmw175 can repress its own synthesis as well as that of the other IE proteins (O'Hare and Hayward, 1985b; DeLuca and Schaffer, 1985). Whilst most of the IE proteins have been shown to bind non-specifically to DNA (Hay and Hay, 1980), Vmw175 is the only one known to interact with a specific target sequence. It binds to the sequence 5'-ATCGTC-3' which was identified within the IE-3 promoter (Muller, 1987; Faber and Wilcox, 1986, 1988). It is generally believed that self-repression occurs by binding of Vmw175 to this sequence at its own transcription start site (Roberts *et al.*, 1988; DeLuca and Schaffer, 1988). Stimulation of gene expression mediated by Vmw175 is thought to result from Vmw175 binding to sites within the promoter region (Tedder *et al.*, 1989). The region of Vmw175 involved in DNA binding, transactivation and repression was located near the N-terminus of the protein, between amino acid residue 275 and 495. Mutations in this region of Vmw175 were shown to greatly

affect these functions (Paterson and Everett, 1988a; 1988b; Shepard *et al.*, 1989; Paterson *et al.*, 1990). A polypeptide corresponding to this region retains the sequence specific DNA-binding properties of the whole protein (Wu and Wilcox, 1991; Everett *et al.*, 1990; 1991a).

4.2 Transactivation Of IE Gene Expression By Vmw65

The transactivation of the IE viral genes is mediated by the UL48 gene product termed Vmw65 (α-TIF, VP16) (Batterson and Roizman, 1983; Campbell *et al.*, 1984; Dalrymple *et al.*, 1985; Pellet *et al.*, 1985). Vmw65 is a 65kDa phosphoprotein located within the tegument of the virion particle (Roizman and Furlong, 1974; Marsden *et al.*, 1978; Szilagyi and Cunningham, 1991; McLauchlan and Rixon, 1992). Previous data have suggested a structural function for this protein as well as a role in mediating IE gene transactivation (Post *et al.*, 1981; Campbell *et al.*, 1984; Ace *et al.*, 1989).

An upstream element containing the sequence TAATGARAT (R= purine) which was required for IE gene transactivation, was identified in all of the IE gene promoters (Mackem and Roizman, 1982; Murchie and McGeoch, 1982; Cordingly *et al.*, 1983; Campbell *et al.*, 1984). The functional relevence of the TAATGARAT motif in the transactivation of IE gene expression was questioned following observations that Vmw65 showed no detectable *in vitro* DNA binding properties (Marsden *et al.*, 1987). Thus, attention was focused on the identification of cellular proteins which interact with both Vmw65 and the TAATGARAT motif. A likely candidate protein was subsequently identified as the cellular transcription factor OCT-1 (OFT-1, NFIII, TRF), which interacts with both Vmw65 and the TAATGARAT motif resulting in the formation of an infected cell complex (Preston *et al.*, 1988; O'Hare and Goding, 1988). Vmw65 appears to alter the specificity of OCT-1 so that it binds to the TAATGARAT motif (O'Hare *et al.*, 1988; Gerster and Roeder, 1988; Aphrys *et al.*, 1989). Mutational analysis of the Vmw65 protein led to the identification of eight amino acids within the N-terminal portion of the protein that are involved in its interaction with OCT-1 (Hayes and O'Hare, 1993), whilst the carboxy-terminal 80 residues function in IE gene transactivation (Ace *et al.*, 1988; Triezenberg *et al.*, 1988; Greaves and O'Hare, 1990). At least one other cellular protein, termed CFF, is also required for the formation of the infected cell complex (Katan *et al.*, 1990).

5. HSV-1 DNA Replication

Seven HSV genes: UL5, UL8, UL9, UL29, UL30, UL42 and UL52, are essential for viral DNA replication (reviewed by Challberg, 1991). These genes are all located in the U_L region of the genome. Circularisation of the HSV DNA takes place prior to the onset of viral DNA synthesis (Jean and Ben-Porat, 1976; Jacob *et al.*, 1979; Poffenberger and Roizman, 1985). The "a" sequences, which are present in the same orientation at both ends of the DNA molecule, permit circularisation. At early stages in infection, viral DNA replication occurs at discrete sites within the infected cell nucleus. At later stages, the nucleus becomes filled with replicating viral DNA (Rixon *et al.*, 1983). The initial sites of viral DNA replication have been designated replication compartments (Quinlan *et al.*, 1984; reviewed by Knipe, 1989). Following the onset of viral DNA replication, DNA molecules greater than one unit length start to appear (Jacob and Roizman, 1977; Hirsch *et al.*, 1977). At later stages in viral infection, large masses of DNA are observed (Jacob and Roizman, 1977; Ben-Porat and Rixon, 1979), consisting of multiple head to tail concatemers (Jacob *et al.*, 1979). According to these observations, these workers proposed that HSV DNA synthesis occurs by the rolling circle method of replication, which involves the continuous synthesis of one strand of DNA and discontinuous synthesis of the second strand.

The HSV genome has 3 origins of replication, one copy of Ori_L and two copies of Ori_S (Frenkel *et al.*, 1975; Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). Ori_L is located near the middle of the long unique region whilst a copy of Ori_S lies within each of the inverted repeat segments flanking the U_S region of the genome (Stow, 1982; Stow and McMonagle, 1983; Weller *et al.*, 1985; Quinn and McGeoch, 1985). The Ori_S and Ori_L are probably functionally equivalent on the basis of sequence similarity and complementation assays.

Most studies on the HSV origin sequences have been performed with Ori_S, since plasmids containing the Ori_L sequence are unstable in *E. coli.* (Weller *et al.*, 1985). The Ori_S sequence is divided into the core region, containing UL9 binding sites, and the surrounding sequences which enhance DNA replication (Elias *et al.*, 1986; Elias and Lehman, 1988; Olivo *et al.*, 1988; Weir *et al.*, 1989; Wong and Schaffer, 1991). The core region can be further divided into four domains, three of which are UL9 binding sites and the fourth an AT rich region between two of the sites. All three UL9 binding sites are necessary for optimum

DNA replication in transient assays (Lockshon and Galloway, 1988; Weir and Stow, 1990; Hernandez *et al.*, 1991). Two of the UL9 recognition sites (sites I and II) cooperatively bind the UL9 protein (Olivo *et al.*, 1988; Elias and Lehman, 1988; Weir *et al.*, 1989; Elias *et al.*, 1990; 1992). Binding of UL9 to sites I and II, results in the looping out and distortion of the DNA (Koff *et al.*, 1991; Fierer and Challberg, 1992).

The portion of the UL9 protein (apparent Mr 83,000), which binds to Ori_S has been located to the carboxyterminal third of the protein (McGeoch *et al.*, 1988a; Weir *et al.*, 1989; Deb and Deb, 1991). Recent data have shown that the DNA binding activity of UL9 is essential for replication (Arbuckle and Stow, 1993; Stow *et al.*, 1993). The UL9 protein also has helicase activity which is required for replication (Stow, 1992; Stow *et al.*, 1993). The UL9 helicase activity may be involved in the unwinding of the DNA prior to replication, although origin-specific unwinding by UL9 has not yet been shown.

Establishment of the replication fork is dependent on the products of the remaining six viral genes essential for viral DNA synthesis. The viral DNA polymerase exists as a heterodimer of the UL30 (Pol gene) and UL42 gene products. Comparative analysis of the catalytic activities of the pol/UL42 complex and the isolated pol protein suggested that the UL42 gene product increased the processivity of the enzyme (Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990). The importance of UL42 interaction with pol was demonstrated by the characterisation of a mutant polymerase, which was unable to bind to the UL42 protein. Although the pol retained enzymatic activity it could not synthesize long DNA products (Digard *et al.*, 1993; Tenney *et al.*, 1993; Stow, 1993). Therefore, it is likely that the UL42 product holds the pol enzyme and the DNA substrate in close proximity, preventing disassociation after each catalytic step.

The helicase and primase activities required for DNA replication have been located to a three subunit enzyme complex composed of the products of the UL5, UL8 and UL52 genes which encode proteins of 97kDa, 70kDa and 120kDa respectively (Crute *et al.*, 1989; Crute and Lehman, 1991). Expression of the UL5 and UL52 proteins in a baculovirus expression system resulted in a dimeric enzyme with similar helicase and primase activities to the native enzyme (Calder and Stow, 1990; Dodson and Lehman, 1991), suggesting that the UL8 product is not required for helicase or primase activity. Further studies suggested that helicase activity could be attributed to the UL5 gene product (Zhu

and Weller, 1992a,b). Thus, primase activity is probably mediated by the UL52 gene product. Recent studies suggest that UL8 may increase the efficiency of primer utilisation by stabilizing the primer-template complex and facilitating nuclear uptake of the helicase/primase complex (Calder *et al.*, 1992)

The UL29 gene encodes a protein of about 130kDa which is the major viral DNA binding protein (DBP) (Bayliss *et al.*, 1975; Purifoy and Powell, 1976; Purifoy *et al.*, 1977). It binds non-specifically to single stranded DNA with high affinity (Ruyechan and Weir, 1984). The protein stimulates the activity of the DNA polymerase and is required for the synthesis of long DNA strands (Ruyechan and Weir, 1984; Hernandez and Lehman, 1990). Further evidence suggested that the UL29 product may be neccessary for the structural organisation of nuclear replication compartments in which viral DNA synthesis occurs, and the correct localisation of pol and other replicative proteins into pre-replicative sites within the nucleus (de Bruyn Kops and Knipe, 1988: Gao and Knipe, 1989; Parris *et al.*, 1988). The DBP has also been shown to stimulate the activity of the DNA polymerase as well as the activity of the UL9 gene product (Hernandez and Lehman, 1990; Boehmer *et al.*, 1993; Boehmer and Lehman, 1993).

5.1 Enzymes Involved In Nucleotide Metabolism

a) Thymidine Kinase

The HSV-encoded pyrimidine deoxyribonuclease kinase enzyme, which is commonly referred to as thymidine kinase (TK), is not required for lytic infection in actively growing cells *in vitro* (Dubbs and Kit, 1964; Elion *et al.*, 1977; Fyfe *et al.*, 1978). However, *tk*$^-$ mutants were shown to grow less efficiently in serum starved cells (Jamieson *et al.*, 1974), suggesting that it may be necessary for virus replication in non-dividing cells. The observation that *tk*$^-$ virus has a low incidence of latent infection (Field and Wildy, 1978) led to the proposal that TK may be necessary for the establishment of latency *in vivo*. However, recent studies showed that latency-associated transcripts (LATs) could be detected in cells infected with *tk*$^-$ virus (Coen *et al.*, 1989; Efstathiou *et al.*, 1989), suggesting that *tk*$^-$ virus was capable of establishing a latent infection. Thus, it was concluded that TK may be required for the reactivation of virus from latency (Coen *et al.*, 1989; Efstathiou *et al.*, 1989).

b) Ribonucleotide Reductase

Ribonucleotide reductase (RR) catalyses the reduction of the four ribonucleotides to the corresponding deoxyribonucleotides, creating a pool of substrates for DNA synthesis. The enzyme is composed of a large (Mr 144,000) and a small (Mr 38,000) sub unit, the products of the UL39 and the UL40 genes respectively (Dutia, 1983; Bacchetti *et al.*, 1984; Preston *et al.*, 1984; Frame *et al.*, 1985). Analysis of the *ts*1207 mutant, which has a lesion in the large sub unit and fails to induce RR activity at the non-permissive temperature, suggested that RR may be required for viral growth and viral DNA synthesis (Dutia, 1983; Preston *et al.*, 1984). Subsequent data indicated that although RR has an essential role in DNA synthesis, it is not essential for virus growth in tissue culture at low temperatures. This suggests that the host cell RR can substitute for the viral enzyme at low temperatures (Goldstein and Weller, 1988a,b,c; Preston *et al.*, 1988).

c) Deoxyuridine 5' triphosphatase (dUTPase)

The viral dUTPase catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab and Francke, 1980), leading to a reduction in the intracellular concentration of dUTP, there by reducing the incorporation of uridine into DNA. The dUTPase was shown not to be essential for virus replication in exponentially growing or serum starved cells *in vitro*, presumably because the cellular counterpart is present in sufficient quantity (Fisher and Preston, 1986). Recent studies have indicated that the dUTPase may be important in neurovirulence and neuroinvasiveness as well as in virus reactivation from latency (Pyles *et al.*, 1992).

d) Uracil-DNA glycosylase

The HSV encoded uracil-DNA glycosylase is the product of the UL2 gene (Caradonna and Cheng, 1981; Caradonna *et al.*, 1987; Mullaney *et al.*, 1989). This enzyme acts to correct the insertion of dUTP and deamination of cytosine residues in DNA. The extremely high G+C content of HSV DNA makes this enzyme an important element of error correction in HSV DNA replication. Analysis of a UL2 deletion mutant showed that this enzyme was dispensable for growth in tissue culture (Mullaney *et al.*, 1989).

Section 1.C Virion Structure

1. The Core

Early observations suggested that packaged viral DNA was wound in a toroidal manner around a central core (Furlong *et al.*, 1972). However, more recent studies using cryoelectron microscopy and image reconstruction have shown that packaged viral DNA assumes a liquid crystalline state and forms a uniformly dense ball (Booy *et al.*, 1991). In this respect, HSV DNA packaging closely resembles that of the double stranded DNA bacteriophages T4 and λ (Lepault *et al.*, 1987). The toroid structure observed by Furlong *et al.* (1972) was probably an artefact of the fixing and drying procedures used in sample preparation (Puvion-Dutilleul *et al.*, 1987).

2. The Capsid

The HSV nucleocapsid is organized into 162 capsomers, of which 150 are hexons and 12 are pentons. The capsomers are organized in a T=16 icosahedral lattice with the pentons positioned at the vertices and the hexons forming the faces and edges (Wildy *et al.*, 1960; Schrag *et al.*, 1989). VP5, the major capsid protein (apparent Mr 155kDa) is thought to be the major component of both the hexons and pentons (Steven *et al.*, 1986; Newcomb *et al.*, 1993). Three capsid forms have been identified within the infected cell nucleus. The A (empty), B (intermediate) and C (full) capsids can be distinguished by their appearance in electron micrographs and by their protein content. The A capsids appear lack an internal structure and are composed of VP5, VP19c, VP23, VP24 and VP26 (Marsden *et al.*, 1978; Morse *et al.*, 1978; Rixon *et al.*, 1990; Davison *et al.*, 1992; McNabb and Courtney, 1992a). B capsids have internal components comprising VP21 and VP22a (Gibson and Roizman, 1972; Rixon *et al.*, 1988). C capsids have an identical protein content to A capsids but unlike A capsids, they contain the viral genome.

3. The Tegument

The HSV-1 tegument appears as an amorphous layer between the envelope and capsid in electron micrographs and is the most poorly characterized part of the virion. Proteins found in the virion are assigned to the tegument on the basis that they are not capsid or envelope proteins. Although over half of the proteins encoded by HSV-1 have been assigned to the tegument, little is known about their structural organization or indeed the role of these proteins in the viral lytic cycle. Some tegument proteins, in particular Vmw65 and the *vhs* protein, have been shown to influence the course of infection (see section 1B4.2 and 1B3.1 respectively). It was proposed that the vertices of the capsid serve as anchors for the tegument (Vernon *et al.*, 1982). Subsequent reports, however, indicate that condensation and organization of proteins into the tegument can occur in the absence of the capsid (McLauchlan and Rixon, 1992), suggesting that the viral factors responsible for the initiation of tegumentation are located within the tegument or envelope.

Alternatively, the viral factors required for the initiation of tegumentation may not be retained in the mature virion.

4. The Envelope

The HSV-1 envelope is a complex structure, derived from the host cell membrane, which is modified by the presence of viral proteins. To data, 9 viral glycoproteins have been identified in the virion envelope and five of these (gB, gD, gH, gK and gL) are necessary for virus entry into cultured cells (Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992; Hutchison *et al.*, 1992a,b; Roop *et al.*, 1993). Several putative transmembrane proteins, predicted to cross the membrane several times, have been identified from analysis of HSV-1 sequence data (McGeoch *et al.*, 1988b). These proteins are the products of the UL10, UL20 and UL43 genes respectively.

Section 1.D Virus Assembly

1. Capsid Morphogenesis

Capsid assembly takes place within the nuclear matrix of the infected cell (Morgan *et al.*, 1954; Bibor-Hardy *et al.*, 1982) and probably involves interactions between VP22a and the protease, resulting in the condensation of the capsid proteins to form B capsids. The transient association of VP22a with the capsid structure during assembly, led to the suggestion that this protein represents the herpesvirus equivalent of the bacteriophage scaffolding proteins (Casjens and King, 1975). The VP22a polypeptide has been shown to assemble into 60nm diameter structures, which could act as templates for the assembly of the capsid shell (Newcomb and Brown, 1991).

The protease and VP22a are encoded by UL26 and UL26.5 respectively (Preston *et al.*, 1983; Liu and Roizman, 1991a,b; Figure 4). The relationship between VP22a and the protease is unusual, in that they arise from two separate but overlapping ORFs. Thus, the protease is an N-terminal extension of VP22a. The UL26.5 protein product is cleaved at the carboxy terminal end by the protease to form VP22a, which is the major component of the core of B capsids (Liu and Roizman, 1991b; Preston *et al.*, 1992). The protease also undergoes self cleavage at the same site, and at a second site upstream of the UL26.5 ORF (Davison *et al.*, 1992; Liu and Roizman, 1993). Cleavage of the protease at both sites results in the formation of two polypeptides which have been identified as capsid components, the N-terminal portion of the protease corresponding to VP24 and the C-terminal portion to VP21 (Davison *et al.*, 1992). The proteolytic activity responsible for cleavage of the UL26.5 gene product and the protease itself has been assigned to VP24 (Liu and Roizman, 1992). The *ts*1201 mutant, which has a lesion in the N-terminal portion of UL26 affecting proteolytic activity (Preston *et al.*, 1983), has proved invaluable in elucidating the steps involved in capsid morphogenesis. At the non-permissive temperature, *ts*1201-infection results in the accumulation of large cored B capsids within the infected cell nucleus (Preston *et al.*, 1983). After downshift to the permissive temperature, proteolytic activity is restored and A, C and small cored B capsids are formed (Rixon *et al.*, 1988). These results suggest that cleavage of the UL26.5

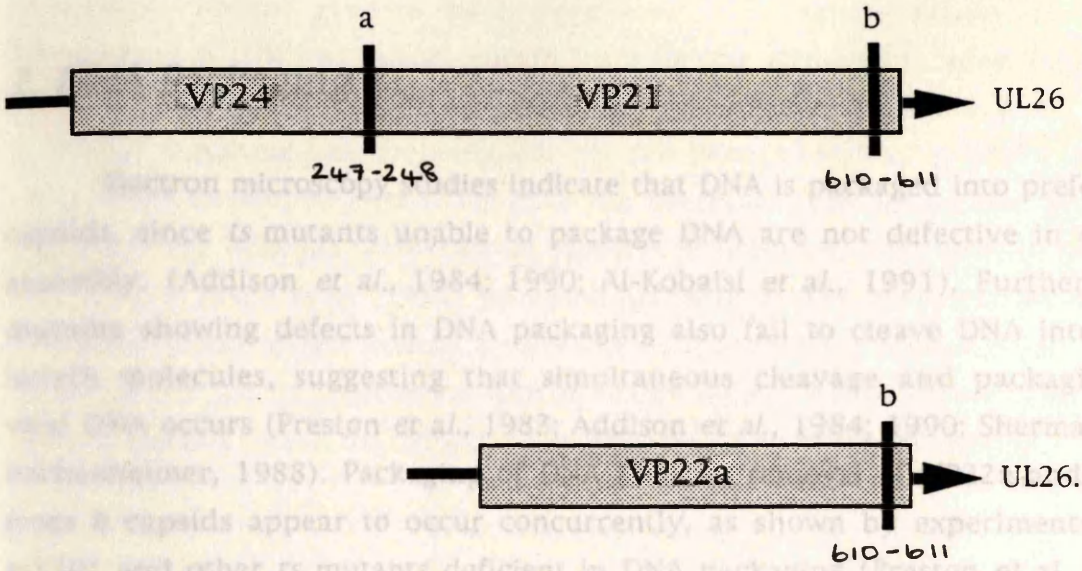


Figure 4. Organisation Of The HSV-1 Genes UL26 And UL26.5.

The 3' coterminal transcripts are shown by arrows, which are overlayed by open boxes indicating the position of the open reading frames specifying the UL26 and UL26.5 gene products. Proteolytic cleavage sites *a* and *b* are indicated by vertical lines.* Cleavage of the UL26.5 gene product at site *b* gives rise to VP22a, whilst cleavage of the protease (UL26) at site *a* and *b* releases the capsid proteins VP21 and VP24. Reproduced from Rixon (1993).

*The codon numbers corresponding to the proteolytic cleavage sites a and b are also indicated.

gene product is not required for the formation of intermediate capsids and that cleavage can occur after capsid assembly.

2. DNA Packaging

Electron microscopy studies indicate that DNA is packaged into preformed capsids, since *ts* mutants unable to package DNA are not defective in capsid assembly. (Addison *et al.*, 1984; 1990; Al-Kobaisi *et al.*, 1991). Furthermore, mutants showing defects in DNA packaging also fail to cleave DNA into unit length molecules, suggesting that simultaneous cleavage and packaging of viral DNA occurs (Preston *et al.*, 1983; Addison *et al.*, 1984; 1990; Sherman and Bachenheimer, 1988). Packaging of DNA and the removal of VP22a and VP21 from B capsids appear to occur concurrently, as shown by experiments with *ts*1201 and other *ts* mutants deficient in DNA packaging (Preston *et al.*, 1983; 1992; Gibson and Roizman, 1972). Empty A capsids are considered to be the end products of abortive DNA packaging. Replication of viral DNA results in the formation of high molecular weight concatemeric DNA which must be cleaved into unit length genomes prior to packaging. The signals required for DNA cleavage and packaging are located within the *a* sequence (Stow *et al.*, 1983; Varmuza and Smiley, 1985; Deiss *et al.*, 1986; Deiss and Frenkel, 1986).

Two viral gene products, Vmw273 and an unidentified viral protein, have been shown to interact with the *a* sequence, and functions in the DNA cleavage/packaging process have been suggested for these two proteins (Chou and Roizman, 1989). Previous data suggested a role for Vmw273 in the release of viral DNA from the capsid (Batterson *et al.*, 1983). The UL6 (Weller *et al.*, 1987), UL12 (Shao *et al.*, 1993), UL15 (Poon and Roizman, 1993), UL21 (deWind *et al.*, 1992), UL25 (Addison *et al.*, 1984), UL26 (Preston *et al.*, 1983), UL28 (Addison *et al.*, 1990; Tengelsen *et al.*, 1993), UL32 (Coen *et al.*, 1984); UL33 (Al-Kobaisi *et al.*, 1991) gene products have been shown to be essential for the assembly of full capsids, inasmuch as mutants in these genes form partially cored capsids which fail to package viral DNA

3. Tegumentation

The intracellular sites within which tegumentation occurs have not been identified. Recent reports have suggested that tegumentation of human herpesvirus-6 (HHV-6) occurs within intranuclear structures called tegusomes derived from cytoplasmic invaginations into the nucleus (Roffman *et al.*, 1990). A similar tegusome-like compartment has not been identified in HSV-1 infected cells. Since enveloped L-particles have not been observed within the inner and outer nuclear lamellae, as would be expected if tegumentation were to occur in the nucleus, Rixon *et al.* (1992) proposed that assembly of the tegument occured in the cytoplasm either around a capsid or as independent condensations. These observations are in accordance with previous reports by Stackpole (1969) who showed capsids free in the cytoplasm at different stages of tegumentation.

4. Envelopment and Egress

It is generally believed that initial envelopment of DNA-containing capsids occurs at modified patches of the inner lamellae of the nuclear membrane (Darlington and Moss, 1969; Nii *et al.*, 1968). The observation of naked capsids within the cytoplasm of infected cells led some investigators to propose that capsids undergo a series of sequential envelopments and de-envelopments as they proceed from the nucleus to the plasma membrane (Morgan *et al.*, 1954; Nii *etal.*, 1968; Cheung *et al.*, 1991; Whealy *et al.*, 1991). However, other authors argued that naked cytoplasmic capsids represent virions whose envelopes have fused with cytoplasmic membranes and are arrested in the process of egress (Campadelli-Fiume *et al.*, 1991).

Although, many investigators fail to agree on the exact sequence of events involved in virion envelopment and egress, they have provided unquestionable evidence implicating the Golgi apparatus and Golgi-associated vesicles in virion maturation and transport to the cell exterior. Disruption of the host cell secretory apparatus with monensin or Brefeldin A prevents viral glycoprotein maturation, export to cytoplasmic membranes and virion egress (Johnson and Spear, 1982; Cheung *et al.*, 1991; Whealy *et al.*, 1991). Furthermore, virus maturation and egress were shown to be greatly impaired in cells which have defective Golgi glycosyltransferases (Campadelli-Fiume, 1982; Serafini-

Cessi, 1983). The possibility that the egress of virions is directed, and mediated in part by viral proteins was recently suggested by Baines *et al.* (1991). These workers identified an HSV-1 putative membrane protein, the UL20 gene product, which is required for the efficient transport of virions from between the inner and outer lamellae into the extracellular space.

Section 1.E Alternative Products of HSV-1 Infection

During Ficoll gradient centrifugation of HSV-1, two distinct bands were observed. The lower band comprised HSV-1 virions, whilst the upper, more diffuse band consisted of previously uncharacterized HSV-1 particles termed light particles (L-particles) (Szilaygi and Cunningham, 1991). L-particles were later shown to lack capsids and viral DNA, consisting predominantly of tegument and envelope proteins. Further studies revealed that certain proteins which do not exist in virions are present in L-particles. In particular, L-particles contain at least three phosphoproteins, including the IE protein Vmw175, that are not detectable in virions (Szilaygi and Cunningham, 1991; McLauchlan and Rixon, 1992). Since L-particles contain proteins which are absent from virions, it is unlikely that they are the result of virions losing their capsids during sample preparation.

To determine whether L-particles and virions share similar pathways of assembly, workers analysed the HSV-1 mutant *ts*1201 which at the NPT fails to produce virions and its capsids are restricted to the nucleus (Preston *et al.*, 1983). Since *ts*1201 was able to produce L-particles at the NPT in quantities similar to those produced by *wt* virus (Rixon *et al.*, 1992), these studies illustrated that L-particle formation is independent of virus assembly. Thus, the factors involved in the assembly of the tegument and acquisition of the envelope are likely to be components of L-particles.

The role of L-particles in viral infectivity is unclear. Some authors have suggested that L-particles provide additional proteins which may facilitate infection. Indeed, recent studies have shown that L-particles are as effective as virions at supplying functional Vmw65 and *vhs* protein into cells (McLauchlan *et al.*, 1992).

2. Materials and Methods

Section 2.A Materials

1. Viruses

The HSV-1 *wt* virus used in this study was strain 17*syn*$^+$ which has a non-syncytial plaque morphology (Brown *et al.*, 1973). Two HSV-1 temperature sensitive (*ts*) mutants of strain 17*syn*$^+$, 17*ts*1204 and 17*ts*1213 were characterised. *Ts*1204 and *ts*1213 were each isolated from an experiment in which a UV-mutagenized HSV-1 DNA fragment was recombined into *wt* HSV-1 DNA. Since the *ts*1204 lesion did not map in the region of the genome corresponding to the mutagenized fragment it was considered to have arisen spontaneously (Matz *et al.*,1983, V.G.Preston unpublished results). The *ts* mutation in *ts*1213, on the other hand, mapped within the same region of the genome that was mutagenized.

2. Tissue Culture Cells

Baby hamster kidney (BHK) 21 clone 13 cells, a fibroblastic line established by MacPherson and Stoker (1962), and low passage HFL cells (Flow 2002) were used throughout this study. Cell lines were obtained from Dr V.G. Preston.

3. Tissue Culture Media

BHK cells were grown in 1x Glasgow modified Eagle's medium (Busby *et al.*, 1964) (supplied as a 10x concentrate by Gibco Ltd.), supplemented with 100 units/ml penicillin, 100µg/ml streptomycin, 10% new born calf serum, and 10% tryptose phosphate. HFL cells were cultured in the same medium except that 10% foetal calf serum and 1% non-essential amino acids were used instead of calf serum and tryptose phosphate. The following modified media supplemented with antibiotics were also used:

Eagles medium containing n% new born calf serum	-ECn
Eagles medium containing n% foetal calf serum	-EFn
Eagles medium containing n% human serum	-EHn

4. Bacteria

Escherichia coli strain DH5α(F^-, ϕ 80d lacZ M15, recA1, endA1, gyrA96, thi-1, hsdr17 (r^-_k, m^+_k), supE44, relA1, deoR, (lacZYA-argF) U169 was used for propagating plasmids.

5. Plasmids

The multifunctional phagemid, pT7T3 18U, was purchased from Pharmacia P-L Biochemicals, Milton Keynes, UK.

6. Chemicals

Chemicals were obtained from the following sources: BDH chemicals, Poole, England; Bio-Rad Laboratories, California, USA; Pharmacia Fine Chemicals, Uppsala, Sweden; and Sigma Chemical Co. Ltd., Dorset, UK. Reagents for electron microscopy were obtained from Agar Aids, Stanstead, Essex and Taab Laboratories, Emmer Green, Reading. Analytical grade reagents were used wherever possible.

7. Enzymes

Restriction and DNA modifying enzymes were supplied by Boehringer Mannheim Ltd, Germany. Lysozyme, DNase, and RNase were purchased from Sigma Chemical Co. Ltd.

8. Oligonucleotides

EcoRI oligonucleotide linkers, $pd(N)_6$ random primers, oligo dT_{12-18}, M13 universal and reverse sequencing primers were all supplied by Pharmacia P-L Biochemicals, Milton Keynes, UK.

9. Antibodies

A polyclonal antiserum, specific to the UL25 gene product, was previously raised in a rabbit to an oligopeptide of 15 amino acid residues (David Smith unpublished results). The oligopeptide represented the region on the UL25 protein from amino acid residues 135 to 150. A polyclonal rabbit oligopeptide antiserum, specific for the major DBP, was supplied by Dr Nigel Stow. TBS was generously supplied by Dr J. Macnab within the Institute. Isothiocyanate-conjugated goat anti-rabbit immunoglobulin was purchased from Nordic Immunological Laboratories Ltd, Berkshire, UK.

10. Immunological Reagents

A 10% w/v suspension of formalin fixed *Staphylococcus aureus* was provided by Dr V.G.Preston.

11. Radiochemicals

All radiochemicals were obtained from Amersham International Plc.

12. Miscellaneous

The rat aspartate amino-transferase cDNA (MasPAT) was kindly supplied by Dr J. Macnab from within the Institute. Hybond-Map Messenger Affinity Paper and cDNA Synthesis System Plus were supplied by Amersham International Plc, Buckinghamshire UK. Nuclease treated rabbit reticulocyte lysate and the TNT T7 Coupled Reticulocyte Lysate System were supplied by Promega. Photographic film was obtained from Kodak Ltd., London, England. Plastic petri-dishes and 96-well microtitre plates were purchased from Nunclon Ltd. Plastic 850cm^2 roller bottles used in cell culture were supplied by Becton Dickinson Ltd. Genescreen Plus nylon membrane was obtained from DuPont, Boston USA.

13. Standard solutions

Tissue Culture Reagents and Buffers.

Phosphate buffered saline (PBS)	170mM NaCl, 3.4mM KCl, 10mM Na_2 HPO_4 and 2mM KH_2 PO_4 pH 7.2 (Dulbecco and Vogt, 1954)
Tris-saline	140mM NaCl, 30mM KCl, 28mM Na_2 HPO_4 , 1mg/ml glucose, 100 units/ml penicillin, 100µg/ml streptomycin, 25mM Tris-HCl pH 7.4.
Trypsin	0.25% (w/v) trypsin (Difco) in tris-saline, containing 0.002% (w/v) phenol red.

Versene	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red.
DNA Gel Electrophoresis Buffers	
Formyl dye	0.1% (w/v) Bromophenol blue, 0.1% (w/v) Xylene-cyanol, 20mM Na_2 EDTA in deionized formamide.
Alkaline loading buffer (6X)	300mM NaOH, 6mM EDTA, 18% (w/v) Ficoll, 0.15% (w/v) bromophenol blue and 0.25% xylene-cyanol.
Alkaline electrophoresis buffer	1mM EDTA pH 8.0, 50mM NaOH.
TAE buffer	40mM Tris-acetate, 1mM EDTA pH 8.0
Sequencing tank buffer (10X)	890mM Tris-borate, 890mM boric acid, 20mM EDTA pH 8.3
TE buffer	10mM Tris-HCl pH7.4, 0.1mM EDTA.
SDS-PAGE Solutions	
Destain	5% methanol, 7% acetic acid.
Fix	50% methanol, 7% acetic acid.
Resolving gel buffer(4X)	1500mM Tris-HCl pH 8.9, 0.4% (w/v) SDS.
Stacking gel buffer (4X)	490mM Tris-HCl pH 6.7, 0.4% (w/v) SDS
Boiling mix	2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 50mM Tris-HCl pH 6.7, 0.004% (w/v) bromophenol blue.
Tank buffer	53mM Tris, 53mM glycine, 0.1% (w/v) SDS.

Hybridisation Buffers

Denhardt's buffer (5X)	0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrollidone, 0.1% (w/v) BSA.
0.8M Sodium phosphate buffer (pre-hybridisation buffer)	0.7M Na_2HPO_4 ,0.15M $NaH_2PO_4.2H_2O$ (pH7.4).
SSC buffer (10X)	3M NaCl, 300mM tri-sodium citrate, adjusted to pH 7.5 with 300mM citric acid
RNA:DNA hybridisation buffer	6X SSC, 2X Denhardt's buffer, 0.1% (w/v) SDS.
Neutralising Buffer	1.5M NaCl, 1M Tris-HCl pH 7.5
Denaturing buffer	1.5M NaCl, 0.5M NaOH.

Immunoprecipitation Buffers

RIPA buffer	0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) NP40, 1mM EDTA, 150mM NaCl, 10mM Tris-HCL pH 7.4.
Wash buffer	600mM LiCl, 100mM Tris-HCl pH 7.4, 1% (v/v) β-mercaptoethanol.

Bacterial Growth and Cloning Solutions

L-broth	170mM NaCl, 5g/l yeast extract, 10g/l Difco Bactotryptone, supplemented with 25-50μg/ml of the appropriate antibiotic.
Ligation buffer (10X)	200mM Tris-HCl pH 7.5, 100mM $MgCl_2$ 100mM dithiotheritol (DTT) and 5mM ATP.

STET buffer	8% (w/v) sucrose, 5% (v/v) NP40, 50mM EDTA, 50mM Tris-HCl pH 8.0.
SOB	2% Difco Bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 10mM $MgSO_4$. SOB was sterilized by filtration.
SOC	SOB containing 20mM glucose, sterilized by filtration
TFB	10mM K-MES, pH 6.2, 100mM RbCl, 45mM $MnCl_2$, 10mM $CaCl_2$ and 3mM HACO.Cl_3. and sterilized by filtration.
DTT buffer	2.25M in 40mM potassium acetate pH6.0. The solution was sterilized by filtration

RNA Purification Buffers

Lysis buffer	0.2M Tris-HCl pH 8.5, 0.14M NaCl, 2mM $MgCl_2$, 5% NP40.
TSE buffer	10mM Tris-HCl pH 7.4, 1mM EDTA and 0.5% (w/v) SDS.

Miscellaneous

Elution buffer	50mM Tris-HCl pH 7.5, 100mM NaCl and 0.5% (w/v) SDS.
Sequencing gel top mix	150ml 40% polyacrylamide:N,N'-methylene bis acrylamide (20:1), 540g urea dissolved in water and made up to 1litre with 50ml 10X TBE. For 100ml of , gel mix 160µl 25% ammonium persulphate and 160µl TEMED were added.

Oligonucleotide labelling buffer (5X)	250mM Tris-HCl pH 8.0, 25mM $MgCl_2$, 5mM β-mercaptoethanol, 2mM each of dATP, dGTP and dTTP, 1M HEPES pH 6.6 and $pd(N)_6$ random primers (1mg/ml).

Section 2.B Methods

1. Cell Culture

1.1 Growth Of Cells

BHK cells were grown at 37^0 in rotating plastic 850cm^2 culture bottles containing 200ml ETC10 in an atmosphere of 5% CO_2, 95% air. A confluent monolayer of approximately 3 x 10^8 cells was sufficient to seed five 850cm^2 bottles. Before harvesting the cell monolayers were washed once with 20ml versene followed by 40ml trypsin:versene (1:1 v/v). The detached cells were resuspended in tissue culture medium.

HFL cells were grown in a similar manner to BHK cells using EF10 as culture medium. Non-essential amino acids (1% v/v) were added to EF10 every second cell passage. A confluent monolayer of 1x10^8 cells was sufficient to seed four 850cm^2 culture bottles.

Both BHK and HFL cells were seeded onto 90mm, 50mm, and 35mm petri dishes at densities of 1.5x10^7, 2x10^6, and 1x10^6 cells per dish respectively, to obtain 75% confluent monolayers in 24 hrs.

1.2 Cell Storage

All cell lines were stored at -140^0. Cells were harvested as previously described, pelleted and resuspended in tissue culture medium containing 10% (v/v) DMSO, at a concentration of 10^7 cells/ml storage medium. Cells, stored in vials, were frozen slowly to -140^0.

Cells were recovered by thawing the contents of the vial rapidly, washing the cells in culture medium to remove the DMSO and finally resuspending the cells in fresh culture medium.

2. Virus Culture And Purification

2.1 Production Of Virus Stocks.

A 90% confluent monolayer of BHK cells in a 850cm^2 plastic bottle was infected with virus at a moi of 0.003 pfu/cell in 20mls of EC10 and incubated for 2-4 days at 31^0 until the cells exhibited obvious CPE. The infected cells were pelleted by centrifugation at 1500 rpm at 4^0 for 10 min. Cell-associated virus was prepared by sonicating the cell pellet until it was homogenous. Debris was removed by centrifugation at 1500rpm at 4^0 for 10 min. The cell associated virus stock was divided into aliquots and frozen at -70^0.

The clarified culture medium was centrifuged in a Sorvall GSA rotor at 12000rpm at 4^0 for 2 hrs to pellet cell-released virus. The cell-released virus was resuspended in 2ml EC10 by sonication, divided into aliquots and stored at -70^0.

2.2 Virus Sterility

The sterility of virus stocks was checked by streaking a sample onto blood agar plates and incubating the plates at 31^0 for 3-5 days. Any virus stocks containing bacteria which grew on blood agar were discarded.

2.3 Titration Of Virus Stocks

Monolayers of BHK cells, seeded with 2x10^6 cells per 50mm petri dish, were infected in duplicate with serial 10-fold dilutions of virus in 100µl PBS, 4% calf serum. Titrations of the *ts* mutants used in this study were carried out at 31^0, the permissive temperature (PT), and 38.5^0, the non-permissive temperature (NPT). After 1 hr incubation at the required temperature, the plates were overlaid with 4ml EH5 to prevent secondary plaque formation. Infected monolayers were incubated for 3 days at 31^0 or 2 days at 38.5^0 and then stained with Giemsa stain. The plates were washed with water after 30 min and the plaques counted using a dissection microscope.

2.4 Preparation Of Purified Radiolabelled Virions And L- Particles.

Confluent BHK monolayers in 850cm^2 roller bottles were infected with a moi of 0.002 pfu of virus per cell in 20ml EC10. At 7 hrs post-infection at 31^0 the culture medium was removed and replaced with EC1 containing 1/5 the normal concentration of methionine and the cells incubated for a further 2 hrs. [^{35}S]-methionine (500µCi) was added to each roller bottle and incubation at 31^0 was continued overnight. A further 500µCi/ml of [^{35}S]-methionine was added to each roller bottle and the incubation continued for another 24 hrs.

The infected cells were pelleted at 1500 rpm at 4^0 for 10 min and the supernatant centrifuged at 12000 rpm at 4^0 for 2 hrs in a Sorvall GSA rotor to concentrate cell-released virus particles. The pelleted virus was resuspended in EC5 without phenol-red.

Virus and L-particles were purified by centrifugation through a 5-15% Ficoll 400 gradient as described by Szilagyi and Cunningham (1991). Gradients (35ml) were prepared in EC1 without phenol-red in cellulose nitrate centrifugation tubes. The crude virus preparation was layered gently onto the gradient surface and spun at 12000rpm at 15^0 for 2 hrs in a Sorval OTD-50 Ultracentrifuge using an AH629 rotor. Following centrifugation, the virion band (lower) and the L-particle band (upper) were visualized by shining a high intensity light from above and removed by insertion of a wide bore needle through the wall of the tube. The virion and L-particles were pelleted in EC1 without phenol-red at 18000 rpm at 4^0 for 1 hr in a TST41 rotor. The virus and L-particle pellets were resuspended in EC1 without phenol-red, containing 10mM Hepes pH7.4 and stored at -70^0.

2.5 Virus Particle Counts

A 5ul aliquot of a virus particle preparation was mixed with equal volumes of a latex bead suspension (of known concentration) and phosphotungstic acid. A sample was spotted onto a parlodium coated copper grid, air dried and visualised under an electron microscope. The number of virus particles relative to latex beads were determined from at least 10 fields of the grid and the number of virus particles per ml calculated.

3. Handling ts Mutant Infections

Infections with *ts* mutants used in this study were carried out at 31^0 (PT) and 38.5^0/ 39.7^0 (NPT). Cell monolayers were placed at the required temperature for at least 20 min prior to infection. All media for cell monolayer manipulations were prewarmed to the required temperature before use. Manipulations involving virus-infected cells were carried out as rapidly as possible, and incubator temperatures monitored regularly.

4. Polyethylene Glycol Treatment Of Virus-Infected Cells

Polyethylene glycol (PEG) treatment of virus-infected cells was performed essentially as previously described by Sarmiento *et al.* (1979) with modifications described by Addison *et al.* (1984). Briefly, HFL cells seeded sparsely on 13mm coverslips were infected with 5 pfu of virus per cell at the required temperature. After 1 hr adsorbtion, unbound virus was removed by washing the monolayer once with PBS. The virus-infected cells were treated with PBS containing 50% (w/v) PEG 6000 for 1 min. Further washes with EC$_0$ containing decreasing amounts of PEG were carried out as described by Sarmiento *et al.*, (1979). Following this treatment the cells were incubated for a further 4 hrs in growth medium before being fixed in methanol:acetone (3:1 v/v) for immunofluorescence assay.

5. Detergent Extraction Of Virion Particles

NP-40, at a final concentration of 1%, was added to purified λvirus resuspended in EC1 without phenol red. The sample was incubated on ice for 30 min then spun in a microfuge at 4^0 for 10 min. The supernatant and the pelleted fractions were made up to equal volumes in Eagles medium without phenol-red, then mixed thoroughly with an equal volume of 2x RIPA buffer. The fractions were sonicated and stored at -70^0.

6. Analysis Of Virus-Induced Polypeptides

6.1 Preparation Of Radiolabelled Virus-Infected Cell Extracts.

Confluent BHK or HFL monolayers were infected with virus at a moi of 20 pfu per cell at the required temperature. After 1 hr incubation to allow the virus to adsorb to the cells, the monolayers were overlaid with growth medium until the appropriate labelling time.

6.2 Preparation Of Radiolabelled Early Virus-Infected Cell Extracts

Virus-infected cell polypeptides, synthesized in the absence of viral DNA replication, were prepared by treating virus-infected cells with phosphonoacetic acid (PAA). Cells were infected with virus at a moi of 20 pfu per cell. After 1 hr the monolayers were overlaid with growth medium until the appropriate labelling time. Virus absorbtion and the subsequent incubation steps were carried out in the continuous presence of PAA.

6.3 Pulse Labelling Virus-Infected Cell Polypeptides With [^{35}S]-Methionine.

At the required labelling time, growth medium was removed from the cell monolayers, cells were washed twice with PBS and incubated in PBS containing 100uCi/ml [^{35}S]-methionine. After 15 min or 60 min, the cells were washed 3x in PBS and harvested immediately in the appropriate lysis buffer or incubated further in tissue culture medium.

6.4 Harvesting Virus-Infected Cell Extracts.

Virus-infected cell extracts were harvested in 500µl of lysis buffer per 35mm dish and the samples boiled for 5 min immediately prior to SDS-polyacrylamide gel electrophoresis. Extracts for immunoprecipitation

analysis were prepared as described in section 2B7.1. All radiolabelled infected cell extracts were stored at -20^0 prior to analysis.

7. Immunological Analysis Of Viral Polypeptides

7.1 Immunoprecipitation

Radiolabelled cell monolayers were harvested into 500µl of RIPA buffer, transferred to glass vials and sonicated until homogenous. The extracts were centrifuged in 2ml Beckman TLA.100 tubes at 50000 rpm for 30 min at 4^0 in a Beckman TL-100 centrifuge to remove cell debris and protein aggregates. The supernatant was stored on ice prior to analysis. The immunoprecipitation procedure was based on a method described by Kessler (1975). A volume of labelled cell extract ranging from 5x10^5 to 1x10^6 cpm, was incubated with the required dilution of rabbit polyclonal antibody or mouse monoclonal antibody for 1 hr at 4^0. Rabbit polyclonal antiserum was spun at 50000 rpm at 4^0 for 30 min prior to addition to the labelled cell extracts. Typically, 50µl of polyclonal sera, diluted 1 in 10, was added to 200µl of labelled cell extract. A volume of 100µl of prewashed *Staphylococcus aureus* (10% w/v) was added to the tubes to bind the immune complexes and the samples incubated on ice for 1 hr. The bacteria were washed 3x for 5 min in 700µl lithium chloride wash buffer. The pelleted bacteria were resuspended in 40µl of boiling mix, heated to 100^0 for 5 min and centrifuged for 5 min in a microfuge. The immunoprecipitated proteins in the supernatant were analyzed by SDS-PAGE and visualized by fluorography.

7.2 Immunofluorescence

HFL cells on 13mm coverslips were infected with a moi of 5 pfu of virus per cell. At the required time post-infection the coverslips were removed from the growth medium and fixed for 10 min in a solution of methanol: acetone (3:1) at -20^0. The coverslips were washed 3x in PBS before use and not allowed to dry out at any stage of the assay. Virus-infected cells were

incubated with 50µl of monoclonal antibody (diluted 1 in 30) for 30 min at RT. Cells were washed 6x in PBS, then treated with 50µl fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse immunoglobulin (1/40 dilution), and incubated for a further 30 min at RT. The coverslips were finally washed 6x in PBS and mounted in 50% glycerol in PBS on glass slides. Fluorescence was visualised under a Nikon Microphot-SA microscope.

8. SDS-Polyacrylamide Gel Electrophoresis

A stock of 30% polyacrylamide, containing a ratio of acrylamide to cross-linking agent (N,N'-methylene bis-acrylamide or N,N'-diallyltartardiamide) of 40:1, filtered through Whatman no1 filter paper, was used for analysis of proteins. A final concentration of 9% polyacrylamide in resolving gel buffer was prepared. Polymerisation was achieved by the addition of ammonium persulphate (APS) (0.006% w/v) and TEMED (0.004% v/v) to the gel solution just before pouring. The gel was overlayed with resolving gel buffer, to ensure a smooth interface upon polymerisation. A stacking gel, containing 5% polyacrylamide in stacking gel buffer, was prepared shortly before sample loading. Wells were formed with Teflon combs. Protein samples were denatured by boiling for 5 min in boiling mix and centrifuged for 5 min before loading. Denatured proteins were separated by electrophoresis at either 0.45mA/cm^2 for 3-4 hrs or 0.09mA/cm^2 for 18 hrs in freshly prepared electrophoresis buffer.

9. Fluorography

Gels were washed three times in a 1:1 mixture of fix and destain for 20 min each wash then dried immediately or soaked in En^{3}hance (New England Nuclear, Boston. USA) for 1 hr at RT. Gels, soaked in En^{3}hance, were rinsed thoroughly in deionized water for 5 min. Finally, gels were dried under vacuum at 80^0 onto sheets of Whatman 3mm paper and placed in contact with Kodak X-omat XS-1 film in conjunction with an intensifying screen at -70^0.

10. Preparation Of Radiolabelled DNA Probes.

10.1 Synthesis Of Radiolabelled Probes By Primer Extension.

DNA (10ng-200ng) in a volume of 20µl was boiled for 10 min, cooled to RT, then mixed with 1x oligo labelling buffer, BSA (0.4mg/ml), [α-^{32}P]-dCTP (50µCi) and 6 units of Klenow polymerase (LFP-labelling grade) in a final volume of 50µl. The reaction was incubated overnight at RT, boiled for 5 min, then chilled on ice immediately before use. Since 90% of the labelled dNTP is usually incorporated into DNA during the incubation step, the labelled DNA probe was not purified before use.

10.2 Synthesis Of cDNA Probes Complementary To PolyA$^+$ RNA Using Oligo dT$_{12-18}$ As Primer.

PolyA$^+$ RNA (1µg) in RNAse free water was heated to 70^0 for 5 min, then rapidly cooled on ice. The chilled polyA$^+$ RNA was added to a reaction mix containing a final concentration of 0.5u/µl of RNAsin (Promega), 50ng oligo dT$_{12-18}$ primer, 20mM dGTP, dATP and dTTP, 120µM dCTP, 50µCi/ml [^{32}P]-dCTP, 200 units of moloney murine leukaemia virus reverse transcriptase (Pharmacia) in 1x reverse transcription buffer in a volume of 25µl. The reaction was incubated for 1 hr at 37^0 then stopped by the addition of EDTA pH8.0 to a final concentration of 20mM. The radiolabelled probe was separated from the unincorporated dNTPs using Geneclean II described later in section 2B13.4. The radiolabelled cDNA was used without denaturation in hybridisations.

10.3 [^{32}P]-Labelling Of DNA Termini

Plasmid DNA was digested with the desired restriction enzyme in the appropriate buffer. Three of the four dNTPs were added at final concentrations of 80µM, together with 2µCi of the fourth [α-^{32}P]-dNTP and 2 units of T4 DNA polymerase. After incubation for 15 min at RT, the sample was heated to 70^0 for 5 min to terminate the reaction. The end labelled DNA

fragments were separated by DNA gel electrophoresis and the fragments identified by autoradiography.

11. Construction And Screening Of A cDNA Library

11.1 Isolation Of Total Virus-Infected Cell Cytoplasmic RNA.

Total cytoplasmic virus-infected cell RNA was isolated essentially as described previously by Preston *et al.* (1979). HFL cell monolayers in 90mm petri-dishes were infected with virus at a moi of 20 pfu per cell. After 1 hr virus absorbtion, the cells were overlaid with EF10 and incubated at the required temperature for a further 6 hrs. The plates were placed immediately on ice and washed twice with ice cold PBS. The cells were scraped from the plates with rubber policemen and pelleted by centrifugation at 1500rpm at 4^0 for 10 min. Five volumes of cell lysis buffer was added to the pelleted cells and the sample drawn back and forth 4x through a 10ml plastic syringe to disrupt the pellet. The resulting cell lysate was centrifuged at 2500rpm at 4^0 for 10 min and the supernatant added to 5ml TSE, 15ml phenol:chloroform (1:1) and 10ml RNAse free water. The suspension was gently shaken intermittently for 10 min at RT, then centrifuged at 2500rpm at RT for 10 min. The aqueous phase was retained and extracted twice with an equal volume of phenol:chloroform (1:1). Finally, the cytoplasmic RNA was extracted with chloroform and precipitated overnight at -20^0 in the presence of 0.1M NaCl and 2 volumes of ethanol. RNA was pelleted, washed with 70% ethanol and dried before being resuspended in RNAse free water containing RNAsin (Promega) at a final concentration of 0.5units/µl. Total cytoplasmic RNA was stored in aliquots at -70^0. The integrity of the RNA was assessed by translation of the RNA *in vitro* and by analysis of the products of first strand cDNA synthesis described in sections 2B11.3 and 2B11.4 respectively.

11.2 Selection Of PolyA+ RNA From Total Cytoplasmic RNA

PolyA+ RNA was purified from total cytoplasmic RNA using Hybond-mAP-Messenger Affinity Paper (Amersham), according to the protocol supplied which was based on the method described by Wreschner and Herzberg (1984). Briefly, the total RNA preparation was spotted onto an appropriately sized piece of paper and allowed to air dry. The dried paper was washed twice in a solution containing 0.1M NaCl to remove polyA- RNA, followed by a brief wash in 70% ethanol to remove the salt. The paper bound PolyA+ RNA was eluted into RNAse free water by heating to 70^0 for 5 min. RNAsin (Promega) was added to the eluted PolyA+ RNA to a final concentration of 0.5 units/µl, and the RNA was aliquoted and stored at -70^0. Prior to use, all wash solutions were incubated in 0.1% (v/v) DEPC at RT for 12 hrs and then autoclaved. The integrity of the polyA+ RNA was assessed as for total RNA.

11.3 Translation Of PolyA+ RNA In Vitro

The nuclease treated rabbit reticulocyte lysate *in vitro* translation system supplied by Promega was used to translate polyA+ RNA. Up to 5µg of PolyA+ RNA was added to a reaction mixture containing 70% nuclease treated rabbit reticulocyte lysate, 0.02mM of each amino-acid except methionine and 0.8mCi/ml [^{35}S] -methionine in a volume of 25µl. The sample was incubated at 30^0 for 1 hr. The reaction was terminated by the addition of an equal volume of boiling mix and the translation products analysed by SDS-PAGE.

11.4 First Strand cDNA Synthesis

First strand cDNA synthesis, using polyA+ RNA as template and oligo dT_{12-18} as primer, was carried out as described in section 2B10.2. Single stranded DNA samples in 0.2 volumes of formyl dye were analyzed by electrophoresis through a 0.7% alkaline agarose gel.

11.5 Double Stranded cDNA Synthesis

Double stranded cDNA was synthesized from 3μg of polyA$^+$ RNA using the cDNA Synthesis System Plus (Amersham), according to the protocol supplied. A summary of the steps involved in cDNA synthesis are shown in Figure 5. Briefly, 5μg of mRNA was added to a first strand synthesis reaction mix containing oligo dT_{12-18} and 20 units of reverse transcriptase in a volume of 50μl. The reaction was incubated at 42^0 for 1 hr and then chilled on ice. Second strand reaction buffer containing 4 units of *E. coli* ribonuclease H and 115 units of *E. coli* DNA polymerase was added to the chilled sample to a final volume of 250μl and the sample was incubated sequentially at 12^0 for 60 min and 22^0 for 60 min. *E. coli* RNAse H was used to nick the RNA in the RNA:DNA hybrid. The *E. coli* DNA polymerase utilises the nicked RNA as a primer in the synthesis of the second DNA strand. The reaction was terminated by heating the sample to 70^0 for 10 min and then chilled on ice. T4 DNA polymerase (2 units per microgram of original mRNA template) was added to the chilled sample and incubated at 37^0 for 10 min to remove any small remaining 3' overhangs from the first strand cDNA. Finally, EDTA pH8.0 was added to the sample to a final concentration of 10mM to terminate the reaction. The double stranded cDNA was purified through a small Sephadex G50 column.

The success of the first and second cDNA strand synthesis reactions was monitored by incorporating 5μCi of α-[^{32}P]-dCTP into the first and second cDNA synthesis steps. Once each incubation step was complete, small aliquots of the reactions were removed to assess the efficiency of cDNA synthesis in terms of yields of cDNA synthesised and the size of cDNA molecules obtained. The yields of cDNA were determined by calculating the percentage of [^{32}P]-dCTP incorporation into DNA and the size of cDNA molecules obtained estimated by alkaline agarose gel electrophoresis.

11.6 Ligation Of Synthetic EcoRI Linkers To cDNA

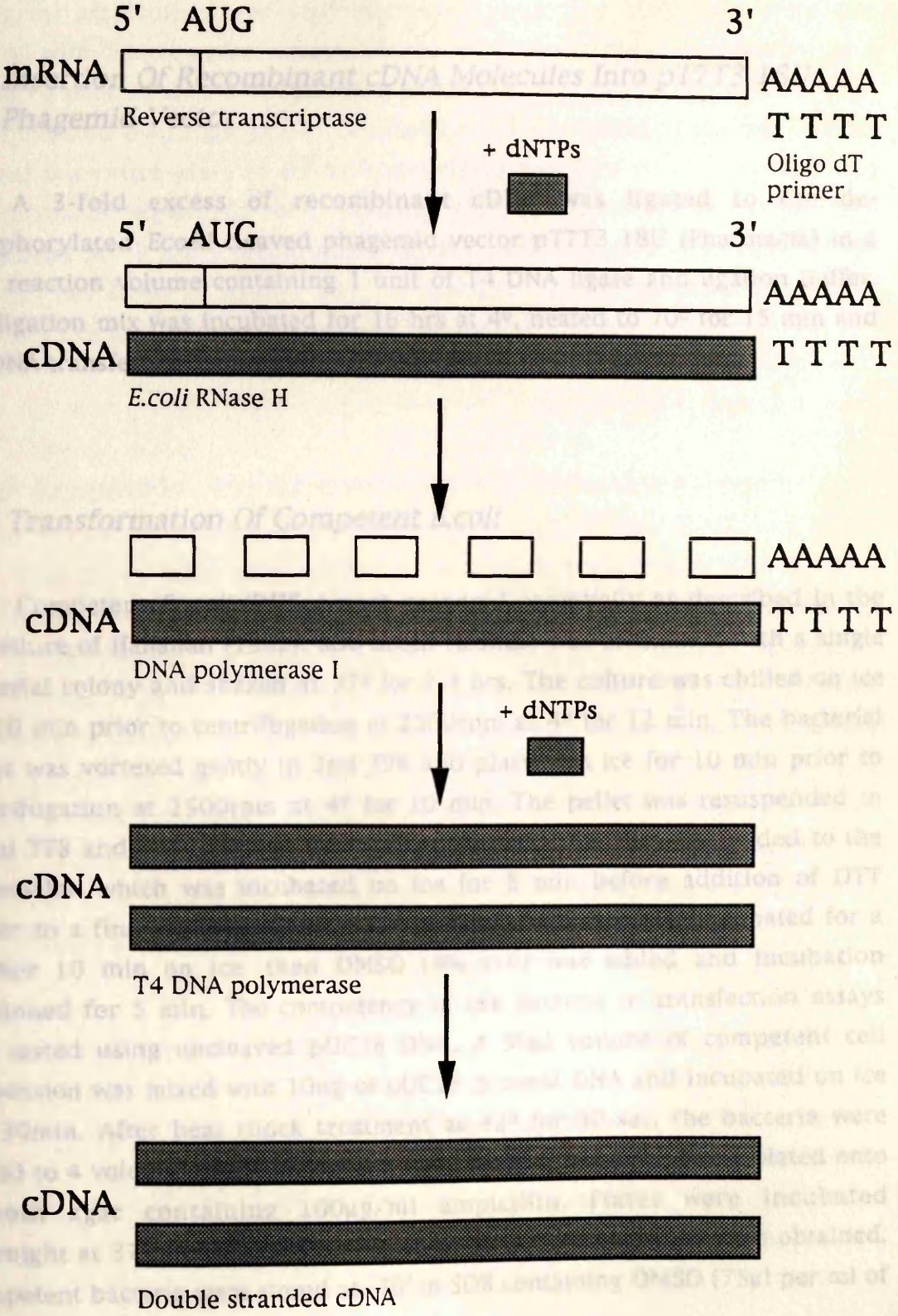
Double stranded cDNA was dissolved in a minimal volume of water. A 100x molar excess of *EcoRI* synthetic phosphorylated linkers (Pharmacia) relative to cDNA was incubated with the cDNA overnight at 14^0 in a 20μl reaction containing 1 unit of T4 DNA ligase in ligation buffer. The ligation reaction was heated to 70^0 for 15 min to denature the ligase, cooled to 37^0 and

Figure 5. Diagrammatic Representation Of The Steps Involved In The Synthesis Of Double Stranded cDNA

1) The first cDNA strand is synthesized by reverse transcriptase using the polyA+ RNA as a template and an oligo dT primer.

2) *E. coli* RNase H is used to produce nicks and gaps in the RNA strand of the RNA:DNA hybrid. The nicked RNA is used as a primer for DNA synthesis and repair by *E. coli* DNA polymerase I.

3) The 3'-5' exonuclease activity of T4 DNA polymerase removes any small remaining 3' protruding ends from the first strand cDNA.


5' AUG
3'
mRNA
AAAAA
Reverse transcriptase
T T T T
+ dNTPs
Oligo dT
primer
5' AUG
3'
AAAAA
cDNA
T T T T
E.coli RNase H
AAAAA
cDNA
T T T T
DNA polymerase I
+ dNTPs
cDNA
T4 DNA polymerase
cDNA
Double stranded cDNA

the cDNA digested with *EcoRI* in the appropriate buffer. Recombinant cDNA molecules were purified from the excess linkers using Geneclean II described in section 2B13.4.

11.7 Insertion Of Recombinant cDNA Molecules Into pT7T3 18U Phagemid Vector

A 3-fold excess of recombinant cDNA was ligated to the de-phosphorylated *EcoRI* cleaved phagemid vector pT7T3 18U (Pharmacia) in a 20µl reaction volume containing 1 unit of T4 DNA ligase and ligation buffer. The ligation mix was incubated for 16 hrs at 4^0, heated to 70^0 for 15 min and the DNA transfected into competent *E. coli* .

11.8 Transformation Of Competent E.coli

Competent *E. coli* (DH5α) were prepared essentially as described in the procedure of Hanahan (1983). SOB broth (6.5mls) was inoculated with a single bacterial colony and shaken at 37^0 for 2-3 hrs. The culture was chilled on ice for 10 min prior to centrifugation at 2500rpm at 4^0 for 12 min. The bacterial pellet was vortexed gently in 2ml TFB and placed on ice for 10 min prior to centrifugation at 2500rpm at 4^0 for 10 min. The pellet was resuspended in 0.5ml TFB and incubated on ice for 5 min. DMSO (4% v/v) was added to the suspension which was incubated on ice for 5 min before addition of DTT buffer to a final concentration of 75mM. The suspension was incubated for a further 10 min on ice, then DMSO (4% v/v) was added and incubation continued for 5 min. The competency of the bacteria in transfection assays was tested using uncleaved pUC18 DNA. A 50µl volume of competent cell suspension was mixed with 10ng of pUC18 plasmid DNA and incubated on ice for 30min. After heat shock treatment at 42^0 for 90 sec, the bacteria were added to 4 volumes of SOC and shaken at 37^0 for 1 hr before being plated onto L-broth agar containing 100µg/ml ampicillin. Plates were incubated overnight at 37^0. Routinely, $1x10^9$ colonies per µg plasmid DNA were obtained. Competent bacteria were stored at -70^0 in SOB containing DMSO (75µl per ml of bacterial suspension).

11.9 Amplification And Storage Of Phagmid cDNA Library

Transformed bacterial colonies, grown on L-broth agar plates, were picked individually with sterile toothpicks into 200µl L-broth containing 100µg/ml ampicillin in 96-well microtitre plate. The microtitre plate was covered with Titertek plate sealers (Flow) to prevent cross contamination, and incubated overnight at 37^0. Following incubation, a duplicate plate was seeded and incubated overnight at 37^0. Sterile glycerol was added to the wells of the original microtitre plate to a final concentration of 15% (v/v). The plate was sealed, shaken vigorously then frozen at -70^0. The duplicate microtitre plate was stored at 4^0 until recombinant clones were screened according to the protocol described in section 2B13.1c and 2B13.3. This procedure generally produces a million fold amplification of the library.

11.10 Coupled In Vitro Transcription And Translation System

The cDNA molecules, cloned downstream from the T7 or T3 RNA polymerase promoter in pT7T3 18U multifunctional phagemid (Pharmacia), were transcribed and translated *in vitro* as previously described by Krieg *et al.*, (1984), using the TNT Coupled Reticulocyte Lysate System (Promega) according to the protocol supplied. Briefly, circular recombinant pT7T3 18U DNA (1µg per reaction) was added to a total reaction volume of 50µl containing 50% TNT rabbit reticulocyte lysate, 40 units of T7 or T3 RNA polymerase, 20mM of each amino acid except methionine and 0.8mCi/ml of [^{35}S]-methionine and incubated at 30^0 for 2 hrs. The protein products were analysed by SDS-PAGE and autoradiography.

12. Nucleic Acid Filter Hybridisation

12.1 Transfer Of DNA

a) Southern Transfer

The procedure followed was essentially that of Southern (1975) in which single stranded DNA was mobilised onto Genescreen Plus nylon membrane (DuPont). The agarose gel containing the separated DNA fragments was shaken gently in DNA denaturing buffer for 30 min at RT and neutralized in DNA neutralizing buffer for 30 min at RT with gentle agitation. The gel was then transferred onto 2 sheets of Whatman 3mm filter paper which were in contact with, but not covered by, 10x SSC buffer. A sheet of Genescreen Plus nylon membrane, the exact size of the gel, was moistened with deionized water, soaked in 10x SSC for 15 min, and then placed on top of the gel. Two sheets of 3mm paper, cut 2mm smaller than the gel, were placed on top of the membrane. Finally, a weighted stack of cut paper towels, approximately 5cm deep, was laid on top of the Whatman paper. At least 12 hrs later, the membrane was removed, rinsed in 2x SSC and air dried. Efficient transfer of DNA fragments from the gel was monitored by visualising the ethidium bromide restained blotted gel under UV light. The DNA immobilized on the membrane was then hybridized to the appropriate ^{32}P-labelled DNA probe.

b) Dot Blot Transfer of DNA

A piece of Genescreen Plus nylon membrane and filter paper pad were cut to fit either a dot or slot blot manifold. The membrane and filter pad were soaked in 0.4M Tris-HCl pH7.5 for 30 min at RT. Meanwhile, the DNA was denatured in 0.25N NaOH for 10 min at RT, chilled on ice, then diluted to the desired concentration in 0.125N NaOH, 0.125x SSC. The pre-soaked membrane and filter pad were fitted into the manifold and the DNA added to the appropriate wells. The DNA solution was left in the wells for 30 min, after which time a gentle suction was applied to the manifold for 30 sec. The membrane was removed from the manifold and air dried. The filters were hybridized to the appropriate ^{32}P-labelled DNA probe.

c) Lysis of Bacteria and Binding of Bacterial DNA to Nylon Membranes.

A piece of Genescreen Plus nylon membrane and filter pad were cut to fit a 96 well dot blot manifold (Schleicher and Schnell) and moistened with water. The membrane and filter pad were placed in the manifold. A volume of 50µl of bacterial suspension was added to each well of the manifold. The bacterial suspension was allowed to remain in the wells without suction for 30 min, after which time a gentle suction was applied for 1 min. The membrane was removed from the manifold and placed colony side up for 3 min on a stack of 3mm paper impregnated with 10% SDS (w/v). This treatment limited the diffusion of the DNA during denaturation and neutralisation steps, resulting in a sharper hybridization signal. The membrane was placed onto 3mm paper saturated with denaturing solution and the bacteria allowed to lyse for 5 min. The membrane was then transferred colony side up to 3mm paper soaked in neutralising solution for 5 min. Finally, the membrane was placed on 3mm paper saturated with 2 x SSC and left for a further 5 min. The air dried membrane was washed briefly in chloroform to remove bacterial debris, rinsed in 2 x SSC and air dried again. The membranes were then hybridized to the appropriate ^{32}P-labelled DNA probe.

12.2 Transfer Of RNA

A piece of Genescreen Plus nylon membrane and filter pad of appropriate size for a slot blot manifold (Schleicher and Schnell) were soaked in deionized water for 15 min. RNA was dissolved in 50% deionized formamide, 6% formaldehyde, and incubated at 50^0 for 1 hr to denature the RNA. The RNA was chilled on ice, diluted in deionized water to the desired concentration and added to the wells of the RNA slot blot manifold, containing the pre-soaked membrane and filter pad. The RNA was allowed to to remain on the membrane for 30 min before a gentle suction was applied. After 30 sec the membrane was removed from the manifold, air dried and baked at 80^0 for 2 hrs to remove the formaldehyde. Hybridisation with an appropriate ^{32}P-labelled probe was then performed.

12.3 DNA:DNA Hybridization

DNA:DNA hybridizations were carried out in sealed plastic bags submerged in a shaking water bath. The membranes were incubated in pre-hybridisation buffer (0.5M sodium phosphate buffer, 7% SDS (w/v)) for 2 hrs at 65^0. The ^{32}P-labelled DNA probe was boiled for 5 min to denature the DNA, then chilled rapidly on ice. The denatured DNA probe was added directly to the pre-hybridisation buffer. The membranes were incubated overnight at 65^0 with continuous shaking. Between $2x10^5$ and $1x10^6$ cpm of ^{32}P-labelled probe (specific activity $>5x10^7$ cpm/µg DNA) was used per ml of hybridisation buffer. Following hybridisation, the membranes were removed from the plastic bags and washed 3x in 0.01M sodium phosphate buffer containing 0.1% SDS (w/v) at 65^0 for 45 min each wash. After the final wash the membranes were rinsed briefly in deionized water then air dried. Membranes, covered with cling film, were placed in contact with Xomat XS-1 film (Kodak) in conjunction with a DuPont phosphotungstate intensifying screen at -70^0.

12.4 DNA:RNA Hybridisation

RNA, transferred to nylon membranes, was hybridized to the desired ^{32}P-labelled DNA probe. Membranes were incubated for 2 hrs at 68^0 in pre-hybridisation buffer (6X SSC containing 2X Denhardts buffer, 0.1% SDS (w/v)) in sealed plastic bags submerged in a shaking water bath. ^{32}P-labelled double stranded DNA probes were boiled for 5 min, cooled rapidly on ice, and added directly to the pre-hybridisation buffer. After an overnight hybridisation at 68^0, membranes were removed from the bags and washed once in 1x SSC for 20 min at RT, then washed three times in 0.2x SSC for 20 min at 68^0. All washes contained 0.1% SDS (w/v). Membranes were rinsed briefly in deionized water, air dried and placed in contact with Kodak X-omat XS-1 film in conjunction with a DuPont phosphotungstate intensifying screen at -70^0.

13. Recombinant DNA Techniques

13.1 Restriction Enzyme Digestion Of DNA

Restriction endonuclease digestions were carried out under the conditions specified by the suppliers for each enzyme.

13.2 Dephosphorylation Of Linearised Vector DNA

Vector DNA was linearized with the appropriate restriction enzyme and treated with calf intestinal phosphatase at a concentration of 5units/ µg DNA in the presence of 20mM Tris.HCl pH8.0 and 40mM NaCl. The reaction was incubated at 37^0 for 1 hr. Linear, dephosphorylated vector DNA was purified using Geneclean II.

13.3 Construction Of Recombinant Phagemids

Linear, dephosphorylated vector DNA was incubated with a 3-fold molar excess of the required DNA fragment overnight at 14^0 in a 20µl ligation reaction containing 1 unit of T4 DNA ligase in ligase buffer. Blunt ended ligations were performed in a similar manner except that a 2-4 fold molar excess of linear vector relative to the purified insert was used and incubations were performed for 4 hrs at RT.

13.4 Purification Of DNA Using Geneclean II Kit

The Geneclean II kit was used to purify "miniprep" plasmid DNA prior to sequencing and to elute DNA fragments from agarose gel slices. Briefly, three volumes of sodium iodide solution supplied with the kit was added to the DNA preparation. A volume of 5µl of glass bead matrix ("Glassmilk") per µg of DNA was added to the DNA in solution and the sample incubated on ice for 5 min. The glass bead matrix binds to single stranded and double stranded DNA

without binding DNA contaminants. The bound DNA was washed 3x with wash buffer, then eluted in TE by heating to 55^0 for 5 min.

13.5 Partial Digestion Of ^{32}P End-Labelled DNA

Approximately 1x10^5 cpm of ^{32}P-labelled DNA fragment was digested with decreasing concentrations of the desired enzyme (10-0.25 units) in a final reaction volume of 20µl. The digests were incubated at 37^0 for 15 min, pooled and analyzed on a 5% non-denaturing polyacrylamide gel. After electrophoresis, the gel was dried under vacuum and placed in contact with Kodak X-omat XS-1 film at -70^0 to obtain an autoradiographic image of the digest.

13.6 Small Scale Preparation Of Plasmid DNA

Small amounts of plasmid DNA were prepared using the method of Chowdhury (1991). Single bacterial colonies were inoculated into 2ml L-broth containing 35-50µg/ml ampicillin and incubated in an orbital shaker at 37^0 for 16-18 hrs. A volume of 500µl of bacterial suspension was vortexed vigorously with an equal volume of a mixture of phenol:chloroform:isoamylalcohol (25:24:1) and the aqueous phase separated from the phenol phase by centrifugation in a microfuge. The phenol was saturated with TE prior to mixing with chloroform and isoamylalcohol. Plasmid DNA was precipitated with isopropanol, lyophilized and resuspended in TE pH8.0 containing 20µg/ml RNAse A. About 5-10 µl of the DNA solution was cleaved with the appropriate restriction enzyme.

13.7 Large Scale Preparation Of Plasmid DNA

A single bacterial colony was inoculated into 5ml L-broth, containing 100µg/ml ampicillin, and grown overnight at 37^0 in an orbital shaker. The overnight culture was added to 350ml L-broth containing ampicillin in 2 litre flasks and the culture shaken at 37^0. When the culture had reached an OD_{650} of

approximately 0.8, chloramphenicol was added to a final concentration of 100µg/ml and incubation continued overnight. Plasmid DNA was prepared by the "maxi-boiling" technique of Holmes and Quigley (1981). Cells were pelleted at 7000 rpm for 3 min (Sorvall GS3 rotor) and resuspended in 20ml STET buffer. Lysozyme, at a final concentration of 1mg/ml, was added and the sample incubated at RT for 30 sec prior to boiling for 45 sec. The lysate was clarified by centrifugation at 18000 rpm at 4^0 for 1 hr in a Sorvall SS34 rotor. Isopropanol (0.9 volumes) was added to the supernatant and the DNA pelleted at 2500 rpm at 4^0 for 10 min. The drained pellet was resuspended in 5ml TE and caesium chloride added to a final density of 1.6mg/ml and ethidium bromide to 0.5mg/ml. The solution was left on ice for 1 hr, spun at 2500 rpm for 10 min to remove debris and transferred to plastic TV865 ultracentrifuge tubes. The tubes were sealed and centrifuged on a vertical TV865 rotor at 40000 rpm at 15^0 for 16 hr. The DNA was visualised by daylight or long wave UV illumination and the lower supercoiled plasmid DNA band removed with a large bore needle and syringe. The DNA was extracted twice with butan-1-ol (TE saturated), and dialyzed against TE at RT for 2 hrs before being treated with 50µg/ml RNAse for 1 hr at 65^0, followed by 50µg/ml proteinase K for 1 hr at 37^0. DNA was extracted with an equal volume of phenol:chloroform, then chloroform and precipitated twice with ethanol. The DNA was pelleted, washed with 70% ethanol, then lyophilized before being resuspended in TE.

14. DNA Sequencing

14.1 Double Stranded DNA Sequencing Reactions

Double stranded DNA was sequenced using "Sequenase" T7 DNA polymerase, a genetic variant of bacteriophage T7 DNA polymerase, (Tabor and Richardson, 1989) and the sequencing solutions supplied with the Sequenase Version 2 Sequencing Kit (United States Biochemical). Double stranded template DNA (3-5µg) was added to 5ng of sequencing primer* in sterile water to a final volume of 8µl and heated to 95^0 for 4 min to denature the template DNA. The sample was cooled to 37^0 for 15 min to allow the primer to anneal to the template DNA and then chilled on ice. The labelling step was performed by adding diluted labelling mix containing 2 units of Sequenase T7

*(M13 universal and reverse sequencing primers)

DNA polymerase and 50μCi/ml of [^{35}S]-dATP, to the chilled DNA sample to a final volume of 15.5μl, and the reaction incubated for 2-5 min at RT. Following incubation at RT, 3.5μl volumes of the labelled DNA mixture were added to tubes containing ddATP, ddTTP, ddCTP or ddGTP termination mix. The samples were incubated at 37^0 for 5 min after which time, stop solution was added to each of the termination mixtures to stop the reaction. The sequencing products were analysed by denaturing polyacrylamide gel electrophoresis (see section 2B15.4)

15. Gel Analysis Of DNA

15.1 Non-Denaturing Agarose Gels

Concentrations of agarose between 0.7% to 1.4% (w/v) in TAE buffer containing 0.5μg/ml ethidium bromide were used in horizontal gels, depending on the size of the DNA fragments to be resolved. DNA samples in 0.2 volumes of formyl dye were electrophoresed at 10V/cm^2 for 3 hrs or until the dye front had migrated 2/3 the length of the gel. DNA fragments were visualised under short wave UV light. Long wave UV light was used to reduce DNA damage when preparative gels were being analysed.

15.2 Alkaline Agarose Gels

Alkaline agarose gels (McDonnell *et al* ., 1977) were used to analyze single stranded DNA. 0.7% agarose horizontal gels were prepared in 50mM NaOH and 1mM EDTA pH8.0. DNA samples were dissolved in 50mM NaOH, 1mM EDTA and 0.2 volumes of 6x alkaline loading buffer and electrophoresed at 0.25V/cm for 2-3hr using alkaline electrophoresis buffer. Electrophoresis was carried out until the dye had migrated 2/3 the length of the gel. At the end of the run, the gel was soaked in 7% TCA for 30 min at RT, covered with cling film and exposed to Kodak X-omat XS-1 film at RT.

15.3 Non-Denaturing Polyacrylamide Gels

DNA fragments (7000-100bp in size) were separated on vertical polyacrylamide gels containing 5% acrylamide cross-linked with 1 part in 30 w/w of N-N'methylene bisacrylamide in TAE buffer. Polymerisation was achieved by the addition of ammonium persulphate (0.006% w/v) and TEMED (0.004%) to the gel solution just before pouring. Wells were formed with Teflon combs. DNA fragments were separated in 0.2 volumes formyl dye by electrophoresis at 3V/cm for 18 hrs, using TAE buffer. Separated DNA fragments were visualised by either ethidium bromide staining or autoradiography.

15 4 Denaturing Polyacrylamide Gels

Denaturing polyacrylamide gels (0.35mm thick) were used to analyze the products of DNA sequencing reactions. Glass sequencing gel plates were washed in ethanol prior to treatment with Replecote (BDH). Plates were washed again in ethanol and polished thoroughly. The glass plates were taped together and 60ml of single concentration gel mix was poured into the glass plate sandwich using a syringe. Gels were left to polymerise for 1 hr before use. Wells were formed with sharks tooth combs. Boiled sequencing reactions were electrophoresed at 70 Watts for 1/2-4 hrs in TBE tank buffer. After electrophoresis the gel was transferred to 3mm Whatman filter paper and dried under vacuum at 80^0 for 2 hrs. Gels were placed in contact with X-omat XS-1 film.

16. Electron Microscopy

16.1 Preparation Of Samples In Epon Resin

The growth medium was removed from mock and virus-infected HFL cell monolayers in 50mm dishes at 3 hrs post-infection at NPT. The monolayers were washed 3x in ice cold PBS then scraped into 0.5ml PBS, transferred to

beem capsules and pelleted at 3000 rpm for 10 min at 4^0. Glutaraldehyde (2.5% in PBS) was added to the pellets and the samples incubated at 4^0 for 1 hr. The pellets were washed 3x in PBS and fixed with 1% (w/v) O_sO_4 for 1 hr. After fixation, the pellets were again washed 3x in PBS and then subsequently dehydrated through a series of increasing ethanol concentrations (30, 50, 70, 90, 100% v/v in PBS). The pellets were infiltrated with epon resin and incubated overnight at room temperature. Following overnight incubation, the resin was removed from the samples and replaced with fresh epon resin. Polymerization of the resin was achieved by incubating the samples at 65^0 for 3 days.

16.2 Thin Sectioning

Pelleted cells, embedded in polymerized epon resin, were cut with a diamond knife on an Ultra-microtome (Ultracut E, Reichert-Jung), and thin sections were collected on parlodium-coated copper grids. Sections were stained with saturated uranyl acetate in 50% ethanol for 1 hr, rinsed with deionized water and counter-stained with lead citrate for 15 min. The stained thin sections were examined at 80KV in a Jeol 100S electron microscope.

17. Computing and Analysis of Sequence Data

Sequence handling was carried out on a DEC VAX computer. The genetic computer group (GCG) sequence analysis software package version 5.0 and 6.0 was used (Devereux *et al.*, 1984; Devereux, 1989).

3.Results

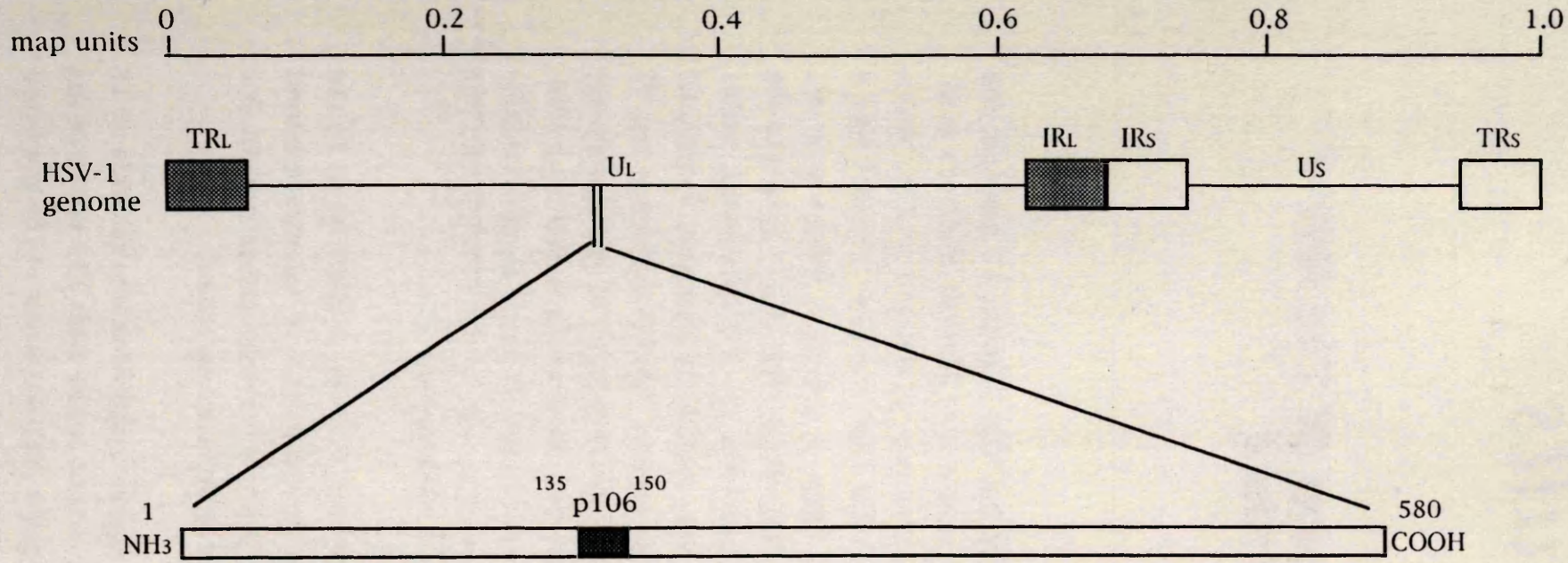
Section 3.A Characterization Of The HSV-1 UL25 Gene Product

1. Introduction

From nucleotide sequence analysis of the HSV-1 strain 17 genome, the HSV-1 UL25 gene was predicted to encode a protein of 62,666MW (McGeoch *et al.*, 1988b). Studies carried out on the HSV-1 *ts* mutants *ts*1204 and *ts*1208, which both have lesions in the UL25 gene, suggested that the gene product was a virion protein required at early and late times in infection (Addison *et al.*, 1984). These conclusions were based upon observations that *ts*1204 bound to the cell surface at the NPT but was unable to penetrate the cell membrane, whilst *ts*1208, although able to enter the cell, was unable to assemble functional capsids. Provided *ts*1204 was allowed to enter cells by brief incubation at the PT prior to growth at the restrictive temperature, the phenotype of the mutant was similar to that of *ts*1208, inasmuch as few capsids, all of which lacked viral DNA, were formed. Since few nuclear capsids were found in *ts*1204- and *ts*1208-infected cells when compared to *wt* virus-infected cells, it was thought that the UL25 gene product may be involved in capsid stabilisation.

Sequence data analysis has indicated that UL25 is unlikely to be in the virus envelope, since it does not have any characteristics of membrane-bound proteins (McGeoch *et al.*, 1988b). Moreover, it is not a known capsid protein. For these reasons, the UL25 protein was thought to be in the tegument.

An antiserum was raised in a rabbit against a synthetic oligopeptide of 15 amino acid residues, termed p106, corresponding to amino acids 135 to 150 of the predicted UL25 ORF. Figure 6 shows a schematic representation of the predicted amino acid sequence of the UL25 protein and the oligopeptide used to make the



1 MDPYCPFDALDVWEHRRFIVADSRNFITPEFPRDFWMSPVFNLPRETAAEQVVVLQAQRTAAAAALENAA 70

71 MQAAELPVDIERRLRPIERNVHEIAGALEALETAAAAAEEADAARGDEPAGGGDGGAPPGLAVAEMEVQI 140

141 VRNDPPLRYDTNLPVDLLHMVYAGRGATGSSGVVFGTWYRTIQDRTITDFPLTTRSADFRDGRMSKTFMT 210

211 ALVLSLQACGRLYVGQRHYSAFECAVLCLYLLYRNTHGAADDSDRAPVTFGDLLGRLPRYLACLAAVIGT 280

281 EGGRPQYRYRDDKLPKTQFAAGGGRYEHGALASHIVIATLMHHGVLPAAPGDVPRDASTHVNPDGVAHHD 350

351 DINRAAAAFLSRGHNLFLWEDQTLLRATANTITALGVIQRLLANGNVYADRLNNRLQLGMLIPGAVPSEA 420

421 IARGASGSDSGAIKSGDNNLEALCANYVLPLYRADPAVELTQLFPGLAALCLDAQAGRPVGSTRRVVDMS 490

491 SGARQAALVRLTALELINRTRTNPTPVGEVIHAHDALAIQYEQGLGLLAQQARIGLGSNTKRFSAFNVSS 560

561 DYDMLYFLCLGFIPQYLSAV 580

Figure 6. A Schematic Representation Of The HSV-1 Genome And The Location Of The UL25 Gene.

The predicted amino acid sequence of the UL25 polypeptide and the location of the oligopeptide p106 (boxed), against which an antiserum was raised, are indicated.

antiserum.Antibodies in the antiserum recognized a virus-specific polypeptide in an immunoprecipitation assay but did not bind to antigen in a western blot analysis (D.Smith unpublished observations).

The studies reported here represent initial efforts to understand the function of the UL25 protein. With the aid of the oligopeptide polyclonal antibody directed against amino acid sequences present in the UL25 gene product, the synthesis, processing and location of the UL25 polypeptide have been examined.

2. Identification Of The UL25 Gene Product

BHK cells were infected with HSV-1 and radiolabelled with [^{35}S]-methionine at 4 hrs post-infection. After a 30 min incubation, cells were harvested and immunoprecipitation reactions were carried out with the serum obtained from the pre-immune rabbit and the anti-UL25 oligopeptide antiserum. The immunoprecipitated proteins were separated on a SDS 9%-polyacrylamide gel and visualized by fluorography (Figure 7). The antiserum recognised a polypeptide of apparent Mr 67000 which was not detected in immunoprecipitations using pre-immune serum. Two other polypeptides with MW of approximately 110kDa and 150kDa, which probably correspond to glycoprotein B and the major capsid protein VP5 respectively, were also present in the immune precipitate. Since similar sized proteins were precipitated from virus-infected cell extracts incubated with the pre-immune sera, the presence of gB and VP5 in the immune precipitates is not due to the specific interaction of VP5 or gB with either the antiserum or the putative UL25 protein.

The specificity of the UL25 antiserum was assessed by performing immunoprecipitations in the absence or presence of 100μg/ml of oligopeptide p106 (Figure 8 lanes 3 and 4 respectively). The results show that the precipitation of the 67,000MW protein was greatly inhibited in the presence of 100μg/ml of oligopeptide p106. Thus, oligopeptide concentrations of 100μg/ml were used in further immunoprecipitation experiments with the UL25 antiserum. A number of polypeptides with molecular weights of approximately 67 kDa were observed in immunoprecipitations performed in the presence of oligopeptide p106. It is likely that these similar sized polypeptides are distinct from the UL25 gene product, since they appear to be slightly larger than

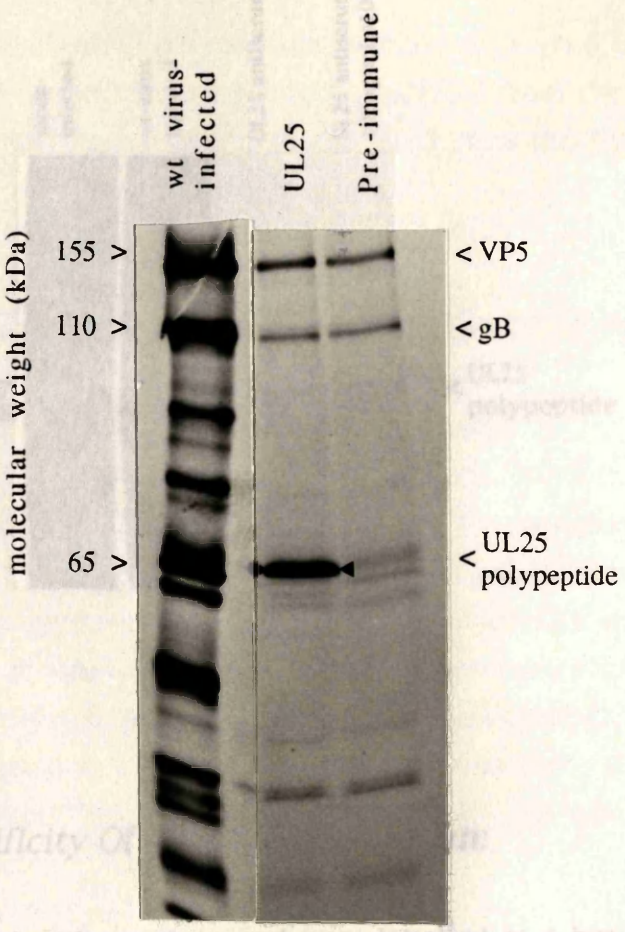


Figure 7. Identification Of The UL25 Gene Product.

BHK cells were infected with *wt* virus at 37^0 and labelled at 4 hrs post-infection with 100μCi/ml of [^{35}S]-methionine. After 30 min the cells were harvested and cell lysates prepared. Immunoprecipitation reactions were carried out using the UL25 specific antiserum (UL25) or pre-immune serum (Pre-immune). The precipitated proteins were separated on a SDS-9% polyacrylamide gel and visualized by fluorography. HSV-1 *wt* virus-infected cell extracts are indicated.

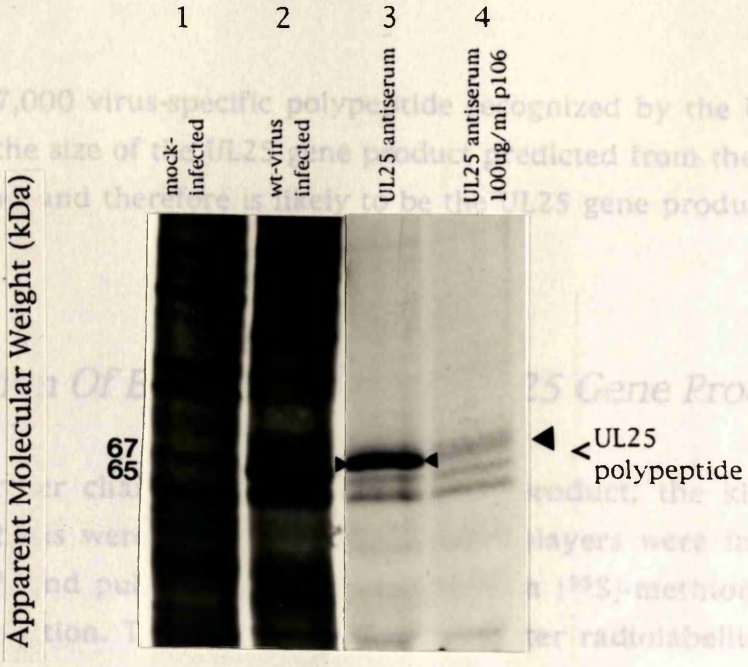
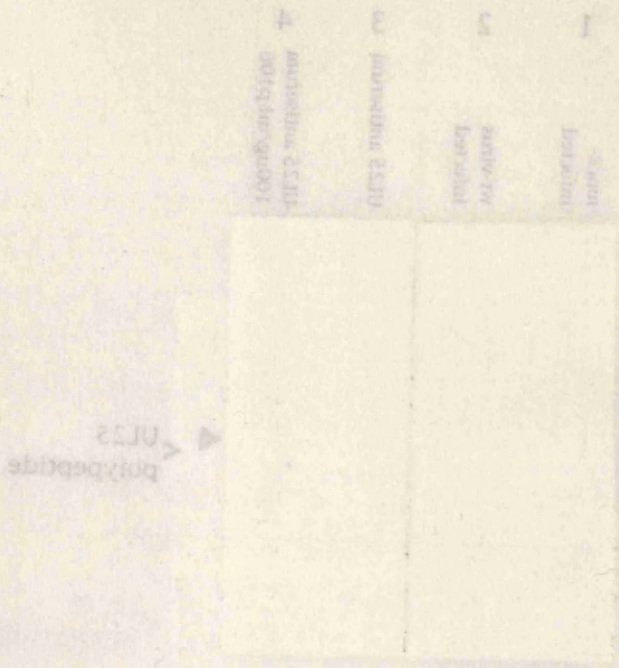


Figure 8. Specificity Of The UL25 Antiserum

Cells were infected with *wt* virus and were labelled at 4 hrs post-infection at 37^0 for 30 min with 100μCi/ml of [^{35}S]-methionine and used in immunoprecipitation reactions with the UL25 antiserum in the absence and presence of 100μg/ml of oligopeptide p106 (Lanes 3 and 4 respectively). The precipitated polypeptides were resolved by electrophoresis through a SDS 9%-polyacrylamide gel and visualized by fluorography. Lanes 1 and 2 represent mock-infected and *wt* HSV-1 infected cell lysates respectively. The polypeptides with MWs of approximately 67 kDa that were observed in immunoprecipitations performed in the presence of oligopeptide p106 are indicated by a filled arrow.



* Additional minor polypeptide bands also appeared to be recognized by the UL25-specific antiserum as indicated in Figure 9, which may be due to internal initiation of translation of the UL25 polyA+ RNA.

polypeptide that was immunoprecipitated with the anti-UL25 oligopeptide antiserum.

The 67,000 virus-specific polypeptide recognized by the UL25 antiserum is similar to the size of the UL25 gene product predicted from the analysis of the HSV-1 genome, and therefore is likely to be the UL25 gene product.

3. Regulation Of Expression Of The UL25 Gene Product.

To further characterize the UL25 gene product, the kinetics of UL25 protein synthesis were analyzed. BHK cell monolayers were infected with *wt* HSV-1 at 37^0, and pulse labelled for 30 min with [^{35}S]-methionine at various times post-infection. The cells were harvested after radiolabelling and the cell lysates used in immunoprecipitation reactions with the UL25 antiserum in the presence or absence of oligopeptide p106. The immunoprecipitated proteins were analyzed by SDS-PAGE and visualized by fluorography (Figure 9). The UL25 protein was detected at 1 hr post-infection and reached a peak of synthesis at 4-5 hrs post-infection. The levels of UL25 protein immunoprecipitated from infected cells appeared to decline slowly thereafter. These results are consistant with UL25 being regulated as a late gene product. Two classes of late gene product, leaky late (γ_1) and true late (γ_2), have been defined, according to their ability to be expressed in the absence of viral DNA replication. Leaky late genes are transcribed in the absence of viral DNA replication. Maximal levels of mRNA synthesis, however, occur only after replication of the viral genome. Transcription of true late genes is more dependent on viral DNA synthesis and less than 5% of mRNA from true late genes in *wt* virus-infected cells is detected under conditions which severely inhibit viral DNA synthesis.

To determine which class of late gene UL25 belonged to, the synthesis of the UL25 protein was analysed in the presence of phosphonoacetic acid (PAA), a viral DNA polymerase inhibitor. Previous studies indicated that 300µg/ml of PAA was sufficient to severely inhibit expression of glycoprotein C, a true late gene product, to less than 5% of levels expressed in untreated cells (Homa *et al.*, 1986). Therefore, BHK cells were infected at 37^0 with HSV-1 in the presence and absence of 100µg/ml and 300µg/ml of PAA. At 4 hrs post-infection the cells were pulse labelled for 30 min with 100µCi/ml of [^{35}S]-methionine and then harvested. The cell lysates were incubated with the UL25 antiserum and

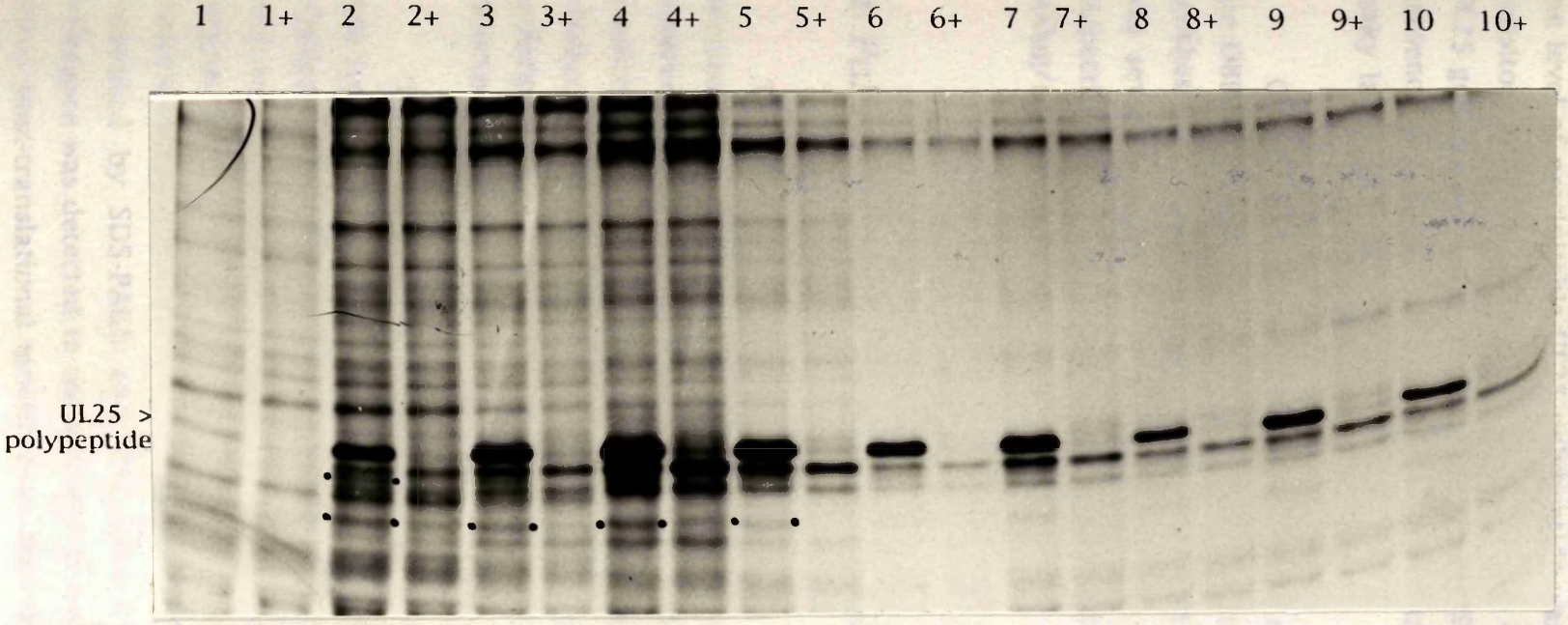


Figure 9. Kinetics Of UL25 Polypeptide Synthesis.

BHK cells were infected with *wt* virus at 37^0 and at various times post-infection the cells were labelled with 100μCi/ml of [^{35}S]-methionine. After 30 min, the cells were harvested and immunoprecipitation reactions were carried out with UL25 specific antiserum. Immunoprecipitated proteins were resolved by electrophoresis through a SDS-9% polyacrylamide gel and visualized by fluorography. The numbers above the lanes indicate hours post-infection at which the cells were harvested. Immunoprecipitations in the presence of 100μg/ml of oligopeptide p106 are indicated by +.

The additional minor polypeptides recognized by the UL25-specific antiserum is indicated by the filled circles.

immunoprecipitation reactions carried out. The immunoprecipitated proteins were analysed by SDS-PAGE and fluorography (Figure 10).

In the absence of PAA, the UL25 polypeptide was detectable at 4 hrs post-infection (lane 3), whilst in virus-infected cells treated with 100μg/ml of PAA the level of precipitable UL25 polypeptide was reduced 5-fold as determined by densitometric analysis of the autoradiograph (lane 5). Similarly, the level of the UL25 gene product was reduced 12-fold in HSV-1 infected cells incubated in the presence of 300μg/ml of PAA. These results indicate that UL25 is regulated as a leaky late viral gene.

Control samples were incubated with monoclonal antibodies specific for the DBP, an early protein, or glycoprotein C (a true late gene). As expected, the synthesis of gC in infected cells incubated in the presence of 300μg/ml of PAA was severely reduced compared to that in untreated cells (lanes 9 and 10 respectively). In contrast synthesis of DBP was not affected in cells treated with 300μg/ml of PAA (lanes 11 and 12).

4. Pulse-Chase Analysis Of The UL25 Polypeptide

The UL25 gene is predicted to encode a protein of molecular weight 62,666 (McGeoch *et al.*, 1988b), which is similar to the apparent size of 67,000kDa observed by SDS-PAGE. The slight discrepancy between the apparent and predicted size of the UL25 polypeptide may be due to the inaccuracy of predicting protein size by SDS-PAGE. Alternatively, the UL25 protein may undergo processing following synthesis to give rise to the 67kDa protein observed by SDS-PAGE.

To determine whether processing of the UL25 polypeptide occurs, BHK cells were infected with *wt* HSV-1 at 37^0 and at 4 hrs post-infection were pulse labelled for 15 min with 100μCi/ml of [^{35}S]-methionine. After labelling, the cells were either harvested immediately or incubated in the absence of radiolabel for various time intervals. Cell lysates were prepared and immunoprecipitation reactions were performed with UL25 antiserum. The precipitated proteins were separated by SDS-PAGE and visualized by fluorography (Figure 11). No difference was detected in the mobility of the UL25 polypeptide, suggesting that either post-translational modification does not occur, or the UL25 polypeptide

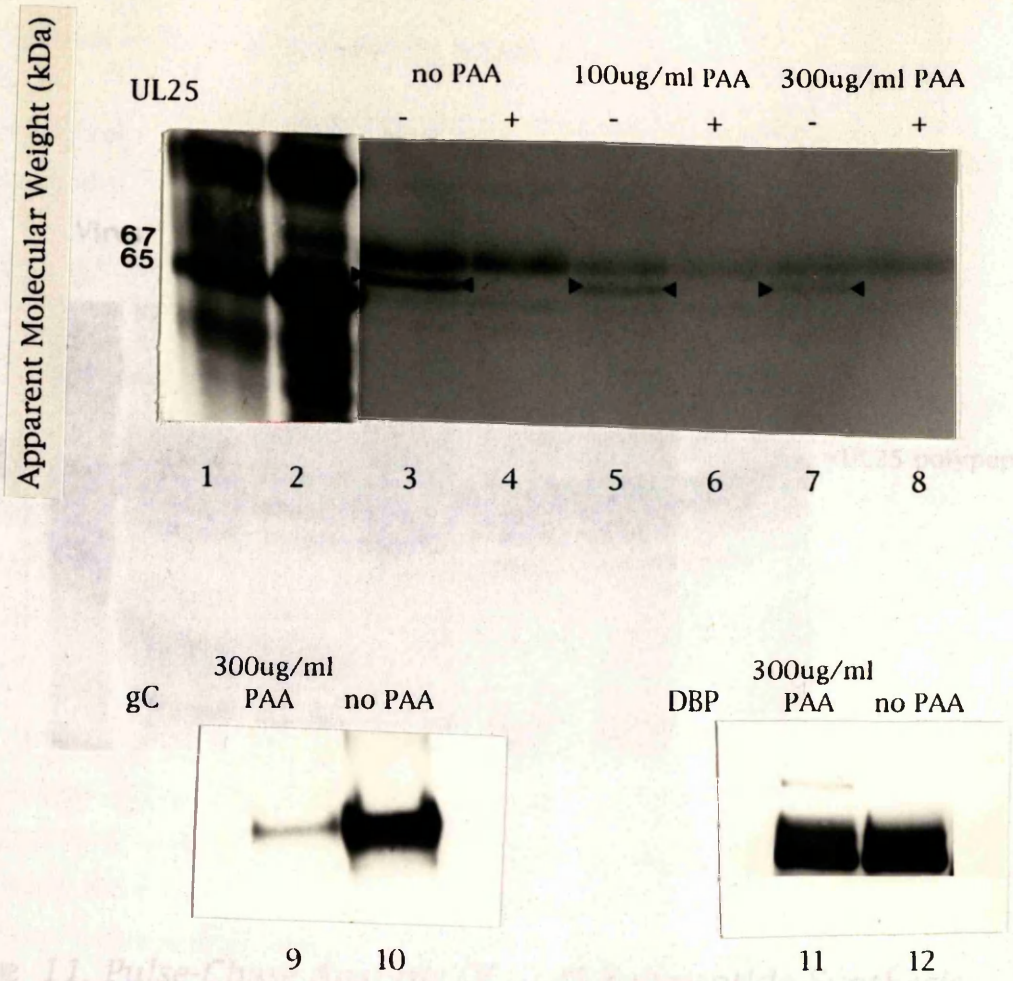


Figure 10. Expression Of UL25 Polypeptide In The Presence And Absence Of HSV-1 DNA Replication.

BHK cells were infected with *wt* virus in the presence and absence of 100μg/ml or 300μg/ml of PAA. At 4 hrs post-infection the cells were pulse-labelleled for 30 min with 100μCi/ml of [^{35}S]-methionine, harvested and the cell lysates used in immunoprecipitation reactions with antiserum directed against the UL25 protein (lanes 3-8). Parallel samples were prepared at 4 and 12 hrs post-infection and used in immunoprecipitations with antisera directed against DBP and gC respectively (lanes 9, 10 and lanes 11, 12). All immunoprecipitation reactions were performed in parallel. The precipitated proteins were analyzed by 9% SDS-PAGE and fluorography. Immunoprecipitations in the presence of 100μg/ml of oligopeptide p106 are indicated by +. Lanes 1 and 2 are purified HSV-1 virions and L-particles respectively.

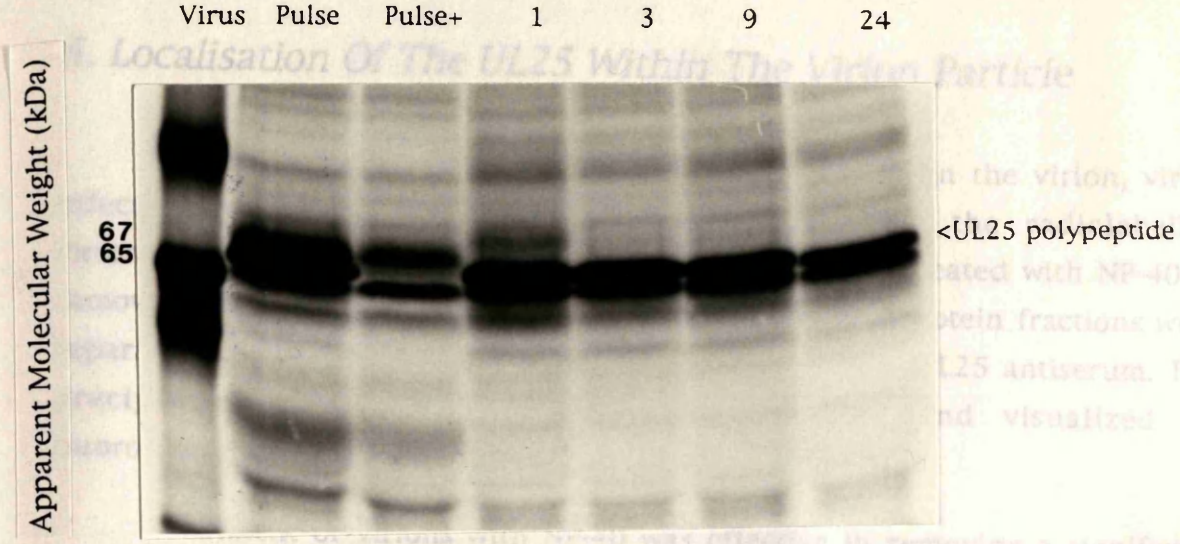


Figure 11. Pulse-Chase Analysis Of UL25 Polypeptide Synthesis.

BHK cells were infected with *wt* virus at 37^0 and at 4 hrs post-infection the cells were pulse-labelled with 100μCi/ml of [^{35}S]-methionine for 15 min. The labelled cells were harvested immediately or incubated for various times post-infection in the absence of radioactive medium. The harvested cell lysates were incubated with antiserum against the UL25 protein. The immunoprecipitated proteins were separated by 9% SDS-PAGE and visualized by fluorography. The pulse and the various times of chase (in hours) are indicated above the lanes. The lane marked Virus, represents purified HSV-1 virions. Immunoprecipitation performed in the presence of 100μg/ml of oligopeptide p106 is indicated by +.

undergoes a form of processing which does not alter its apparent molecular weight.

Alternatively, it is possible that processing is occurring on the nascent polypeptide.

5. Localisation Of The UL25 Within The Virion Particle

To determine the location of the UL25 protein within the virion, virus-infected cells were labelled with [^{35}S]-methionine, and the radiolabelled virions, purified by density gradient centrifugation, were treated with NP-40 to remove the envelope proteins. The soluble and insoluble protein fractions were separated and prepared for immunoprecipitation with the UL25 antiserum. The precipitated proteins were analysed by SDS-PAGE and visualized by fluorography (Figure 12).

Treatment of virions with NP-40 was effective in removing a significant proportion of the envelope proteins and Vmw65 (lane 8). In contrast, the capsid protein VP5, was not solubilized, suggesting that the capsid remained intact following NP-40 treatment (lane 7). Furthermore, the predominant association of many tegument proteins including the UL25 polypeptide (lane 1,3) with the capsid following removal of the envelope is consistent with previously published data, suggesting that the extraction procedure was satisfactory (Roizman and Furlong, 1974).

In conclusion, the data indicate that the UL25 polypeptide is a structural component of the virion and forms a tight association with the tegument/capsid structure, inasmuch as removal of the viral envelope does not result in the loss of a significant amount of the UL25 polypeptide from the virion.

6. Location Of The UL25 Polypeptide Within L-Particles

The previous data suggested that the UL25 protein might be important for capsid stability and form a tight interaction with one or more capsid or tegument components. This being so, the UL25 protein might not be expected to be an integral component of L-particles since they lack the capsid structure. To address this possibility, equivalent numbers of ^{35}S-labelled virions and L-particles, were solubilized and incubated with UL25 antiserum. The

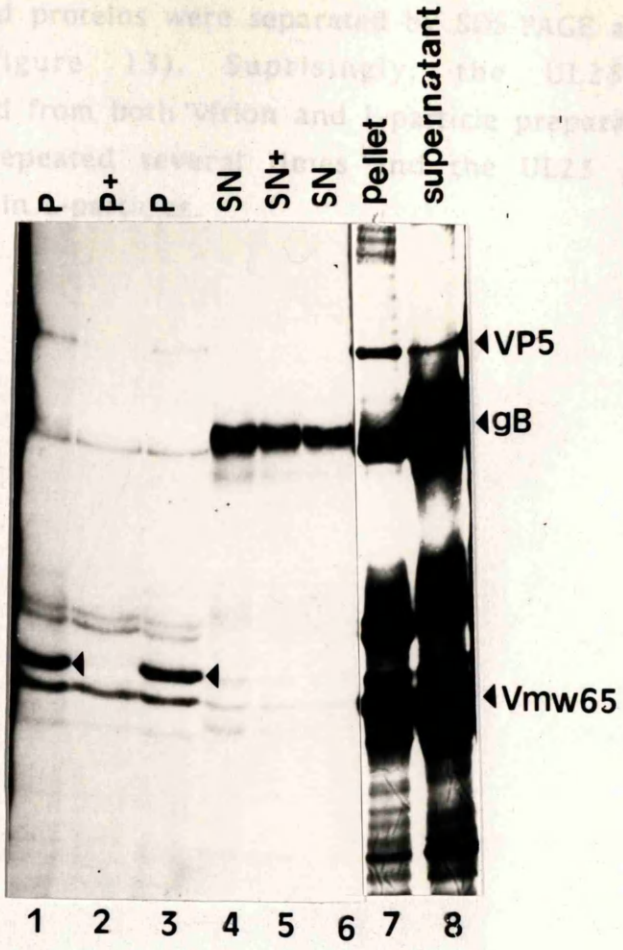
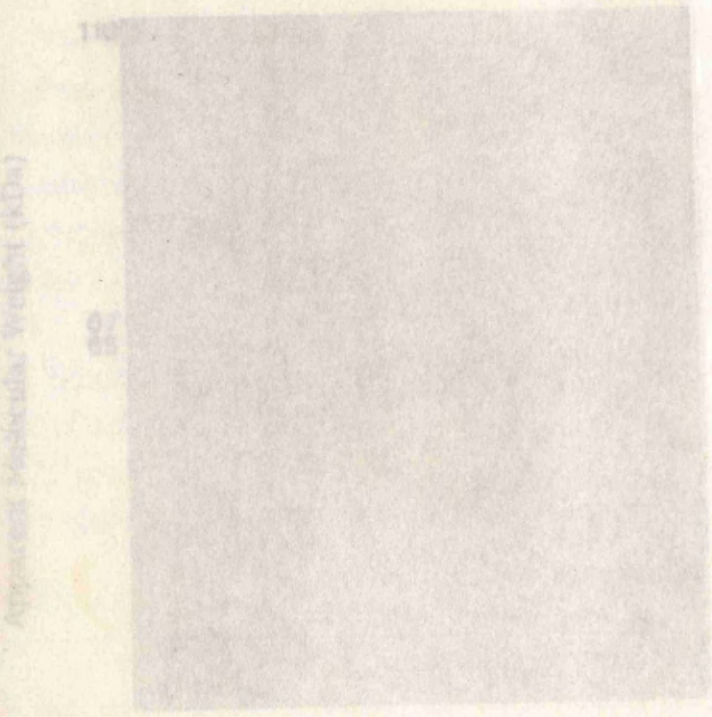


Figure 12. Localisation Of The UL25 Polypeptide Within The HSV-1 Virion.

HSV-1 virions were labelled with [^{35}S]-methionine and purified by density gradient centrifugation. Purified virions were treated with 1% NP-40 and the soluble and insoluble proteins were separated by centrifugation into supernatant (SN) and pelleted (P) fractions respectively. Equivalent amounts of each fraction were immunoprecipitated with antiserum specific for UL25 in the presence (+) and absence of 100μg/ml of oligopeptide p106. The immunoprecipitated proteins were separated on a SDS 9%-polyacrylamide gel and visualized by fluorography. The UL25 polypeptide is indicated by the arrow head. The viral envelope proteins gB and VP5and the tegument protein Vmw65 are indicated. The radiolabelled pelleted and supernatant fractions are indicated.

immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography (Figure 13). Suprisingly, the UL25 protein was immunoprecipitated from both virion and L-particle preparations. The above experiment was repeated several times and the UL25 polypeptide was consistently found in L-particles.



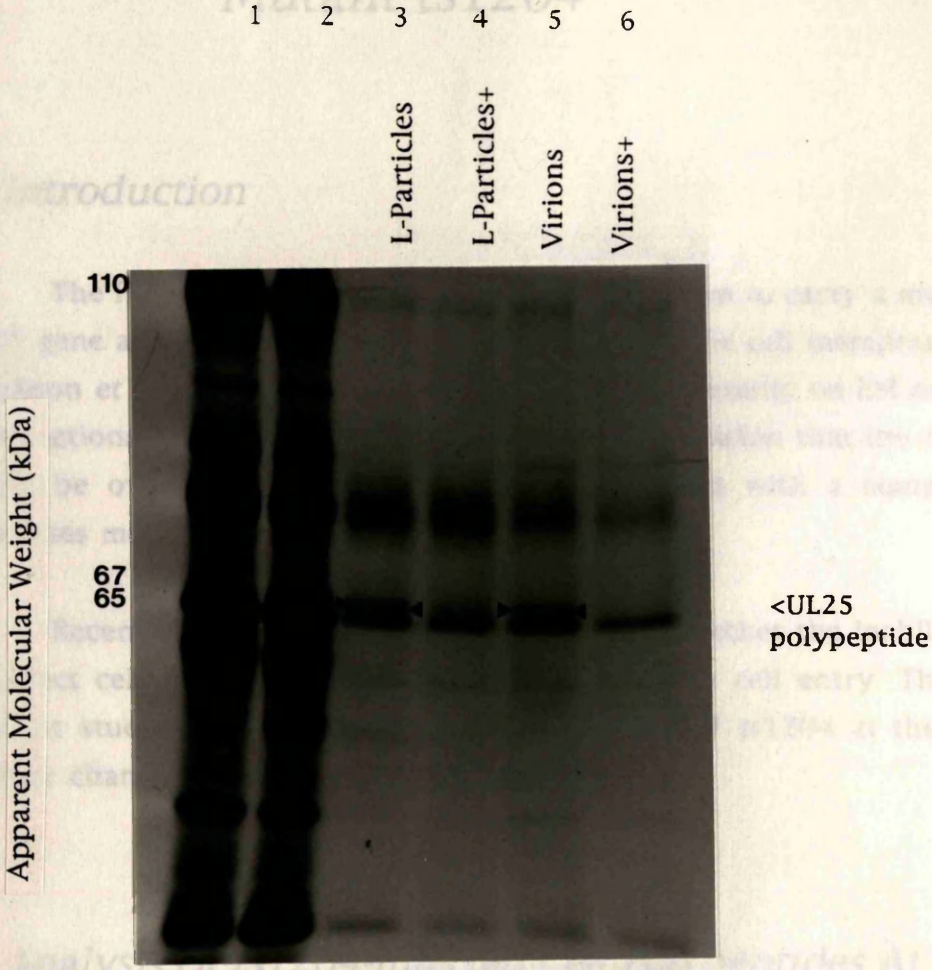


Figure 13. Localisation Of UL25 Polypeptide In L-Particles.

^{35}S-labelled HSV-1 virions and L-particles were purified by density gradient centrifugation. Equivalent numbers of virions and L-particles were solubilized and used in immunoprecipitation reactions with antiserum directed against the UL25 protein in the presence (+) and absence (-) of 100μg/ml of oligopeptide p106. Precipitated proteins were resolved by electrophoresis through a SDS-9% polyacrylamide gel and visualized by fluorography. Purified virions and L-particles are shown in lanes marked 1 and 2 respectively, whilst immunoprecipitated polypeptides are shown in lanes 3,4,5 and 6.

Section 3B Characterisation Of The HSV-1 Mutant ts1204

1. Introduction

The HSV-1 mutant *ts*1204 was previously shown to carry a mutation in the UL25 gene and as a result was unable to penetrate the cell membrane at the NPT (Addison *et al.*, 1984). This conclusion was based primarily on EM examination of thin sections of *ts*1204 infected cells and the observation that the *ts*1204 defect could be overcome by treatment of infected cells with a compound which promotes membrane fusion (ie. PEG)

Recently, there has been some doubt as to whether the inability of *ts*1204 to infect cells at the NPT resulted from a defect in cell entry. The aim of the present study was to re-examine the phenotype of *ts*1204 at the NPT and to further characterize the *ts*1204 defect.

2. Analysis Of ts1204-Infected Cell Polypeptides At The NPT.

To determine the nature of the *ts*1204 defect, HFL cells were either mock-infected or infected with *wt* HSV-1 or *ts*1204 and incubated at 38.5^0. At 7 hrs post-infection the cells were pulse-labelled for 30 min with 100μCi/ml of [^{35}S]-methionine and the cell lysates analyzed by SDS-PAGE and autoradiography (Figure 14). In contrast to *wt* HSV-1, *ts*1204 was unable to induce viral polypeptides at the NPT. Indeed, *ts*1204-infected cells showed a similar protein profile to that of mock-infected cells. Furthermore, *ts*1204 did not appear to induce host cell shut-off of protein synthesis. Consistent with previous findings, *ts*1204 infection of HFL cells at the NPT resulted in the accumulation of a 56,000 MW cellular protein, which was not induced in mock-infected or *wt*-infected cells.

In conclusion, these data clearly support previous findings that *ts*1204 is defective at a step prior to the onset of viral protein synthesis, at the stage of virion adsorption, penetration or uncoating of the viral genome.

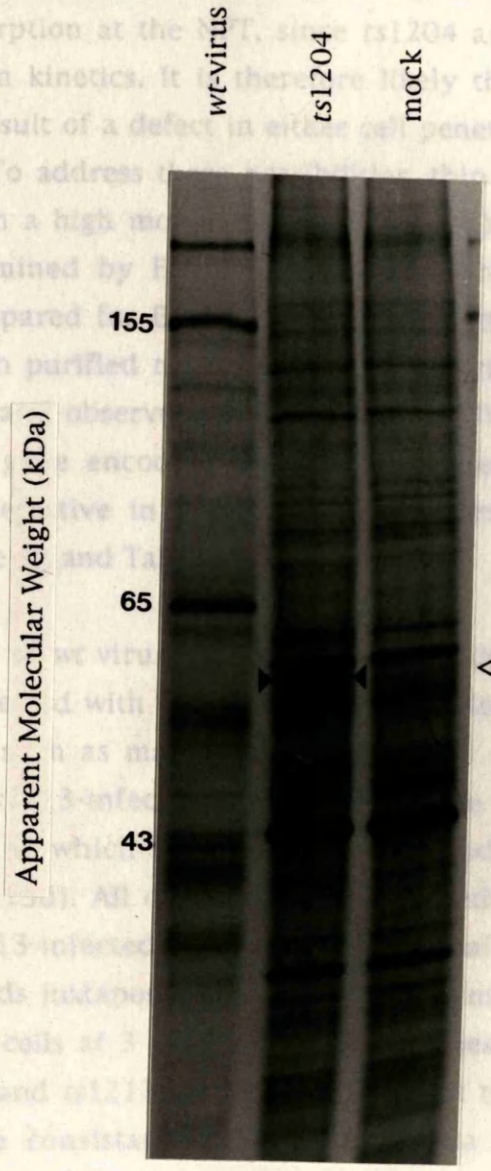


Figure 14. Induction Of Viral Polypeptides By ts1204 At The NPT.

HFL cells were mock-infected or infected with *ts*1204 or *wt* virus* and incubated at the NPT. At 7 hrs post-infection, the cells were pulse-labelled with 100μCi/ml of [^{35}S]-methionine for 30 min before being harvested and the cell lysates analyzed by SDS-PAGE and autoradiography. The cellular polypeptide induced in *ts*1204-infected cells is indicated by the arrow head.

*(moi of 20pfu per cell)

3. EM Analysis Of Thin Sections Of ts1204 Infected Cells

Addison *et al.* (1984) demonstrated previously that *ts*1204 was not defective in adsorption at the NPT, since *ts*1204 and *wt* HSV-1 exhibited very similar adsorption kinetics. It is therefore likely that the failure of *ts*1204 to infect cells is a result of a defect in either cell penetration, uncoating or both of these processes. To address these possibilities, thin section preparations of HFL cells infected with a high moi (about 200pfu/cell) of purified *ts*1204 virus at 39.5^0, were examined by EM. Infected cells were harvested at 3 hrs post-infection and prepared for EM as described in section 2B16. As controls, cells were infected with purified *ts*1213 or *wt* HSV-1 virions and these samples were similarly treated and observed in parallel. The HSV-1 mutant *ts*1213 carries a mutation in the gene encoding the large tegument protein Vmw273 and is believed to be defective in uncoating of the viral genome. The results are indicated in Figure 15 and Table 2.

In contrast to *wt* virus-infected cells, capsids were not observed within nuclei of cells infected with *ts*1204 or *ts*1213. Indeed, these cells showed little sign of infection such as margination of host-cell chromatin. Further analysis of *ts*1204- and *ts*1213-infected cells revealed the presence of capsids in the cytoplasm, some of which were found juxtaposed to the nuclear membrane (Figures 15c and 15d). All of the capsids observed in the 100 thin sections of *ts*1204- and *ts*1213-infected cell cytoplasm, contained viral DNA. In contrast, cytoplasmic capsids juxtaposed to the nuclear membrane were not observed in *wt* virus-infected cells at 3 hrs post-infection. These observations demonstrate that both *ts*1204 and *ts*1213 are unable to uncoat the viral genome at the NPT. These results are consistant with previous data suggesting that functional Vmw273 is required for the release of the viral genome from the nucleocapsid (Batterson *et al.*, 1983).

The present study failed to confirm that *ts*1204 has a defect in membrane penetration, inasmuch as capsids could be detected within the infected cell cytoplasm at the NPT. Although EM examination of thin sections of *ts*1204-infected cells at the NPT revealed numerous enveloped virions attached to the cell membrane, a similar phenomenon was observed in both *ts*1213- and *wt* virus-infected cells.

Figure 15. Electron Micrographs Of Mock, ts1204, ts1213 And wt-Infected HFL Cells At 39.5^0 At 3 Hrs Post-Infection.

HFL cells were infected with a high moi (200pfu per cell) of virus and incubated at 39.5^0. At 3 hrs post-infection the cells were harvested and prepared for electron microscopy as described in section 2B.16.

(C) cytoplasm

(CM) cell membrane

(N) nucleus

The bar represents 0.5μM.

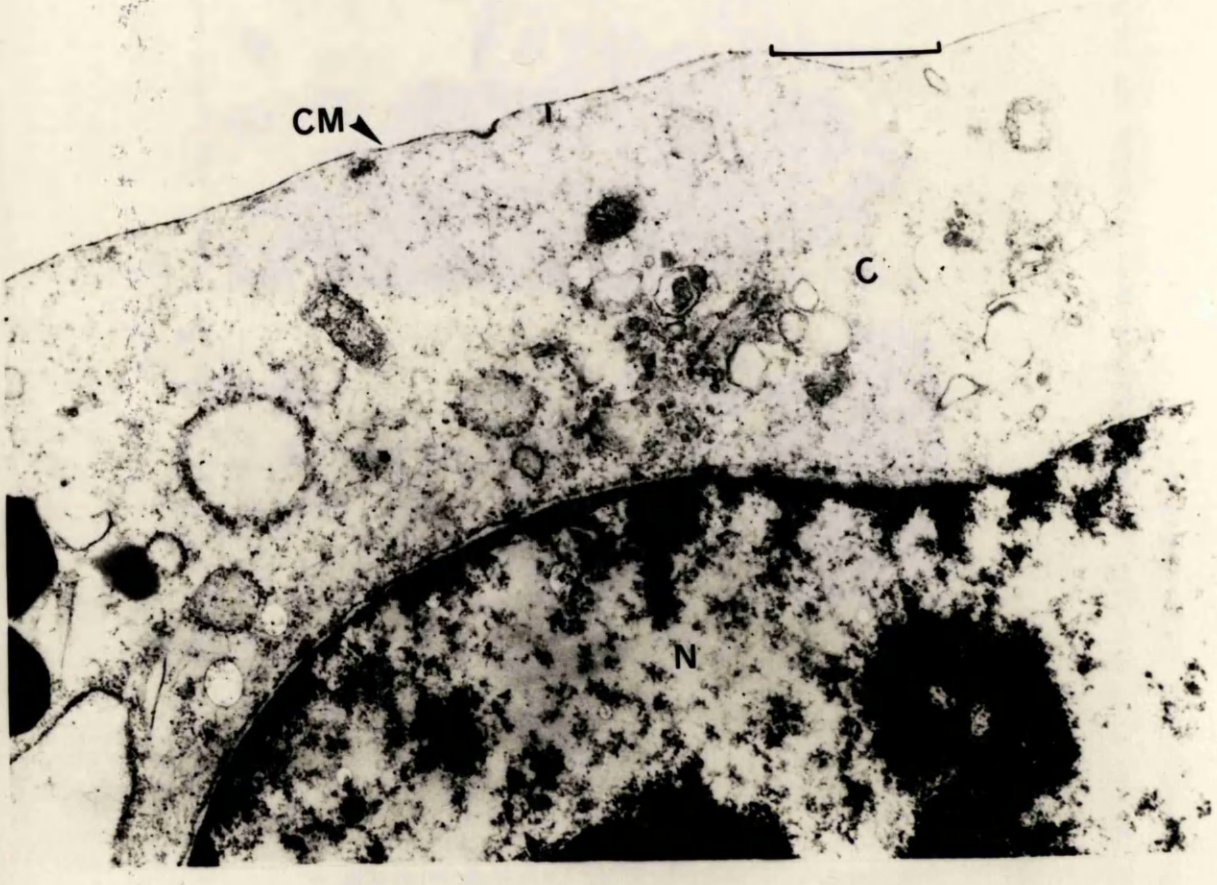


Figure 5a) Mock-Infected Cell

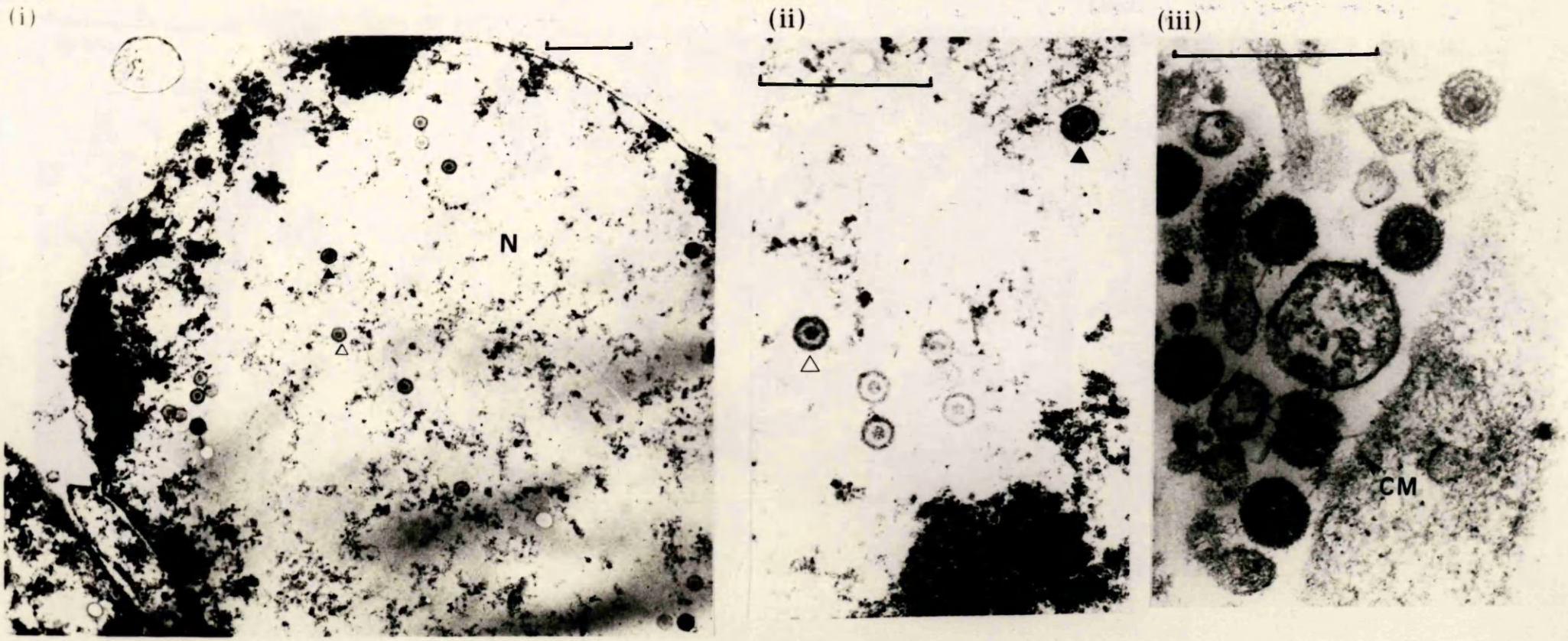


Figure 15b) Nucleus and Surface of wt Virus-Infected Cells.

(i) Empty and full capsids in the nucleus of infected cell. Filled arrows indicate full capsids, whilst empty capsids are indicated by empty arrows; (ii) Cluster of capsids in the nucleus; (iii) Enveloped *wt* virus on the surface of the cell.

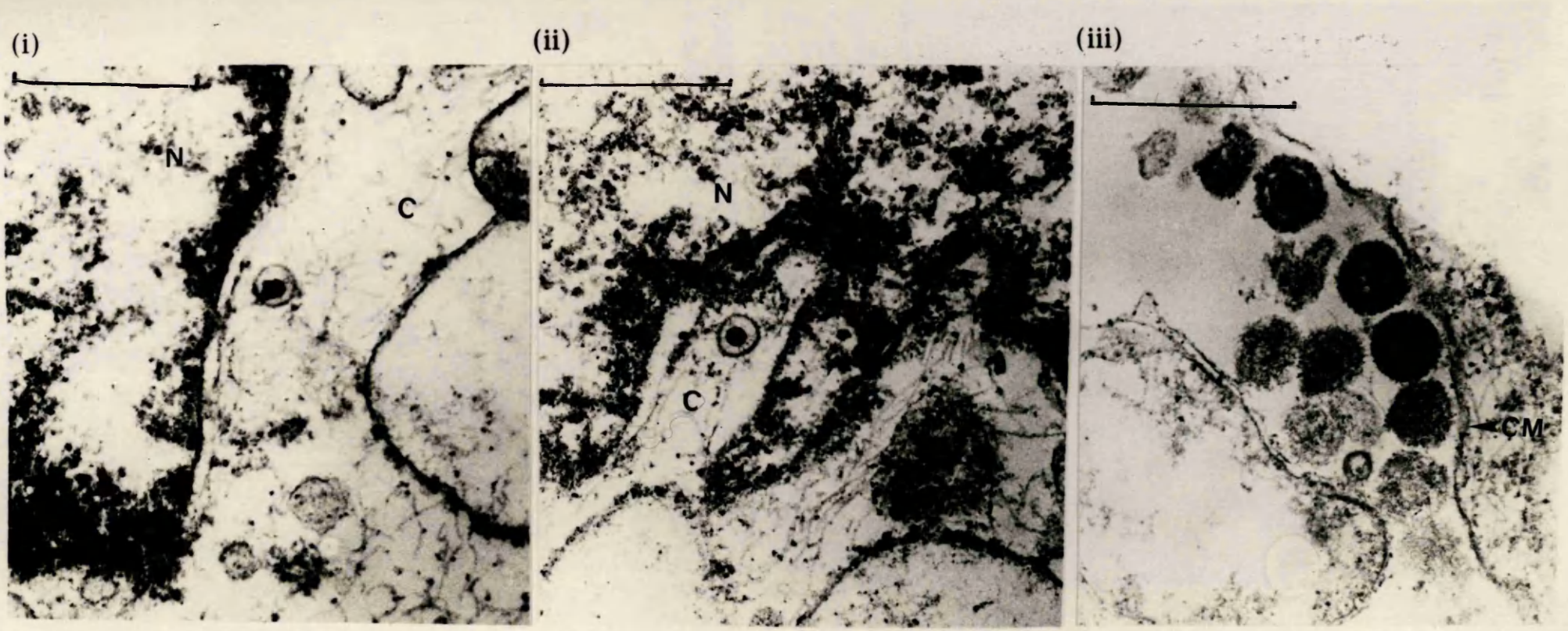


Figure 15c) Surface and Interior of ts1204-Infected Cells.

(i) and (ii) Cytoplasmic capsids juxtaposed to the nuclear membrane; (iii) Enveloped *ts*1204 virions on the surface of the cell.

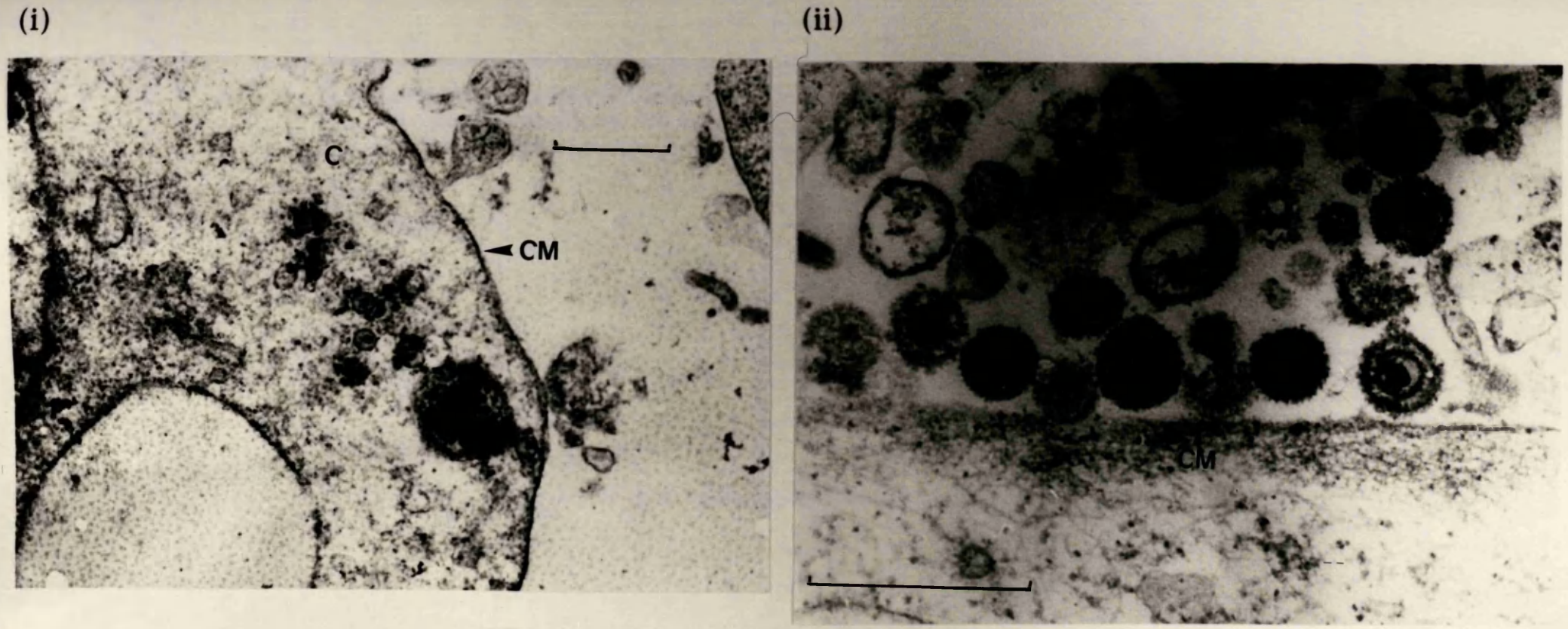


Figure 15d) Surface and Cytoplasm of ts1213-Infected Cells.

(i) Cluster of cytoplasmic capsids containing DNA; (ii) Enveloped *ts*1213 virions on the surface of the cell.

Virus-infected HFL Cells	Number of nuclear capsids per 100 sections	Number of cytoplasmic capsids per 100 sections	Number of sections in which capsids were observed
ts1204	0	11	9
ts1213	0	13	5
mock	0	0	0
wt virus	196	0	32

Table 2. Number Of Capsids In Virus-Infected HFL Cells.

HFL cells were infected with a high moi (200 pfu per cell) of *wt* virus, *ts*1204 or *ts*1213 and incubated at 39.5^{0}. At 3 hrs post-infection the cells were harvested and prepared for EM as described in section 2B.16. The number of cytoplasmic and nuclear capsids were counted in 100 thin sections of each virus-infected cell preparation.

4. PEG Treatment Of ts1204-Infected Cells

To confirm that the inability of *ts*1204 to establish a lytic infection resulted from a failure to release the viral genome from the capsid, virus-infected cells were treated according to the procedure described by Sarmiento *et al.* (1979). In this experiment, *ts*1204, *ts*1213 and *wt* virus-infected HFL cells incubated at 39.5^0, were treated with PEG, a compound which promotes membrane fusion, to allow entry of virus attached to the cell surface. Control samples were treated in a similar manner except PEG was omitted from all solutions. Following PEG treatment, virus-infected cells were incubated for a further 4 hrs at the NPT, and either prepared for indirect immunofluorescence using DBP antiserum and anti-mouse IgG conjugated to FITC (Figure 16) or pulse-labelled with [^{35}S]-methionine and the cell lysates analyzed by SDS-PAGE and autoradiography (Figure 17).

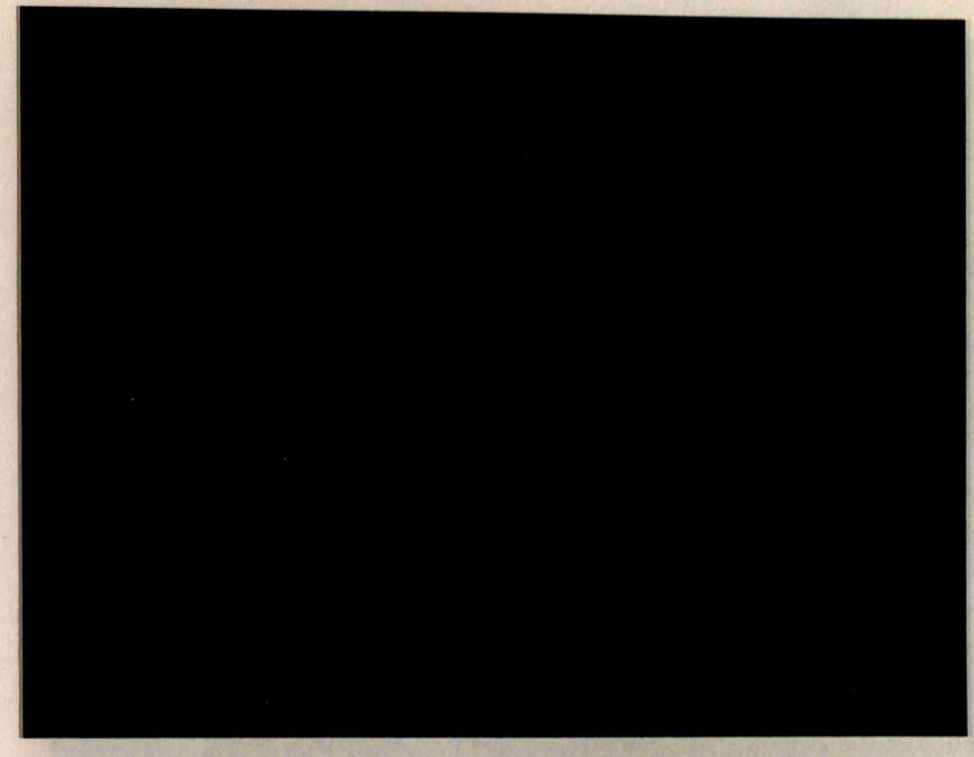
Greater than 95% of *wt* virus-infected cells that were untreated or treated with PEG showed bright nuclear fluorescence, whilst none of the *ts*1204- and *ts*1213-infected cells treated with PEG showed bright nuclear fluorescence. Instead, *ts*1204-infected cells treated with PEG resembled mock-infected cells or untreated *ts*1204 infected cells at the NPT, suggesting that PEG was not sufficient to overcome the *ts*1204 defect and that *ts*1204 was defective at a stage subsequent to membrane fusion ie. uncoating. In the absence of a positive control, it is conceivable that on this occasion PEG treatment was not sufficient to promote membrane fusion. However, the results obtained were consistently reproducible, suggesting that the above procedure was satisfactory.

SDS-PAGE analysis of PEG treated and untreated cell extracts confirmed data from the immunofluorescence assays, inasmuch as PEG treatment failed to overcome the *ts*1204 defect.

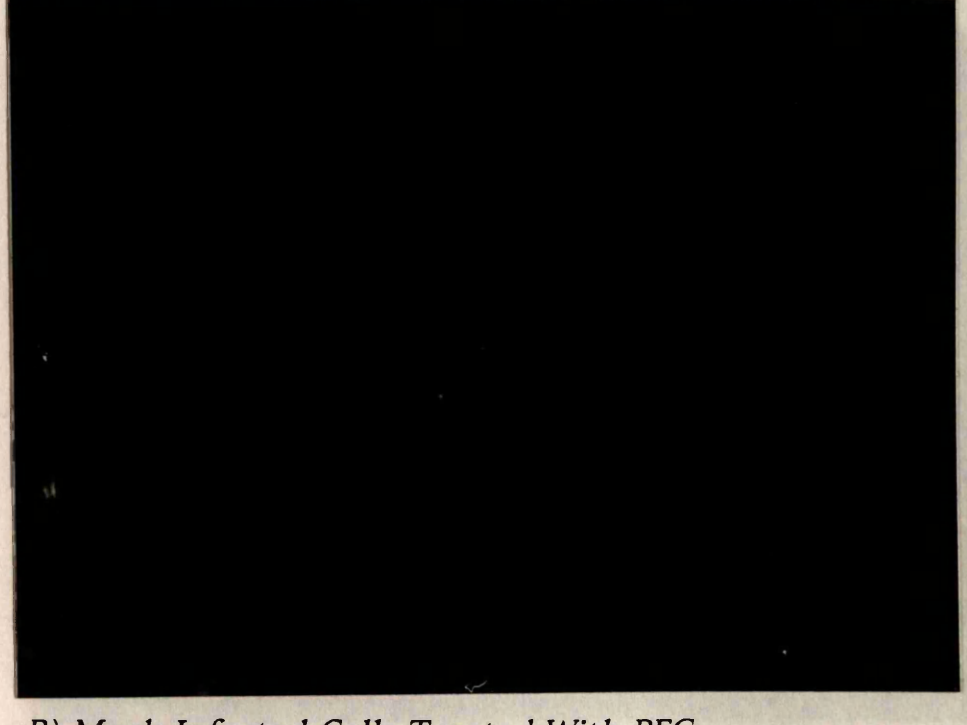
In conclusion, the results presented here indicate that *ts*1204 is unable to uncoat the viral genome at the NPT. These results fail to confirm data presented by Addison *et al.* (1984) who suggested that *ts*1204 was defective in cell entry. In addition, the work also suggests that the HSV-1 mutant *ts*1213, which contains a lesion in the gene encoding the large tegument protein Vmw273, is also defective in uncoating. This finding is in agreement with previous experiments showing that Vmw273 is required for the release of viral DNA from the capsid (Batterson *et al.*, 1983).

Figure 16. Analysis Of The Effects Of PEG Treatment On HSV-1 Infected Cells Using An Indirect Immunofluorescence Assay.

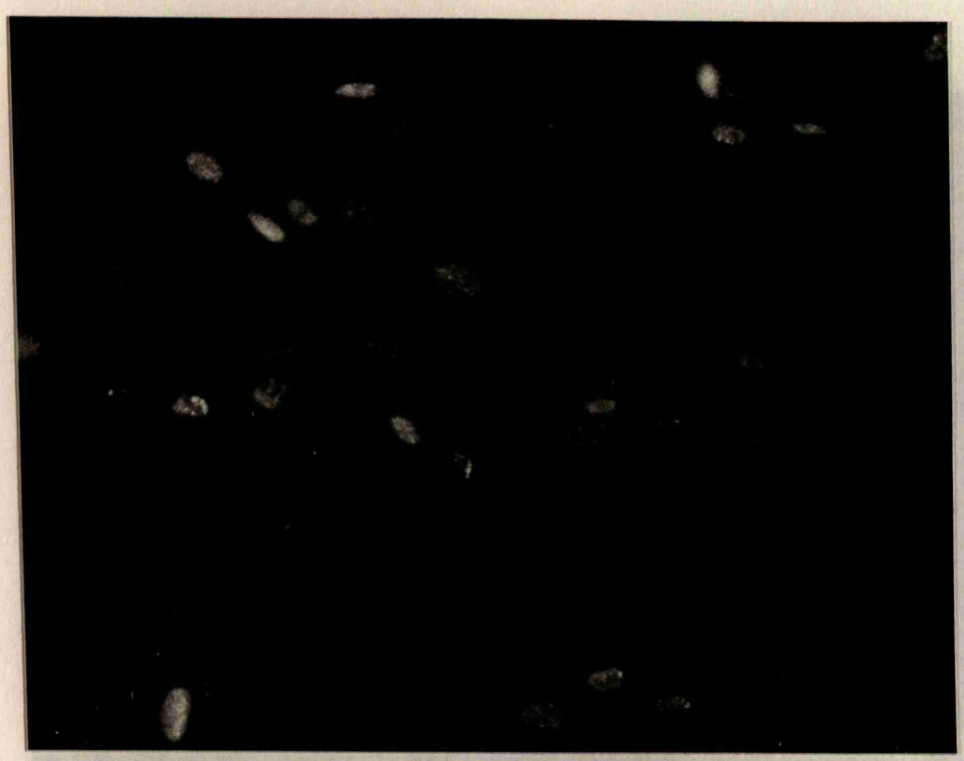
HFL cells were infected with 5pfu per cell of *ts*1204, *ts*1213 or *wt*-HSV-1 at 39.5^0 and treated with PEG according to the procedure described in section 2B.4. Control samples were treated in a similar manner except PEG was omitted from all solutions. Following PEG treatment, the virus-infected cells were incubated for a further 4 hrs at 39.5^0 then prepared for immunofluorescence. Immunofluorescence was performed using an antiserum specific for the DBP.



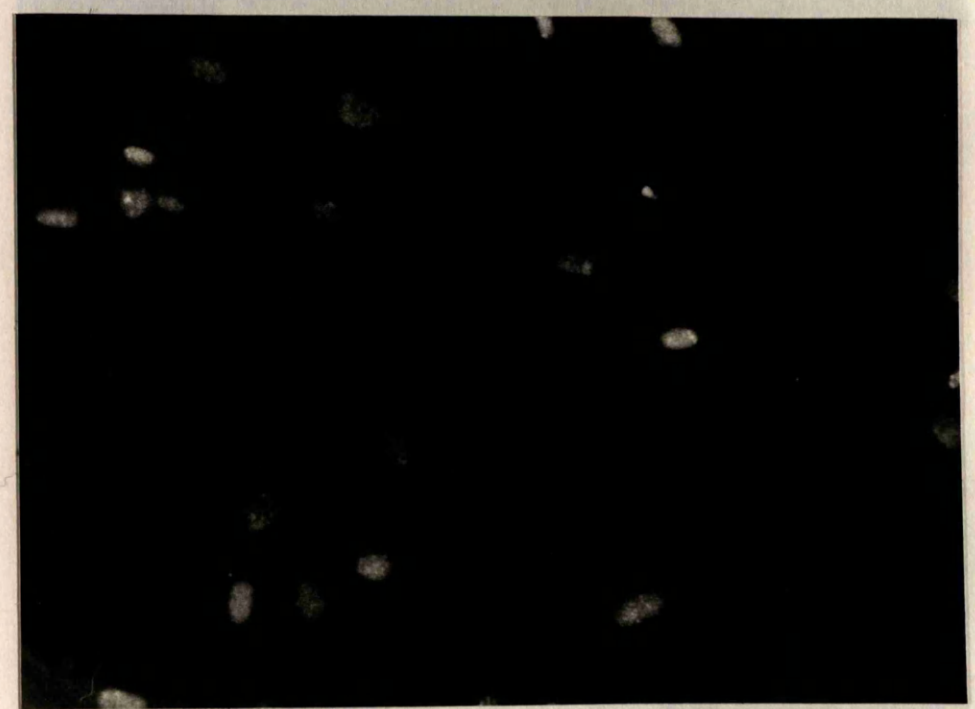
A) Mock-Infected Cells



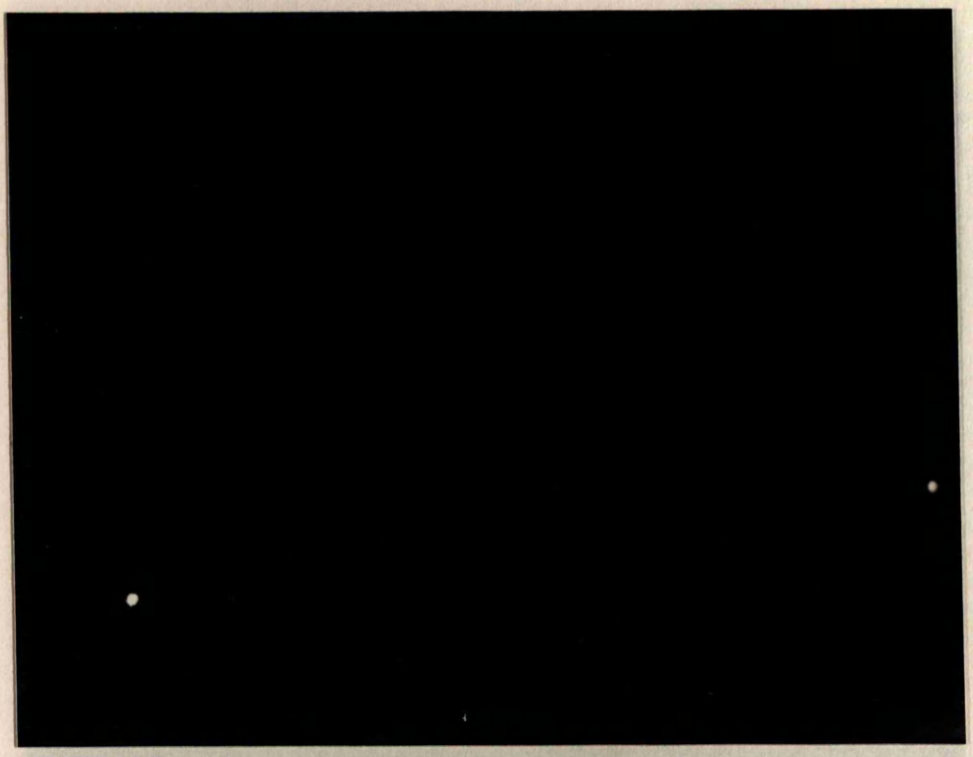
B) Mock-Infected Cells Treated With PEG.



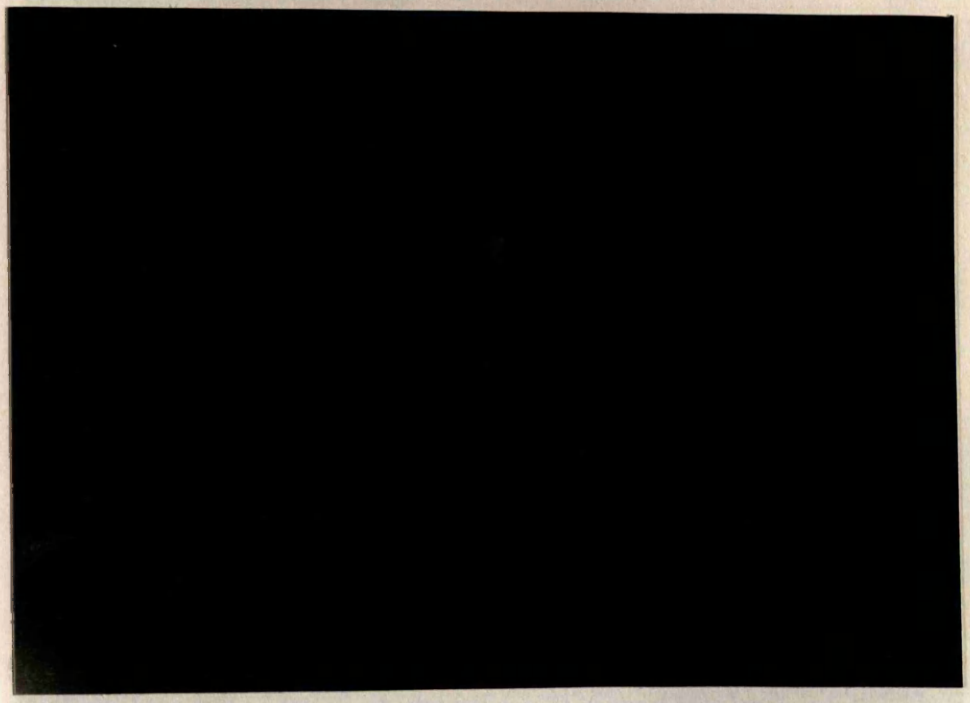
C) Wt Virus-Infected Cells



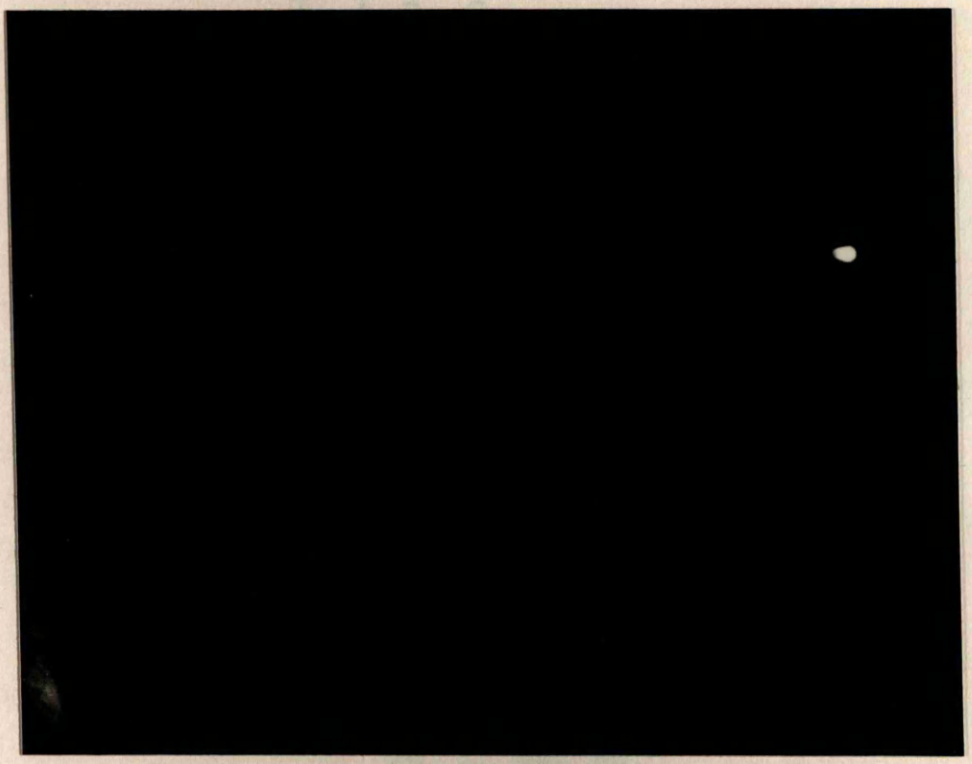
D)Wt Virus-Infected Cells Treated With PEG.



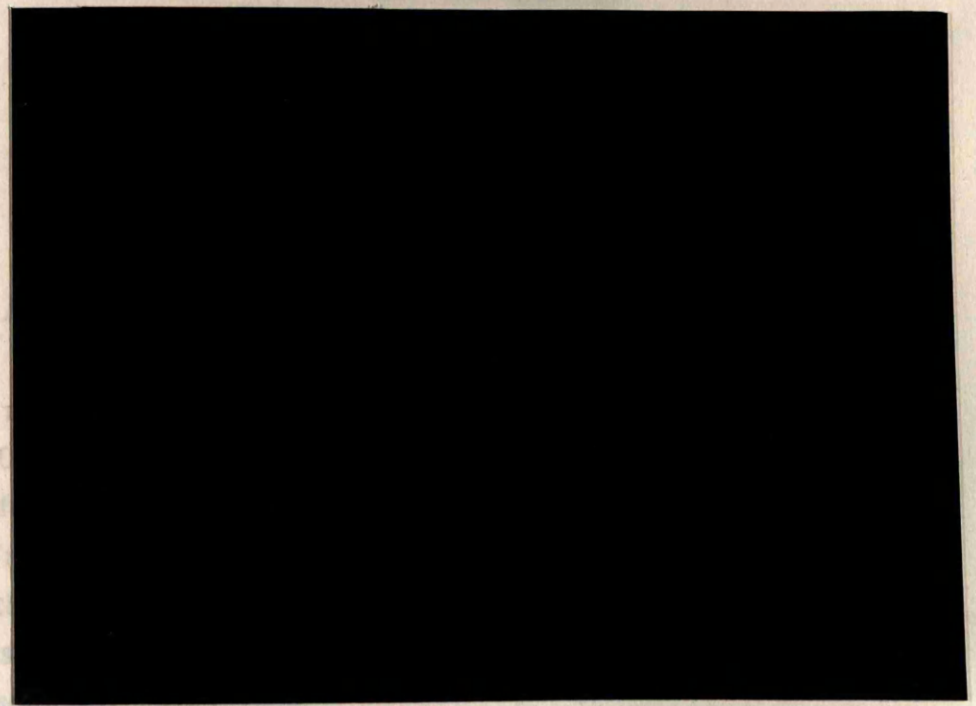
E) ts1204-Infected Cells



F) ts1204-Infected Cells Treated With PEG.



G) ts1213-Infected Cells



H) ts1213-Infected Cells Treated With PEG.

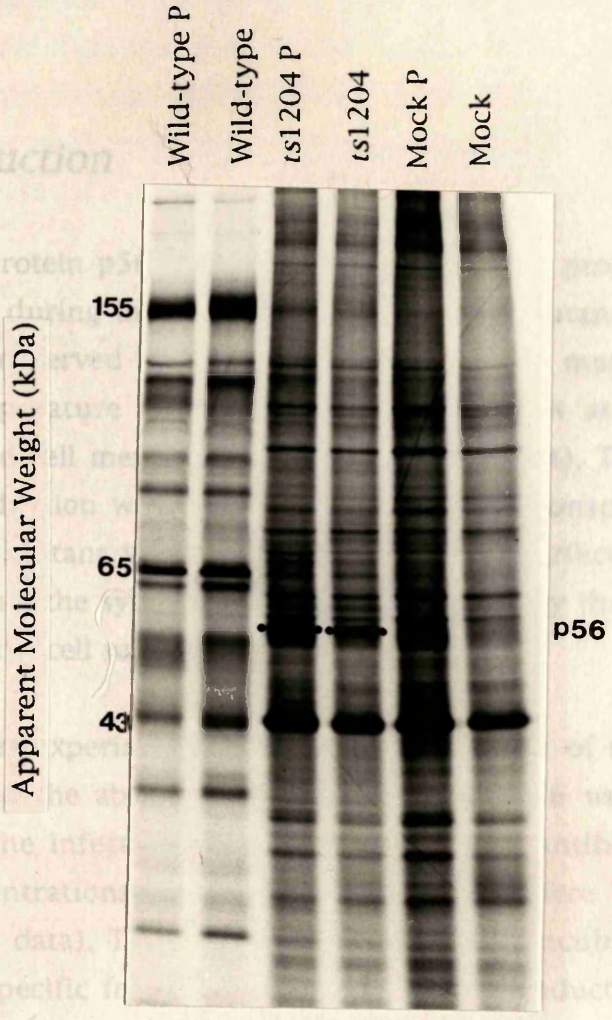


Figure 17. Effect Of PEG Treatment On ts1204 Infection.

HFL cell monolayers were mock-infected or infected with *ts*1204 or *wt* virus at a moi of 20pfu per cell at 39.5^0 ,then treated with PEG as described in section 2B.4. After a further 6 hrs incubation at 39.5^0, the cells were pulse-labelled with 100μCi/ml of [^{35}S]-methionine for 30 min and then harvested. The cell polypeptides were separated by SDS-PAGE and visualized by autoradiography. Control samples were prepared in a similar manner except PEG was omitted from all solutions. Lanes marked P represent cells treated with PEG.

Section 3.C Induction Of p56 In HFL Cells

1. Introduction

The protein p56 is an example of a cellular protein whose synthesis is upregulated during infection with an HSV-1 *ts* mutant (Preston, 1990). This protein was observed in HFL cells infected with the mutant *ts*1204 at the NPT. At this temperature it was believed that *ts*1204 attaches to but fails to penetrate the cell membrane (Addison *et al.*, 1984). These studies suggested that p56 induction was unlikely to result from nonspecific binding of large numbers of mutant virus particles to the cell surface. On this basis it was concluded that the synthesis of p56 was induced by the specific interaction of ts1204 with the cell surface components.

Further experiments to determine the effect of neutralising gH and gD antibodies on the ability of *ts*1204 to induce p56 were performed by pre-incubating the infecting virus with monoclonal antibody specific for gD or gH at concentrations which reduced the virus titre by about 10^4 (Preston unpublished data). The results showed that pre-incubation of the virus with antibodies specific for gH or gD prevented the induction of p56 by *ts*1204. Furthermore, treatment of cells prior to the addition of virus, with purified HSV-1 truncated gD, lacking the carboxyterminal transmembrane domain, also inhibited p56 induction by *ts*1204.

2. The Role Of gC In The Induction Of p56

Previous results have shown that virus neutralised by monoclonal antibody specific for gD, could still attach to cells, presumably via the interaction of gC with cell surface HS (Ligas and Johnson, 1988). Thus the observation that *ts*1204, neutralized by monoclonal antibodies specific to gD, failed to induce p56 at the NPT, suggested that gC was not important for the induction of p56.

To address this possibility, the *E. coli* β- galactosidase gene under the control of the SV40 early promoter was inserted into the unique *XbaI* site

within the cloned HSV-1 strain MP *BamHI* i fragment which contained a frame shift mutation in the gC gene (Preston unpublished data). The mutated fragment was recombined into *ts*1204. Two isolates, gC1*ts*1204 which made a truncated gC protein and gC2*ts*1204 which produced no detectable polypeptide recognized by the monoclonal antibody specific for gC, were analyzed for their ability to induce p56.

HFL cell monolayers were mock-infected or infected with either *ts*1204, gC1*ts*1204, gC2*ts*1204 or *wt* virus at a moi of 20 pfu per cell. The cells were incubated at the NPT for 7 hrs after which time they were pulse-labelled with 100uCi/ml of [^{35}S]-methionine for 30 min. Following incubation, the cells were harvested and the radiolabelled cell proteins were analyzed by SDS-PAGE (Figure 18). The results showed that both gC1*ts*1204 and gC2*ts*1204 were able to induce p56 in HFL cells at the NPT and it can be concluded therefore, that the interaction between gC and the cell surface is not important for the induction of p56.

3. Induction Of p56 By L-Particles

Previous reports suggested that p56 induction resulted from the interaction of *ts*1204 with the cell membrane. If this is the case then it is plausible to argue that the induction of p56 should also be mediated by *ts*1204 L-particles. To address this possibility, virions and L-particles were purified from *ts*1204-infected HFL cells according to the procedure described in section 2B.2.4, except that the infected cells were not incubated with [^{35}S]-methionine. L-particle preparations are normally contaminated with a small proportion of virions. To ensure that the extent of p56 induction could be attributed to attachment of L-particles and not contaminating virions to the cell surface, the percentage of contaminating virions in the L-particle preparation was determined by virus partical counting. Approximately 0.3% of the viral particles in the preparation were virions. In addition, the particle to pfu ratio of the purified virions, determined by particle counting and plaque assay, was calculated as 1 in 20 (results not shown). According to these data, it was assumed that the low numbers of virions contaminating the L-particle preparation would not be able to induce detectable levels of p56 at particle numbers representing a pfu of 20 or less.

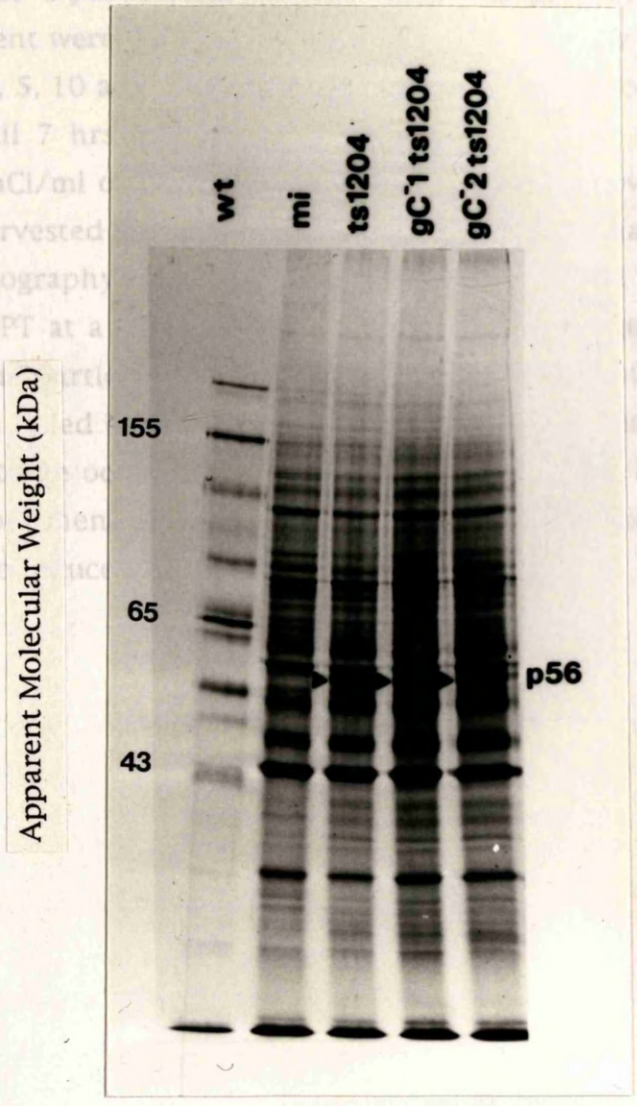


Figure 18. Induction Of p56 By gC Mutant ts1204.

HFL cell monolayers were mock-infected or infected with *wt*-virus, *ts*1204, gC1*ts*1204 or gC2*ts*1204 at a moi of 20 pfu per cell. The isolate, gC1*ts*1204 made a truncated gC protein whilst gC2*ts*1204 produced no detectable gC polypeptide that was recognized by a gC-specific monoclonal antibody (See text for details). The cells were incubated at the NPT for 7 hrs at which time the infected-cells were pulse-labelled with 50μCi/ml of [^{35}S]-methionine for 30 min. Following incubation, the cells were harvested and the radiolabelled proteins analyzed by SDS-PAGE and autoradiography.

HFL cell monolayers were mock-infected or infected with purified *ts*1204 or L-particles at the NPT. The numbers of L-particles used in the experiment were 20, 100, 200 and 400 particles per cell which represented a moi of 1, 5, 10 and 20 pfu per cell respectively. The cells were incubated at the NPT until 7 hrs post-infection, at which time the cells were pulse-labelled with 50uCi/ml of [^{35}S]-methionine for 30 min. Following incubation, the cells were harvested and the radiolabelled proteins analyzed by SDS-PAGE and autoradiography. The results show that the purified virions could induce p56 at the NPT at a moi of 20 pfu per cell, which is equivalent to approximately 400 virus particles per cell (Figure 19). In contrast, a similar number of L-particles failed to induce p56 at the NPT. This experiment was repeated on a further three occasions with identical results. Thus it can be concluded from this experiment that L-particles purified from *ts*1204-infected cells are unable to induce p56 at the NPT.

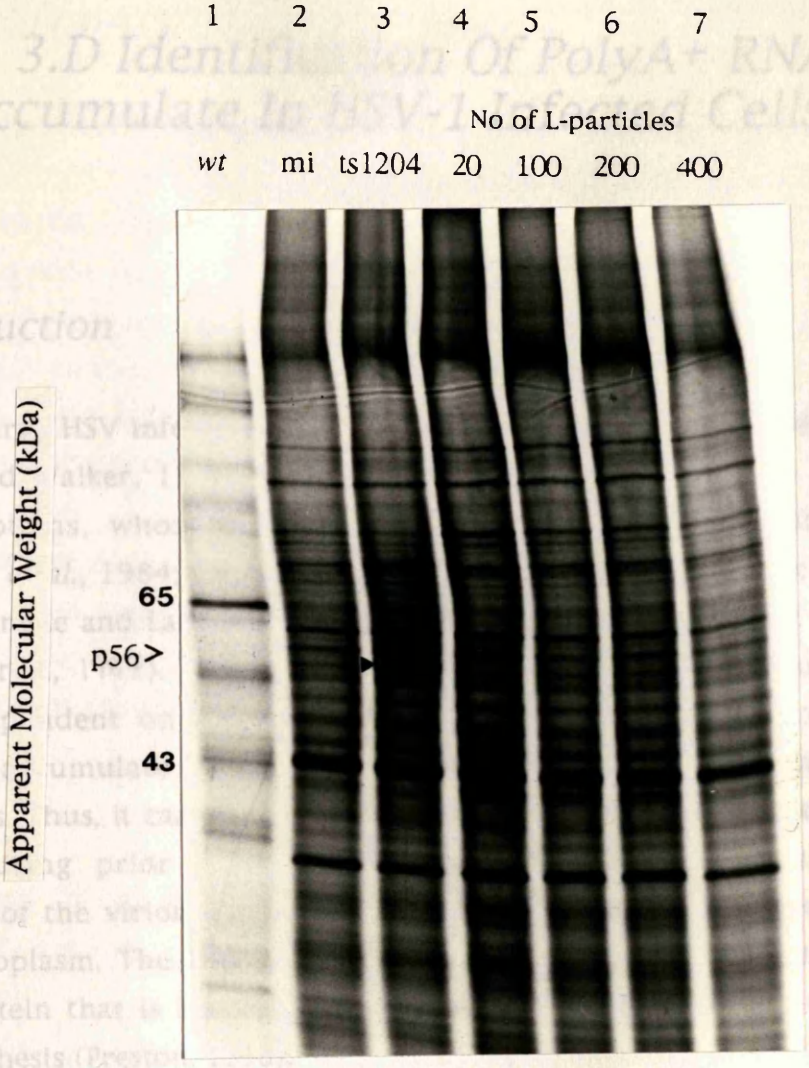


Figure 19. Induction Of p56 By L-Particles

Virions and L-particles were purified from *ts*1204-infected HFL cells as described in section 2B.2.4. HFL cells were mock-infected or infected with *ts*1204, *wt* virus or *ts*1204 L-particles. The number of L-particles added per cell were (lane 4) 20, (lane 5) 100, (lane 6) 200 and (lane 7) 400. The cells were incubated at the NPT until 7 hrs post-infection, at which time the infected cells were pulse-labelled with 50uCi/ml of [^{35}S]-methionine for 30 min. Following incubation the cells were harvested and the radiolabelled proteins were analyzed by SDS-PAGE and autoradiography. The cell protein p56 is indicated in lane 3. Lanes 1 and 2 represent *wt* virus- and mock-infected cell proteins respectively.

Section 3.D Identification Of PolyA+ RNAs That Accumulate In HSV-1 Infected Cells.

1. Introduction

In general, HSV infection leads to a decrease in host cell protein synthesis (Fenwick and Walker, 1978). However, the converse is true for a minority of cellular proteins, whose expression is upregulated in HSV-infected cells (LaThangue *et al.*, 1984; Macnab *et al.*, 1985; Kemp *et al.*, 1986a,b; Patel *et al.*, 1986; LaThangue and Latchman, 1988; Kennedy *et al.*, 1990; Hewitt *et al.*, 1991; Wakakura *et al.*, 1987). Although the expression of many HSV-induced cellular genes is dependent on viral protein synthesis, a minority of cell proteins appear to accumulate in conditions that restrict the induction of HSV polypeptides. Thus, it can be assumed that these cellular proteins are induced by events occuring prior to the onset of viral protein synthesis, such as attachment of the virion to the cell surface or entry of the nucleocapsid into the cell cytoplasm. The HSV-induced host cell protein p56 is an example of a cellular protein that is induced by events occuring prior to the onset of viral protein synthesis (Preston, 1990).

The aim of this investigation was to isolate cDNAs representing polyA$^+$ RNAs that accumulate in *ts*1204-infected HFL cells.

To isolate the full length cDNA clone encoding p56, a phagemid cDNA library was prepared from polyA$^+$ RNA purified from *ts*1204-infected cells. The cDNA library was screened by differential hybridization. Clones that showed preferential hybridization to the cDNA probe synthesized from polyA$^+$ RNA purified from *ts*1204-infected HFL cells rather than a similar probe synthesized from mock-infected cell polyA$^+$ RNA, were selected for further analysis.

2. Construction Of The cDNA Library

The polyA$^+$ RNA used as a template for cDNA synthesis was prepared from *ts*1204-infected cells at 7 hrs post-infection, at which time maximal levels of p56 synthesis occured (Preston, 1990). HFL cell monolayers on 90mm petri dishes were infected with *ts*1204 at a moi of 20 pfu per cell at the NPT. At 7 hrs post-infection, the cells were washed twice in ice cold PBS and scraped from the dishes using rubber policemen. The harvested cells were pelleted by centrifugation and polyA$^+$ RNA was extracted from the pelleted cells according to the procedures described in sections 2B.11.1 and 2B.11.2.

The success of cDNA cloning is determined largely by the quality of the polyA$^+$ RNA used as a template for first strand cDNA synthesis. It was therefore important to check the integrity of the polyA$^+$ RNA preparation. This was done using the following tests:

a) The Ability of the Bulk mRNA Preparation to Direct the Synthesis of Long Molecules of First Strand cDNA.

This was determined by synthesizing first strand cDNA using the polyA$^+$ RNA as a template and oligo $dT_{12\text{-}18}$ as a primer according to the procedure described in section 2B.11.4. The radiolabelled cDNA was analyzed on a 1% alkaline agarose gel and visualized by autoradiography (Figure 20). ^{32}P end-labelled *HindIII* fragments of λ DNA were used as MW markers. The single stranded cDNA appeared on the gel as a continuous smear from less than 300b to more than 5kb. If the polyA$^+$ RNA is high quality then ideally, the majority of the cDNA molecules should be 1-2kb in length. However, the results shown in Figure 20 showed that the the bulk of the cDNA was approximately 500b in length, which suggests that the polyA$^+$ RNA had undergone some degradation. Nonetheless, the ability of the polyA$^+$ RNA to direct the synthesis of significant amounts of long cDNA molecules (5kb) suggested that degradation of the polyA$^+$ RNA preparation was not extensive.

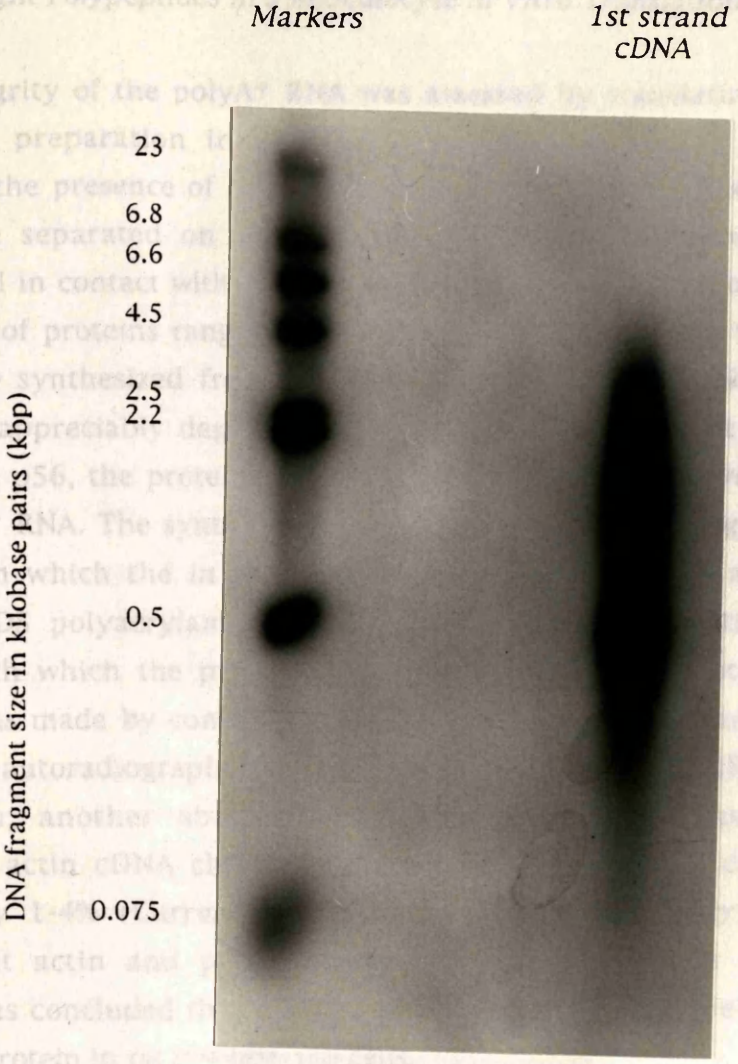


Figure 20. First Strand cDNA Synthesis.

HFL cells were infected with *ts*1204 at a moi of 20pfu per cell and incubated at the NPT for 7 hrs post-infection. The cells were harvested and the total polyA$^+$ RNA was prepared from the infected cells as previously described in sections 2B.11.1 and 2B.11.2. Poly A$^+$ RNA (0.5µg) was used as a template for the synthesis of single stranded cDNA according to the procedure described in section 2B.11.4. The ^{32}P-labelled first strand DNA was analyzed by electrophoresis through an alkaline 1% agarose gel using as markers ^{32}P end-labelled *Hind*III fragments of bacteriophage λ DNA.

b) The Ability of the PolyA$^+$ RNA Preparation to Direct the Synthesis of High Molecular Weight Polypeptides in a Reticulocyte In Vitro Translation System.

The integrity of the polyA$^+$ RNA was assessed by translating 0.5µg of the polyA$^+$ RNA preparation in a cell free rabbit reticulocyte lysate system (Promega) in the presence of 0.8mCi/ml of [^{35}S]-methionine. The radiolabelled proteins were separated on a SDS 9%-polyacrylamide gel and detected by placing the gel in contact with pre-flashed Kodak Xomat XS-1 film (Figure 21). A large number of proteins ranging in size from less than 10 kDa to greater than 100 kDa were synthesized from the polyA$^+$ RNA, confirming that the polyA$^+$ RNA was not appreciably degraded. In addition, the abundance of the polyA$^+$ RNA encoding p56, the protein of interest, was checked by *in vitro* translation of this polyA$^+$ RNA. The synthesis of cDNA was performed using a polyA$^+$ RNA preparation in which the *in vitro* synthesis of p56 appeared as a prominent band on a SDS polyacrylamide gel (Figure 21). A crude estimation of the frequency with which the p56 polyA$^+$ RNA occured in the total polyA$^+$ RNA population was made by comparing the intensity of the p56 polypeptide band shown in the autoradiograph of *ts*1204-infected cell proteins (Figure 21) with that of actin, another abundant cellular protein by densitometry. The frequency of actin cDNA clones in a fibroblastoid cell line cDNA library is approximately 1-4% (Current Protocols in Molecular Biology, 1987). Thus, assuming that actin and p56 each have an equal number of methionine residues, it was concluded that the p56 protein comprised between 1 and 5% of the total cell protein in *ts*1204-infected cells.

2.1 Strategy For cDNA Cloning

Previous studies have reported that about 2x10^5 recombinants must be screened (using nucleic acid probes) to detect a low abundance polyA$^+$ RNA (Sambrook *et al.*, 1989). In this study it was estimated that the polyA$^+$ RNA encoding p56 is relatively abundant, comprising at least 1% of the total polyA$^+$ RNA population used to construct the cDNA library. Thus, on this basis it is conceivable that for every 100 recombinants generated by cDNA cloning, at least one of these will represent the polyA$^+$ RNA encoding p56. However, it is usually regarded as sensible to aim for a library that contains at least 5 times more recombinants than the total indicated by the lowest abundance estimate.

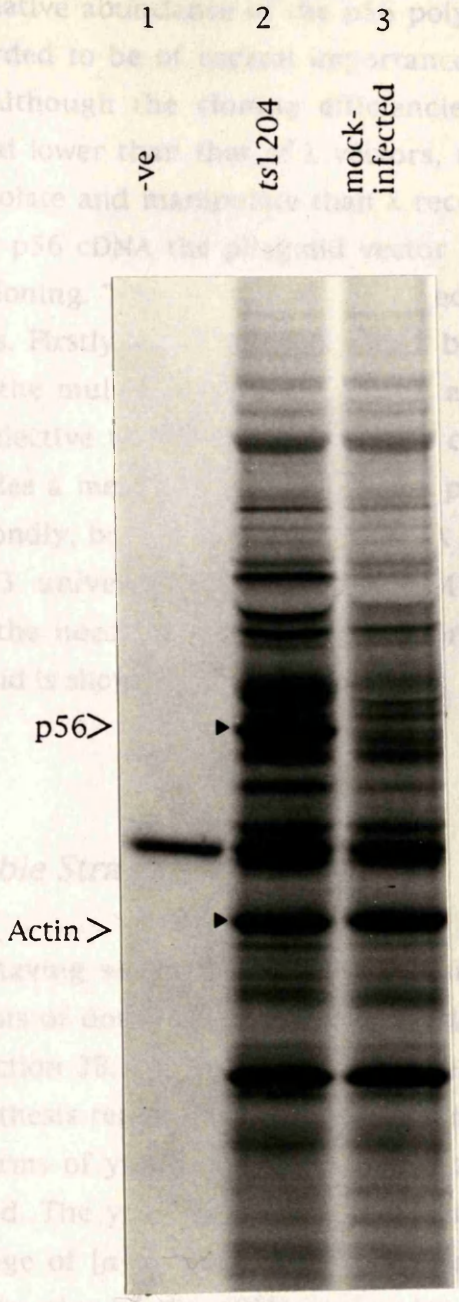


Figure 21. Translation Of PolyA+ RNA In Reticulocyte Lysates.

HFL cells were mock-infected or infected with *ts*1204 at a moi of 20pfu per cell and incubated at the NPT. At 7 hrs post-infection, the cells were harvested and cytoplasmic RNA was extracted from the infected cells essentially as described by Preston *et al.*, (1979) (refer to section 2B.11.1). PolyA+ RNA was selected from the cytoplasmic RNA using Hybond-mAP Messenger Affinity Paper. The polyA+ RNA was translated *in vitro* as described in section 2B.11.3. The translated proteins were separated on a SDS-9% polyacrylamide gel and visualized by autoradiography. In the *ts*1204-infected cell track the position of the cellular protein p56 is indicated by the arrow head. Lane 1 represents a negative RNA control, whilst lanes 2 and 3 show the *in vitro* translation products of *ts*1204-infected cell RNA and mock-infected cell RNA respectively.

Considering the relative abundance of the p56 polyA$^+$ RNA, a high cloning efficiency was not regarded to be of central importance in the isolation of the corresponding cDNA. Although the cloning efficiencies of phagemid vectors are usually 10 to 50-fold lower than that of λ vectors, the resulting cDNAs are considered simpler to isolate and manipulate than λ recombinants. Thus for the purpose of isolating the p56 cDNA the phagmid vector pT7T3 18U (Pharmacia) was chosen for cDNA cloning. This phagemid was selected as a cloning vector for a number of reasons. Firstly, pT7T3 18U contains both the T7 and T3 RNA promoters which flank the multiple cloning site. The arrangement of the RNA promoters allows the selective transcription of either cDNA strand to produce RNA *in vitro* and provides a means of identifying the protein product encoded by the cDNA insert. Secondly, both strands of the cDNA insert can be sequenced directly using the M13 universal primer or the M13 reverse sequencing primer, thus, avoiding the need for further cloning procedures. A diagram of the pT7T3U 18U phagemid is shown in Figure 22.

2.2 Synthesis Of Double Stranded cDNA.

The polyA$^+$ RNA, having satisfied the above requirements, was used as a template for the synthesis of double stranded cDNA using the Amersham cDNA synthesis system (see section 2B.11.5 and Figure 5). The incorporation of [α^{32}P]-dCTP into the cDNA synthesis reactions allowed the monitoring of the efficiency of cDNA synthesis in terms of yields of cDNA synthesized, and the size of the cDNA molecules obtained. The yields of cDNA synthesized were determined by calculating the percentage of [α^{32}P]-dCTP incorporation into DNA (results not shown). To determine the size of the cDNA molecules produced during cDNA synthesis, small aliquots of the first and second cDNA reaction mixtures were analyzed by electrophoresis through a 1% alkaline agarose gel using as markers ^{32}P end-labelled *HindIII* fragments of λ. The radiolabelled cDNA molecules were visualized by autoradiography (Figure 23). The results indicated that synthesis of first strand cDNA using oligo dT_{12-18} and the polyA$^+$ RNA preparation as a template had occured, shown by the smear of radiolabelled first strand cDNA which ranged in size from less than 300bp to more than 2.5kb in length. Similarly, second strand cDNA synthesis made by self priming, appeared as a smear of radiolabelled cDNA from less than 500bp to approximately 6kbp in length.

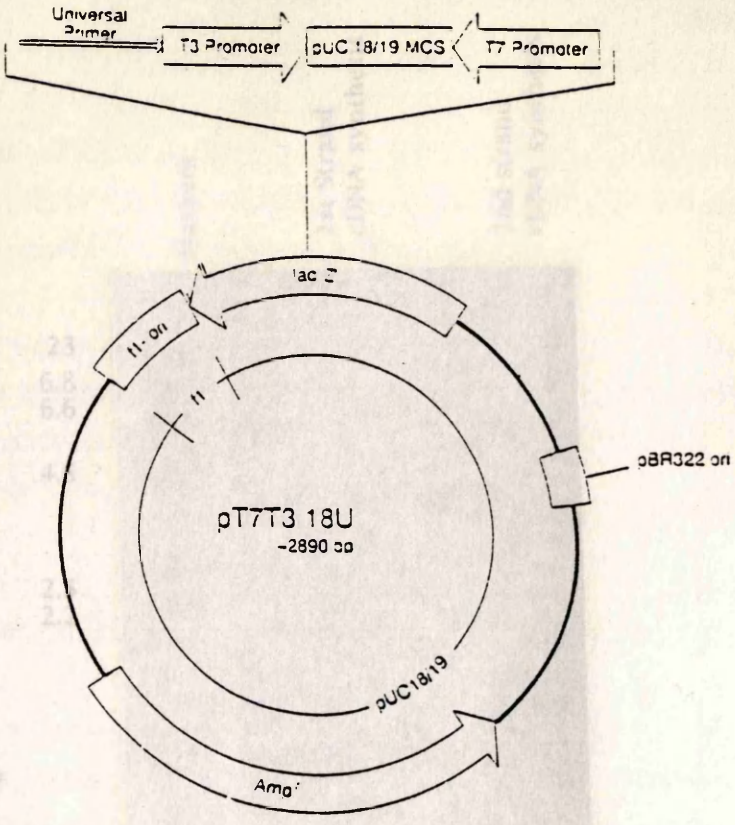


Figure 22. A Diagrammatic Representation Of The pT7T3 18U Cloning Vector.

The pT7T3 18U multifunctional phagemid is shown containing the T7 and T3 RNA promoters flanking the pUC18 multiple cloning site in opposite orientation. This arrangement allows transcripts to be generated from either strand of cDNA *in vitro* translation experiments. The multiple cloning site is located within the *lacZ'* gene. The position of the *Amp*^{r} gene and the origins of replication are indicated.

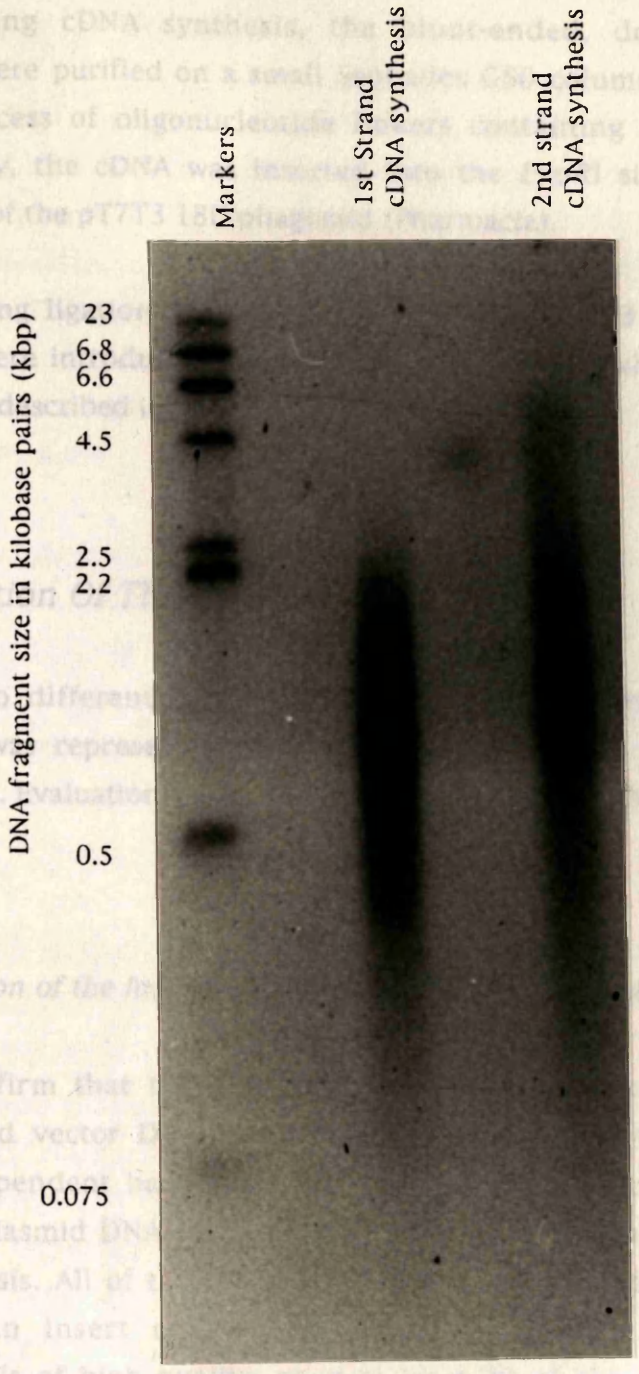


Figure 23. First And Second Strand cDNA Synthesis.

Poly A$^+$ RNA (3µg), extracted from *ts*1204-infected HFL cells as previously described, was used to synthesize ^{32}P-labelled double stranded cDNA molecules using the Amersham cDNA Synthesis System according to the protocol described in section 2B.11.5. The products of the first and second cDNA synthesis reactions were analyzed by electrophoresis through a 1% alkaline agarose gel and visualized by autoradiography. ^{32}P end-labelled *Hind*III fragments of bacteriophage λ DNA were used as molecular weight markers.

Following cDNA synthesis, the blunt-ended, double stranded cDNA molecules were purified on a small Sephadex G50 column and then ligated to a 100-fold excess of oligonucleotide linkers containing phosphorylated *EcoR*I ends. Finally, the cDNA was inserted into the *EcoR*I site within the multiple cloning site of the pT7T3 18U phagemid (Pharmacia).

Following ligation of the cDNA inserts into pT7T3 18U, the recombinant molecules were introduced into competent *E. coli* (DH5α). The cDNA library was amplified as described in section 2B.11.9.

2.3 Evaluation Of The cDNA Library

Prior to differential screening, the library was evaluated to determine whether it was representitive of the total polyA$^+$ RNA population in *ts*1204-infected cells. Evaluation of the cDNA library was done in two ways.

a) Examination of the Inserts of 24 Randomly Chosen Clones.

To confirm that the bacteria contained recombinant cDNA rather than recircularized vector DNA, "miniprep" DNA was prepared from 24 randomly chosen independent bacterial clones picked from the primary transformation plate. The plasmid DNAs were cleaved with *EcoR*I and analysed by agarose gel electrophoresis. All of the 24 clones contained cDNA inserts, however, only 11 contained an insert of greater than 1kbp. Ideally, if the polyA$^+$ RNA preparation is of high quality, then at least 20 of the 24 recombinant clones should have inserts with an average size of 1kb or more. The fact that the majority of the cDNA inserts were less than 1kb in length was not suprising since previous data suggested that the polyA$^+$ RNA preparation used for cDNA synthesis had undergone some degradation.

From the analysis of the above clones, the number of transformants per microgram of polyA$^+$ RNA template was calculated as $1X10^5$.

b) Screening for the Actin cDNA

Since actin comprises approximately 1-4% of the total protein in a fibroblastoid cell line, a similar proportion of recombinant bacterial clones might be expected to hybridize to an actin hybridization probe. Bacterial plasmid DNAs were immobilized onto a nylon membrane and hybridized to a ^{32}P-labelled actin DNA probe. Of the 480 clones screened in this manner, 21 of these hybridized to the radiolabelled actin probe (results not shown). Thus the freqency with which the actin cDNA occurs within the cDNA library is approximately 4.4%.

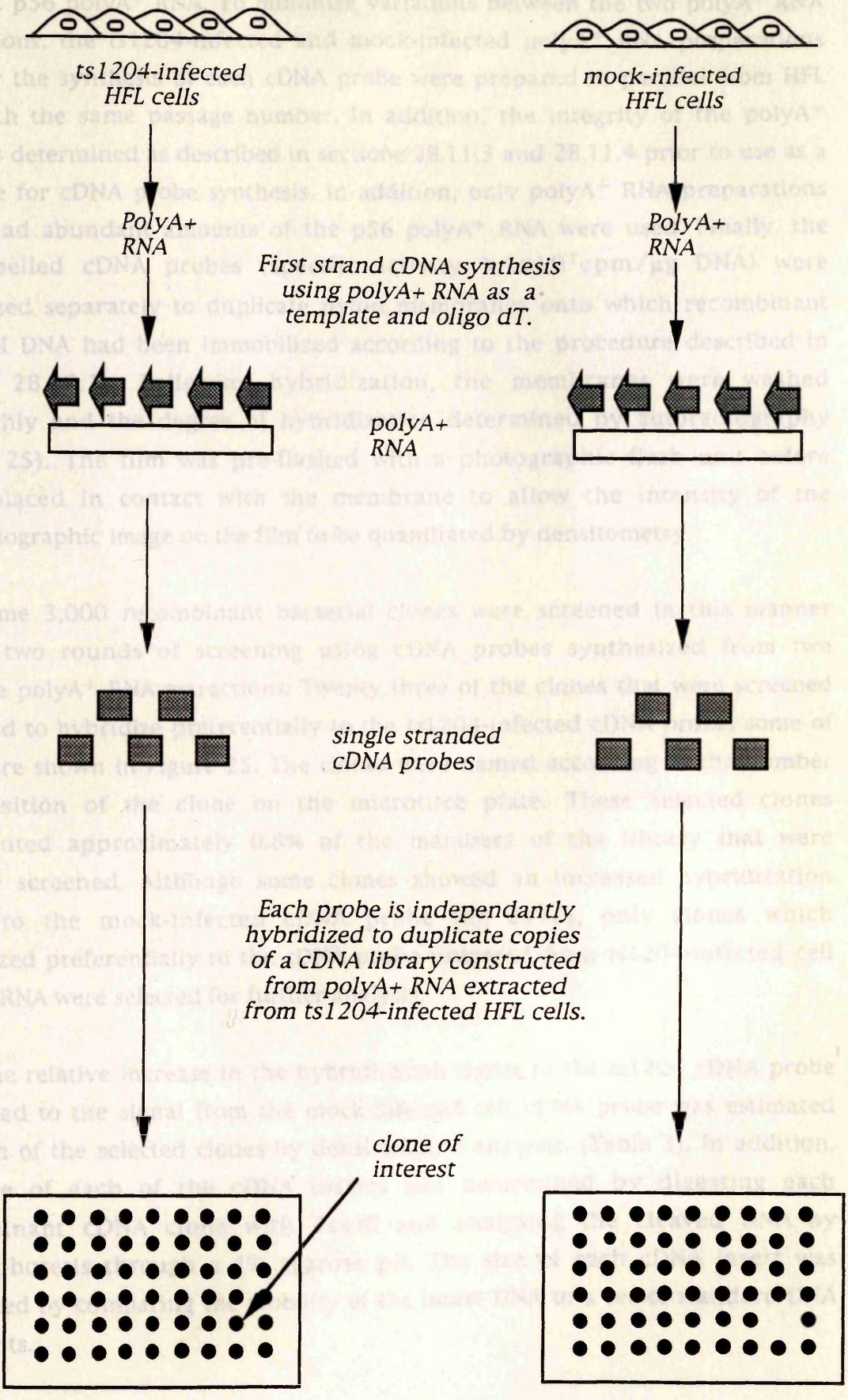
Clearly, degradation of the polyA$^+$ RNA preparation may result in a reduction in the probability of isolating a full length p56 cDNA. However, since *in vitro* translation of the p56 polyA$^+$ RNA resulted in a major band on an SDS-polyacrylamide gel, it is likely that there is an abundance of the full length p56 polyA$^+$ RNA in the total RNA preparation used for cDNA synthesis.

3. Identification Of cDNA Clones Of Interest

The differential filter hybridization method was used to screen the cDNA library because it lended itself perfectly to the isolation of cDNAs representing polyA$^+$ RNAs which accumulate in *ts*1204-infected cells. Usually, the use of this screening method is limited by the abundance of the cDNA of interest within the library. Previous differential screening studies have been successful in detecting cDNAs representing polyA$^+$ RNAs comprising not less than 0.5% of the total polyA$^+$ RNA population (Foster *et al.*, 1982; Schutzbank *et al.*, 1982; Linzer and Nathans, 1983). Since the p56 polyA$^+$ RNA was estimated to occur at a frequency of at least 1% in the total polyA$^+$ RNA population, it was hoped that this screening method would be sufficient for the isolation of the corresponding p56 cDNA.

A summary of the principles of differential hybridization are given below and illustrated in Figure 24. Briefly, ^{32}P-labelled first strand cDNAs were synthesized using polyA$^+$ RNA extracted from mock- and *ts*1204-infected HFL cells as templates. The majority of the cDNA molecules should corresponded to cellular polyA$^+$ RNAs whose concentrations were not appreciably altered by *ts*1204-infection. However, a minority of cDNAs should be copied from polyA$^+$

Figure 24. Differential Screening



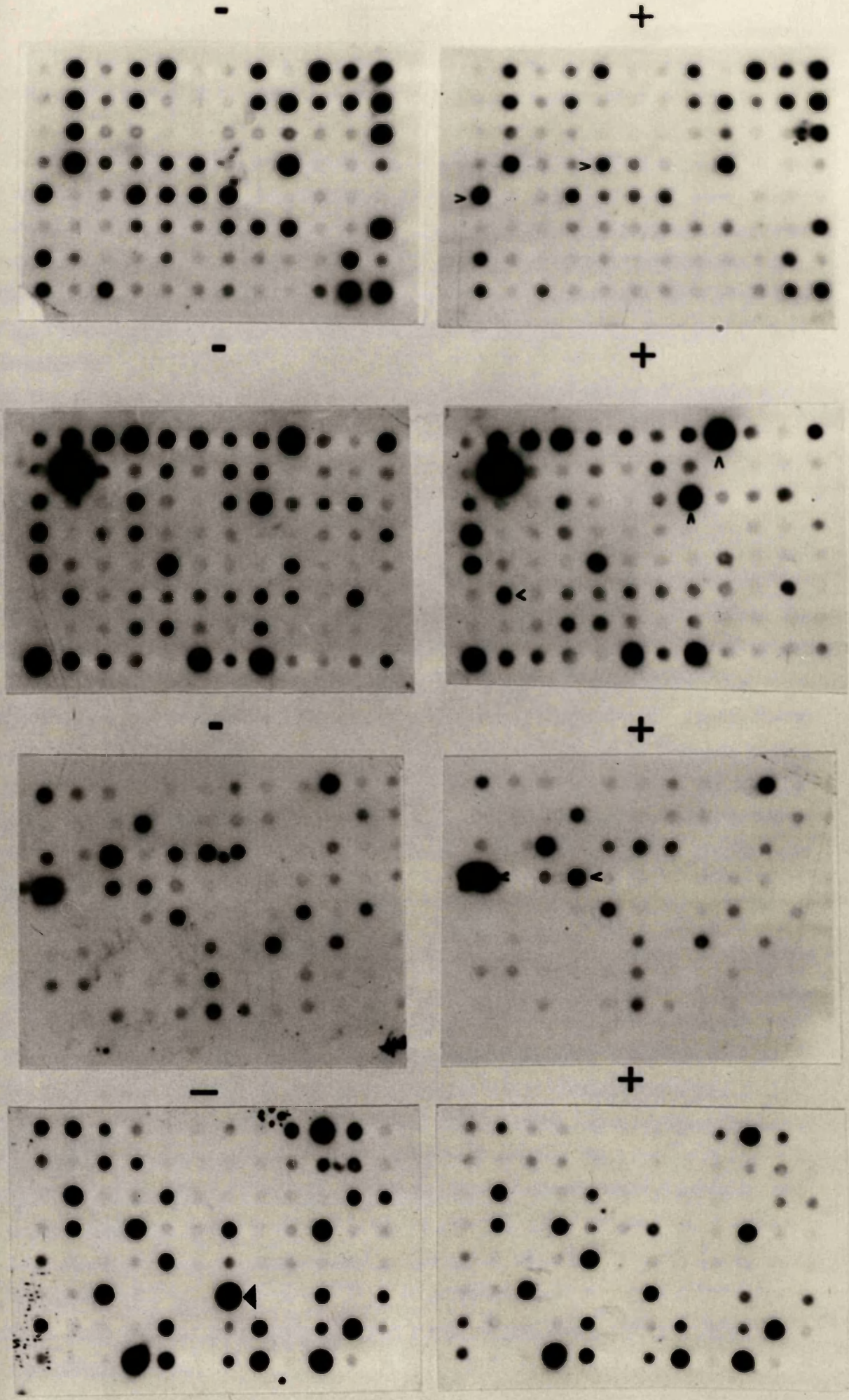
RNAs whose concentrations were significantly increased in *ts*1204-infected HFL cells, eg. p56 polyA$^+$ RNA. To minimize variations between the two polyA$^+$ RNA populations, the *ts*1204-infected and mock-infected polyA$^+$ RNA preparations used for the synthesis of each cDNA probe were prepared in parallel from HFL cells with the same passage number. In addition, the integrity of the polyA$^+$ RNA was determined as described in sections 2B.11.3 and 2B.11.4 prior to use as a template for cDNA probe synthesis. In addition, only polyA$^+$ RNA preparations which had abundant amounts of the p56 polyA$^+$ RNA were used. Finally, the radiolabelled cDNA probes (specific activity $>1x10^7$cpm/μg DNA) were hybridized separately to duplicate nylon membranes onto which recombinant bacterial DNA had been immobilized according to the procedure described in section 2B.13.1c. Following hybridization, the membranes were washed thoroughly and the degree of hybridization determined by autoradiography (Figure 25). The film was pre-flashed with a photographic flash unit before being placed in contact with the membrane to allow the intensity of the autoradiographic image on the film to be quantitated by densitometry.

Some 3,000 recombinant bacterial clones were screened in this manner during two rounds of screening using cDNA probes synthesized from two separate polyA$^+$ RNA extractions. Twenty three of the clones that were screened appeared to hybridize preferentially to the *ts*1204-infected cDNA probe, some of which are shown in Figure 25. The clones were named according to the number and position of the clone on the microtitre plate. These selected clones represented approximately 0.8% of the members of the library that were initially screened. Although some clones showed an increased hybridization signal to the mock-infected cDNA probe (eg 217F), only clones which hybridized preferentially to the cDNA probe prepared from *ts*1204-infected cell polyA$^+$ RNA were selected for further analysis.

The relative increase in the hybridization signal to the *ts*1204 cDNA probe compared to the signal from the mock-infected cell cDNA probe was estimated for each of the selected clones by densitometric analysis. (Table 3). In addition, the size of each of the cDNA inserts was determined by digesting each recombinant cDNA clone with *EcoR*I and analysing the cleaved DNA by electrophoresis through a 1% agarose gel. The size of each cDNA insert was calculated by comparing the mobility of the insert DNA to a set of standard DNA fragments.

Figure 25. Screening Of The cDNA Library

Individual bacterial clones were grown at 37^0 in microtitre wells containing L-broth supplemented with 100μg/ml of ampicillin. A volume of 50μl of each bacterial suspension was spotted onto nylon membranes using a dot blot apparatus. The bacteria were lysed and the plasmid DNA denatured and fixed to the membrane. Recombinant plasmid DNAs from the lysed bacteria were hybridized to ^{32}P-labelled single stranded cDNA probes synthesized from cytoplasmic polyA$^+$ RNA extracted from *ts*1204-infected (+) or mock-infected (-) HFL cells. The clones were named according to the number and position of the clone on the membrane. The arrows indicate a particular clone that demonstrated differential hybridization. Clone 217F which hybridized preferentially to the mock-infected cDNA probe is indicated by the filled arrow.



The relative hybridization of each of the 23 selected clones to the *ts*1204 cDNA probe as well as the size of each cDNA insert are summarized in Table 3.

The majority of the selected clones showed only a marginal increase in the hybridization signal to the *ts*1204-infected cDNA probe. Futhermore, these clones hybridized weakly to the *ts*1204 cDNA probe, which suggests that they represent minor messages within the total cytoplasmic polyA$^+$ RNA population. Interestingly, recombinants that hybridized almost exclusively to the *ts*1204 cDNA probe appeared to represent abundant messages within the polyA$^+$ RNA population.
In the present study, only cDNA clones which hybridized to abundant polyA+ RNAs were analyzed further.

4. Identification Of Homologous cDNA Recombinants

The polyA$^+$ RNA encoding p56 is believed to be an abundant species within the total *ts*1204-infected HFL cell polyA$^+$ RNA population (1-5%). Therefore, the cDNA encoding p56 might be expected to occur at a similar frequency in the cDNA library. Consequently, of the 3000 recombinant bacterial clones that were screened, a conservative estimate for the number of recombinant cDNA clones encoding p56 would be approximately 30. Furthermore, it is feasible that a proportion of the 23 clones that show preferential hybridization to the *ts*1204-cDNA probe may represent identical polyA$^+$ RNAs. To address this possibility, DNA cross-hybridization analysis of each of the 23 selected clones was performed.

Recombinant phagemid DNA was prepared from each of the 23 bacterial clones showing preferential hybridization to the *ts*1204-cDNA probe according to the protocol described in section 2B.13.7. The insert DNA from each recombinant was purified from the vector DNA by cleaving the DNA with *EcoR*I and separating the cleaved DNA fragments by electrophoresis through a 0.7% agarose gel. Following electrophoresis, the cDNA inserts were purified from the agarose gel using Geneclene II (described in section 2B.13.4). Finally, the purified cDNA inserts were used as templates to synthesize individual ^{32}P-labelled single stranded DNA probes by primer extension (section 2B.10.1). Each radiolabelled probe was hybridized to 200ng of each of the 23 recombinant phagemid DNAs immobilized on nylon membranes. Following hybridization, the filters were washed thoroughly and the extent of hybridization was determined by autoradiography.

Clone	Insert size (kbp)	Relative hybridisation
34H	0.7	3.4
32F	0.12	2.8
35H	0.11	1.5
84D	1.2	13.6
131E	2.2	11.4
131H	2.5	15.8
1310H	0.075	17.6
162F	0.2	1.75
168C	1.6	3.2
169A	0.3	4.0
177E	0.6	3.75
1711G	1.1	3.3
191D	0.8	3.1
194D	1.6	4.5
208E	0.4	2.0
2012D	1.6	3.2
218E	1.6	5.1
2212H	1.6	6.0
339E	1.6	5.74
633H	0.8	15.2
634C	0.55	12.3
634E	0.4	10.7
637H	0.325	14.0

Table 3. Summary Of ts1204-Inducible cDNA Clones

Insert sizes were measured on 1% agarose gels by comparing the relative mobilities of DNA fragments generated by *EcoRI* cleavage of the recombinant cDNAs, with a set of standard fragments. The relative hybridisation signal of each clone to the cDNA probe prepared from *ts*1204-infected HFL cell PolyA$^+$ RNA compared to that synthesized from mock-infected polyA$^+$ RNA was estimated by densitometric analysis.

The majority of the recombinant cDNA clones appeared to be unique, inasmuch as they did not cross-hybridize with any of the remaining 22 recombinants (results not shown). Nonetheless, DNA cross-hybridization analysis allowed the identification of two distinct groups of cDNA molecules that represented polyA$^+$ RNAs which appeared to accumulate in *ts*1204-infected HFL cells. According to the number of bacterial clones in each group, the groups were designated C4 and C6 respectively. The degree of cross hybridization between the the C4 and C6 cDNA clones is illustrated in Figures 26 and 27 respectively.

The group of cDNA clones designated C6 consisted of six members, 194D, 168C, 2012D, 2212H, 218E and 339E. With the exception of 339E, all of the clones were isolated during the first round of screening. The individual ^{32}P-radiolabelled probes cross-hybridized with equal intensity to each member of C6, which suggested that the C6 clones shared an identical proportion of homologous sequences in the cDNA. Indeed, digestion of the C6 recombinant cDNAs with *EcoR*I and analysis of the cleaved DNA by electrophoresis through a 1% agarose gel revealed that each of the C6 recombinants contained cDNA insert of approximately 1.5kb in size (Table 3).

Confirmation that each of the above recombinants had homologous cDNA inserts was obtained from sequence data analysis of each clone by the dideoxy chain termination method using M13 universal and reverse primers. The sequencing products were analyzed by denaturing polyacrylamide gel electrophoresis and visualized by placing the dried gel in contact with X-Omat XS-1 film (Figure 28). Figure shows an autoradiograph of the 3' termini DNA sequences of the C6 cDNA clones. The sequence lying upstream of the A-rich region, which probably represents the polyA$^+$ tail, is identical in each of the clones. This finding confirms that the C6 cDNA clones represent identical polyA$^+$ RNAs.

shown in figure 28

Comparison of the 150 bp of the 3' terminus of the C6 cDNA with existing sequences stored in the GenEMBL Database using the FASTA program of the GCG sequence analysis software, failed to establish the identity of the protein encoded by the C6 cDNA. Similarly, the amino acid sequence predicted from translation of the 200bp DNA sequence in all three ORFs did not show any significant homology to sequences stored in the Swiss-Prot Protein Sequence

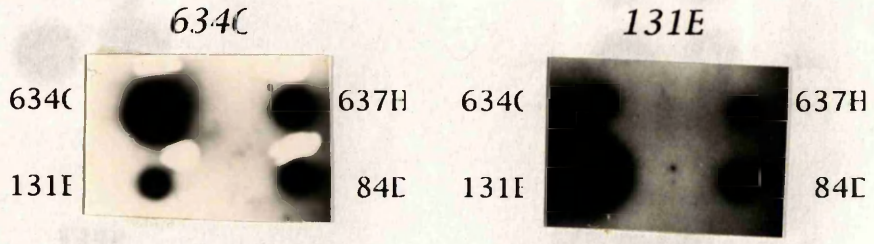


Figure 26. DNA Cross-Hybridisation Analysis Of The C4 cDNA Clones

Recombinant cDNA was prepared from the C4 bacterial clones as described in section 2B.13.7. Approximately 200ng of each of the recombinant cDNAs was denatured and immobilized onto a nylon membrane using a dot-blot manifold as indicated. Each cDNA on the membrane was hybridized to ^{32}P-labelled clone specific DNA probes prepared by primer extension (probes are shown in italics above each membrane). Following hybridisation, the membranes were washed thoroughly and the degree of hybridisation determined by autoradiography. The degree of hybridisation of the 634C- and 131E-specific DNA probes to each of the recombinant C4 cDNAs is shown in this figure.

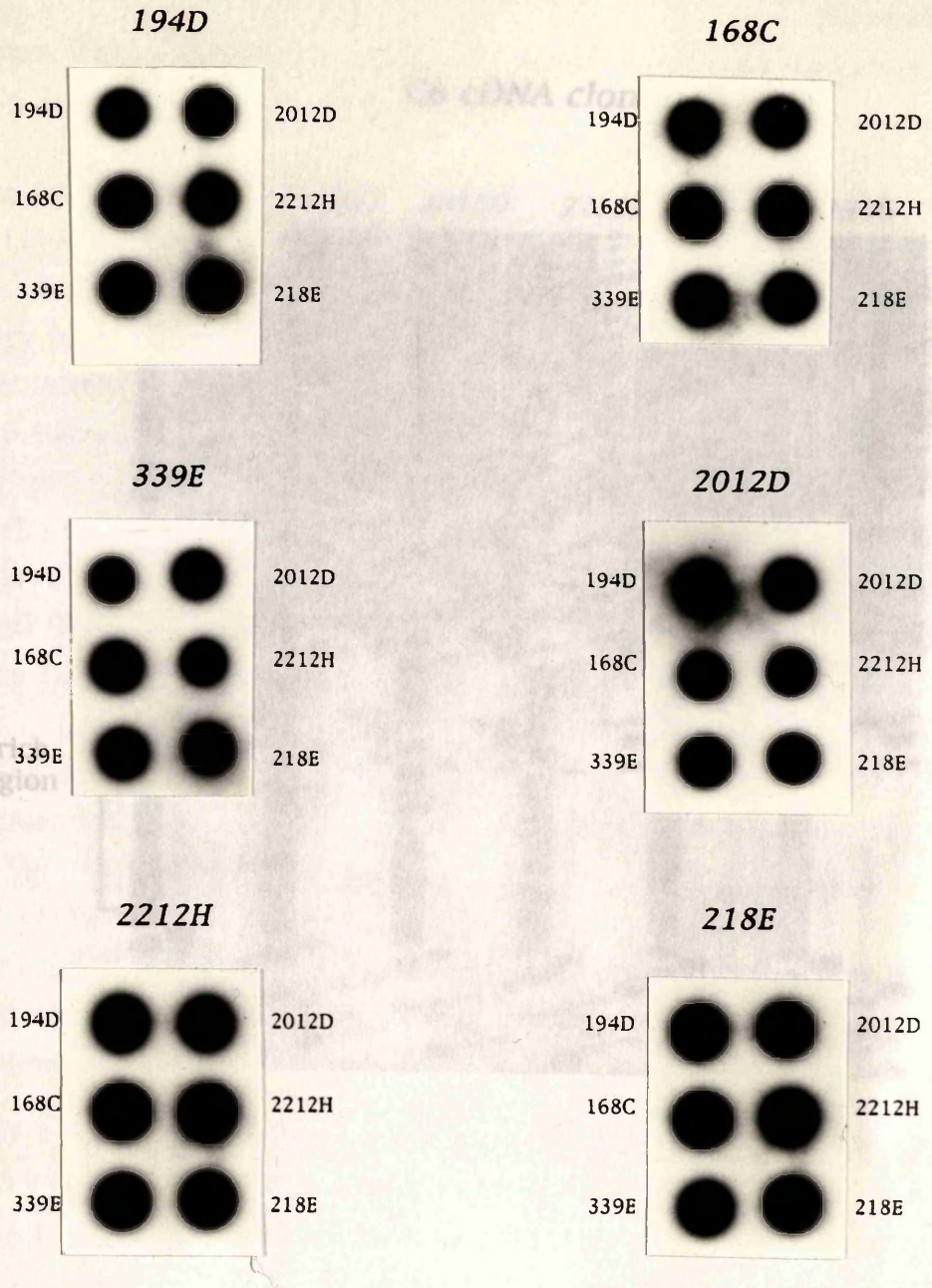


Figure 27. DNA Cross-Hybridisation Analysis Of The C6 cDNA Clones.

Recombinant cDNA was prepared from the bacterial clones 194D, 2012D, 168C, 339E, 2212H and 218E as described in section 2B.13.7. Approximately 200ng of each of the recombinant cDNAs was denatured and immobilized onto a nylon membrane using a dot-blot manifold as indicated. A further 5 membranes were prepared in a similar manner. Each cDNA on the membrane was hybridized to ^{32}P-labelled clone specific DNA probes prepared by primer extension (probes are shown in italics above each membrane). Following hybridisation, the membranes were washed thoroughly and the degree of hybridisation was determined by autoradiography.

[1] AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTAAATTACTAAAA
AAAAAAGTTTATCTTTTTGATTTCTTGACTTGATAGTGTCCTAA
AAGAGTGACGTCTCGACTCTACAGATCTCTCTACTCCTTTAGTAG
CTACCATTATTAGGCTAGTACGAGCA [157]

Figure 28. DNA Sequence Of The 3' Terminus Of The C6 cDNA Clones

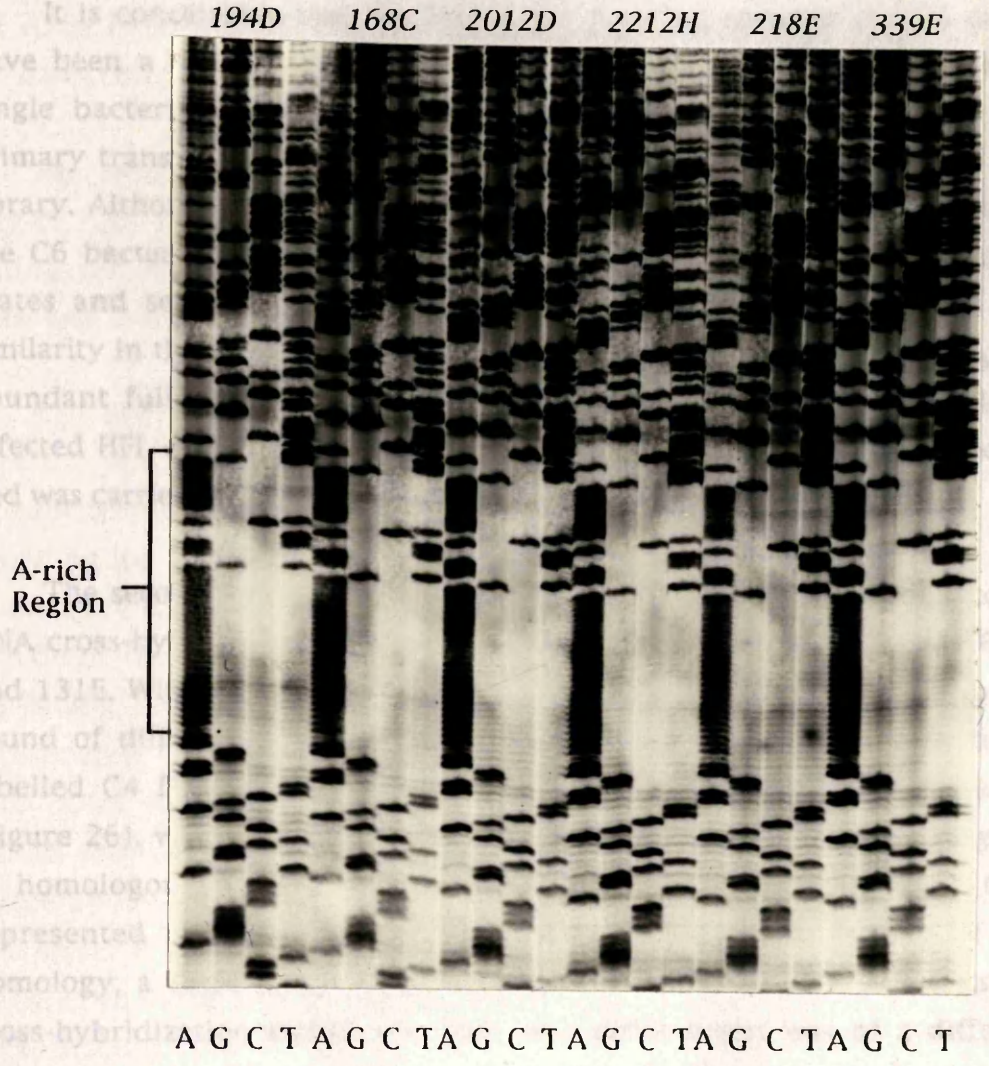


Figure 28. Sequence Analysis Of The C6 cDNA Clones.

Plasmid DNA was prepared from the C6 cDNA clones and sequenced by the dideoxy-chain termination method using "Sequenase" T7 polymerase and the sequencing solutions supplied with the Sequenase System Plus kit. The sequencing products were analyzed by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. The A-rich region of each of the cDNA clones is indicated.

Database. These findings introduce the possibility that the C6 cDNA encodes a previously uncharacterized cellular protein.

It is conceivable that the homology observed between the C6 clones may have been a consequence of cross-contamination of adjacent colonies with a single bacterial colony during the isolation of bacterial colonies from the primary transformation plate or during subsequent manipulations of the cDNA library. Although this possibility cannot be ruled out, it is unlikely since all of the C6 bacterial clones were isolated from individual primary transformation plates and separate microtitre plates. Another explanation for the observed similarity in the size of the C6 cDNA molecules is that the C6 cDNAs represent an abundant full length polyA$^+$ RNA whose expression is upregulated in *ts*1204-infected HFL cells. Further analysis of the C6 clones is described in section 3D.5 and was carried out using clone 194D.

The second group of cDNA clones that appeared to be related according to DNA cross-hybridization analysis, consisted of four members, 634C, 637H, 84D and 131E. With the exception of 84D, these clones were isolated during the first round of differential screening. Unlike the C6 cDNA clones, none of the ^{32}P-labelled C4 DNA probes cross-hybridized with equal intensity to each other (Figure 26), which suggested that the C4 cDNA clones shared only a proportion of homologous DNA sequence. Although it was possible that the C4 cDNAs represented related polyA$^+$ RNAs which shared varying degrees of sequence homology, a more likely explanation for the variations in the intensity of the cross-hybridization signals was that each cDNA insert was of a different size. Digestion of the C4 recombinant cDNAs with *EcoR*I and analysis of the cleaved DNA by electrophoresis through a 1% agarose gel, revealed that the latter proposal was more likely, inasmuch as the C4 cDNA clones 634C, 637H, 84D and 131E contained cDNA inserts sizes of 0.55kbp, 0.325kbp, 1.2kbp and 2.2kbp respectively. Unfortunately, the recombinant bacterial clone 131E which contained the plasmid with the largest cDNA insert was found to be contaminated with bacteriophage, and as a consequence it was discarded. Further analysis of the C4 group of cDNA clones was carried out using clone 84D.

5. Further Analysis Of The C6 cDNA Clones

5.1 RNA Homologous To The C6 cDNA Clones Accumulates In ts1204-Infected HFL Cells.

The results so far report the isolation of a cDNA (C6) corresponding to a polyA$^+$ RNA which accumulates in *ts*1204-infected HFL cells. To confirm this finding, the relative abundance of the 194D specific polyA$^+$ RNA in *ts*1204-, *wt* virus- and mock-infected cells was assessed.

HFL cell monolayers were mock-infected or infected with *ts*1204 or *wt* virus at a moi of 20pfu per cell and incubated at the NPT. At 7 hrs post-infection the cells were harvested and total cytoplasmic RNA was extracted from the cells according to the procedure outlined in section 2B.11.1. Each of the total cytoplasmic RNA preparations (20µg) was incubated with 50% formamide, 6% formaldehyde to denature any RNA secondary structure. The denatured RNA was finally immobilized onto a nylon membrane in a slot blot manifold and hybridized to a ^{32}P-labelled DNA probe prepared by primer extension using the 194D cDNA insert as a template. Following hybridization, the membrane was washed thoroughly and placed in contact with pre-flashed Xomat XS-1 film (Figure 29 lane 1). Since clone 162B demonstrated equal colony hybridization in differential screening, it was used as a control in the present study. The results indicated that the polyA$^+$ RNA represented by the 194D cDNA was detectable in mock-infected cells and accumulated in *ts*1204-infected cells at the NPT, whilst it was barely detectable in cells infected with *wt* virus. Densitometric analysis of the intensity of the autoradiographic images on the film showed that *ts*1204-infection of HFL cells resulted in a 4 fold accumulation of the 194D specific polyA$^+$ RNA. Independent total cytoplasmic RNA populations were tested in a second experiment with identical results (lane C6 2).

5.2 The C6 cDNA Encodes A 40kDa Cell Protein.

The previous data indicated that the C6 cDNA was derived from a polyA$^+$ RNA that accumulated in *ts*1204-infected HFL cells at the NPT. As described previously, the clones designated 194D, 168C, 2212H, 218E, 2012D and 339E, which comprise C6, each have cDNA inserts of approximately 1.5kb, suggesting that

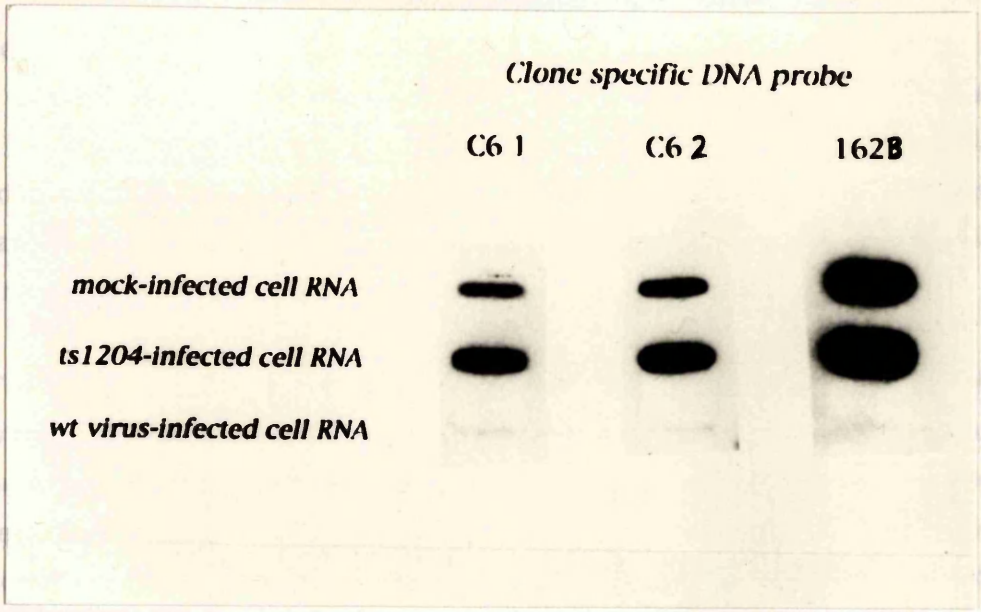


Figure 29. Examination Of HFL Cell Total Cytoplasmic RNA For Sequences Homologous To The C6 cDNA.

HFL cell monolayers were mock-infected or infected with *ts*1204 or *wt* HSV-1 at a moi of 20pfu per cell at the NPT. At 7 hrs post-infection, the cells were harvested and total cytoplasmic RNA was extracted from the harvested cells as described in section 2B.11.1. Approximately 20µg of each of the total RNA preparations was immobilized onto a nylon membrane using a slot blot manifold. The immobilized RNA was hybridized to a ^{32}P-labelled C6-specific DNA probe prepared by primer extension. Following hybridisation, the membrane was washed thoroughly and the degree of hybridisation was determined by autoradiography. Lanes marked 1 and 2 represent identical experiments performed with independent RNA preparations. Clone 162B demonstrated equal colony hybridisation in differential screening, and is presented here as a control.

they may represent full length polyA$^+$ RNA molecules. On this basis it was assumed that the size of the full length C6 protein could be determined by *in vitro* transcription and translation of the C6 cDNA using the TNT Coupled Reticulocyte Lysate System (Promega).

Sequence data analysis of the C6 cDNA clones indicated that the cDNA insert sequences were orientated so that transcription from the T7 promoter would generate mRNA from the correct strand. Recombinant C6 cDNA was therefore transcribed by bacteriophage T7 polymerase which was coupled to translation in the micrococcal nuclease treated rabbit reticulocyte lysate in a single reaction of coupled transcription-translation (see section 2B.11.10).

The protein product encoded by the C6 cDNA was separated by SDS-PAGE and visualized by autoradiography. Rainbow molecular weight markers, supplied by Amersham, were also run on the gel. The efficiency of the TNT coupled Reticulocyte Lysate System in directing the transcription of complementary RNA from DNA as well as the translation of RNA into protein was assessed by performing similar reactions with the luciferase DNA supplied with the kit and mock-infected HFL cell polyA$^+$ RNA.

The results are illustrated in Figure 30. An estimation of the molecular weight of the polypeptide encoded by the C6 cDNA was obtained by measuring the relative mobility of the C6 protein on a 9% SDS-polyacrylamide gel and refering to a standard curve that was constructed by plotting the Log_{10} of the molecular weight of each rainbow marker polypeptides against the distance that the marker had migrated through the gel. Thus, according to the standard curve, the C6 polypeptide was estimated to have a molecular weight of approximately 40 kDa. Although a number of lower molecular weight protein bands could be seen, in addition to the 40kDa C6 protein in lane 2, these probably resulted from the internal initiation of translation.

5.3 C6 cDNA Does Not Encode The Mitochondrial Aspartate Amino-Transferase.

Macnab *et al.*, (1985) identified a series of tumour specific cellular proteins with apparent molecular weights of 200, 90, 40 and 32kDa respectively, that were recognized by antisera raised against HSV-2 infected cells and by TBS.

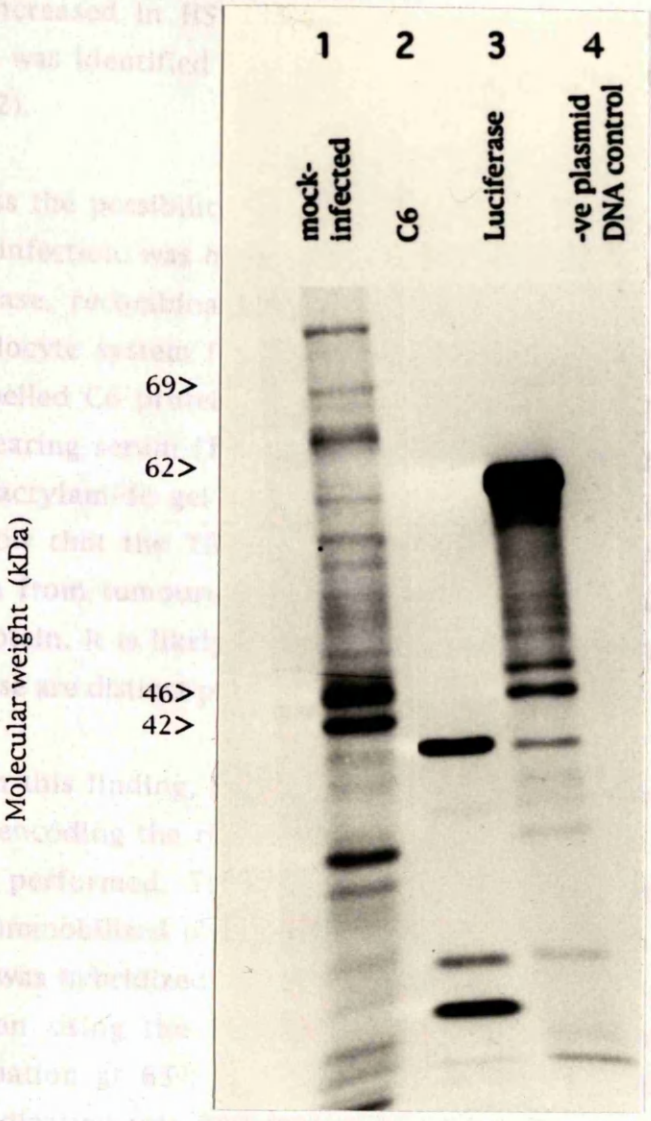


Figure 30. The Gene Product Of The C6 ORF.

The C6 cDNA insert cloned downstream from the T7 RNA polymerase promoter in pT7T3 18U were transcribed and translated *in vitro* using the TNT Coupled Reticulocyte Lysate System (Promega). The radiolabelled proteins were resolved on a 9% SDS-polyacrylamide gel and visualized by autoradiography (lane 2). *In vitro* translated mock-infected HFL cell polyA+ RNA and the 62kDa protein product of the Luciferase RNA supplied with the kit, are indicated in lanes 1 and 3 respectively. The position of the Rainbow molecular weight markers are indicated by the arrows to the left of the figure.

Further analysis of the 40kDa cell protein, confirmed that expression of this protein was increased in HSV-infection (Macnab *et al.*, 1992). Recently, the 40kDa protein was identified as the mitochondrial aspartate amino-transferase (Lucasson, 1992).

To address the possibility that the 40 kDa C6 protein, which accumulates during *ts*1204-infection, was homologous to the 40 kDa mitochondrial aspartate amino-transferase, recombinant cDNA 194D was used as a template in the TNT coupled reticulocyte system for the *in vitro* synthesis of C6 polypeptide. ^{35}S-methionine labelled C6 protein, synthesized *in vitro*, was immunoprecipitated with tumour bearing serum (TBS). The precipitated proteins were separated on a 9% SDS-polyacrylamide gel and visualized by autoradiography (Figure 31). The results show that the TBS immunoprecipitated the [^{35}S]-labelled 40kDa cellular protein from tumourogenic Bn5T cells (Lane 3), but failed to interact with the C6 protein. It is likely therefore that the C6 protein and the aspartate amino-transferase are distinct proteins.

To confirm this finding, DNA cross hybridization analysis of the C6 cDNA with the cDNA encoding the rat aspartate amino-transferase (MasPAT Huyhn *et al.*. 1981) was performed. The C6 and MasPAT cDNA inserts (200ng) were denatured and immobilized onto a nylon membrane using a slot blot manifold. The membrane was hybridized to a ^{32}P-labelled MasPAT DNA probe prepared by primer extension using the MasPAT cDNA insert as a template. Following overnight incubation at 65^0, the membrane was washed thoroughly and the degree of hybridization was determined by autoradiography (Figure 32). The MasPAT DNA probe was unable to hybridize to the C6 cDNA, confirming previous data that the C6 and MasPAT cDNAs encode distinct proteins.

6. Further Characterisation Of The C4 cDNA Clones

6.1 RNA Homologous To The C4 cDNA Clones Accumulates In ts1204-Infected HFL Cells.

The previous section described the isolation of a cDNA (C4) representing a polyA$^+$ RNA which accumulated in *ts*1204-infected HFL cells, inasmuch as the C4 cDNA appeared to show preferential hybridization to the *ts*1204-cDNA probe

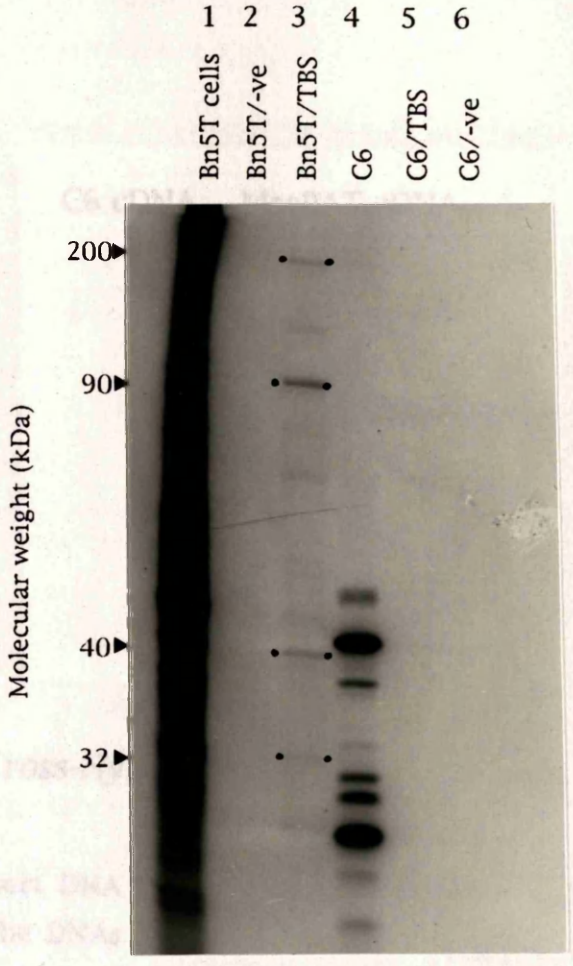


Figure 31. Immunoprecipitation Of The C6 Protein Product With TBS.

The ^{35}S-methionine-labelled C6 protein product was synthesized *in vitro* using the TNT Coupled Reticulocyte Lysate System as described in section 2B.11.10. Approximately 1x10^{6}cpm of the radiolabelled C6 protein was added to 200µl of RIPA buffer and used in immunoprecipitations with TBS. The precipitated proteins were separated on a SDS 9%-polyacrylamide gel and visualized by autoradiography. The 200, 90, 40 and 32kDa proteins that were immunoprecipitated from ^{35}S-methionine labelled Bn5T cells with TBS (lane 3) are indicated by dots. Lanes 1) ^{35}S-methionine labelled Bn5T cell extracts; 2) Bn5T cells immunoprecipitated without TBS ;3) Bn5T cells immunoprecipitated with TBS; 4) C6 protein product; 5) C6 protein immunoprecipitated with TBS; 6) C6 protein immunoprecipitated without TBS.

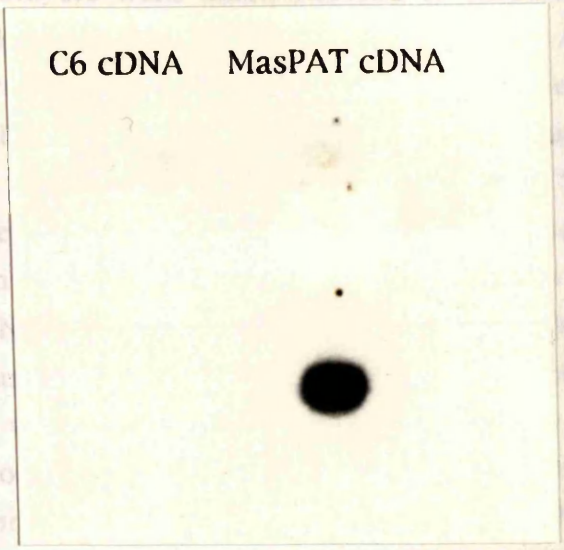


Figure 32. DNA Cross-Hybridisation Analysis Of C6 With MasPAT.

C6 and MasPAT insert DNA (200ng) were denatured and immobilized onto a nylon membrane. The DNAs were hybridized overnight at 65^0 to a ^{32}P-labelled MasPAT DNA probe prepared by primer extension using ^{32}[P]-dCTP. Following hybridisation, the membrane was washed thoroughly and the extent of hybridisation determined by autoradiography.

* The DNA sequence of the 5' terminal end of clone 84D is shown in Figure 34.b).

during rounds of differential screening. To confirm these findings, attempts were made to detect the RNA homologous to the 84D cDNA in *ts*1204-infected HFL cells. For comparison, the relative level of 84D polyA$^+$ RNA in mock- and *wt*-virus infected HFL cells were determined in parallel.

HFL cell monolayers were mock-infected or infected with *ts*1204 or *wt* virus at a moi of 20pfu per cell and incubated at the NPT. At 7 hrs post-infection the cells were harvested and total cytoplasmic RNA was extracted according to the procedure outlined in section 2B.11.1. A total of 20ug of each of the total cytoplasmic RNA preparations was incubated with 50% formamide, 6% formaldehyde to denature possible RNA secondary structure and the denatured RNA was finally immobilized onto a nylon membrane in a slot blot manifold. The immobilized RNA was hybridized to a ^{32}P-labelled DNA probe prepared by primer extension using the 84D cDNA as a template. Following hybridization, the membrane was washed thoroughly and the membrane was placed in contact with pre-flashed Xomat XS-1 film (Figure 33). Since clone 638E demonstrated equal colony hybridization to mock- and *ts*1204-infected cell cDNA in differential screening, it was used as a control in the present study. The results indicate that the polyA$^+$ RNA represented by the 84D cDNA was barely detectable in mock- or *wt* virus-infected cells incubated at the NPT, but accumulated in cells infected with *ts*1204 under the same conditions. Densitometric analysis of the intensity of the autoradiographic images on the film indicated that *ts*1204-infection of HFL cells resulted in a 16 fold increase in the concentration of the polyA$^+$ RNA represented by the 84D cDNA clone. Independent total cytoplasmic RNA preparations were tested in a second experiment (lane C4 2). On this occasion a 5-fold increase in the relative concentration of the 84D polyA$^+$ RNA in *ts*1204-infected cells was apparent. The significance in the variation in 84D polyA$^+$ RNA accumulation in *ts*1204-infected cells is discussed in section 4.

6.2 Identification Of An Alu Repetitive Sequence In 84D.

Approximately 200bp of the 5' terminal end of clone 84D was sequenced using M13 reverse primer according to the procedure described in section 2B.14. Comparison of this 200bp region with other known nucleotide sequences that comprise the Genbank and EMBL databanks was performed using the GCG sequence analysis software package version 5.0 and 6.0 (Devereux, 1989). This

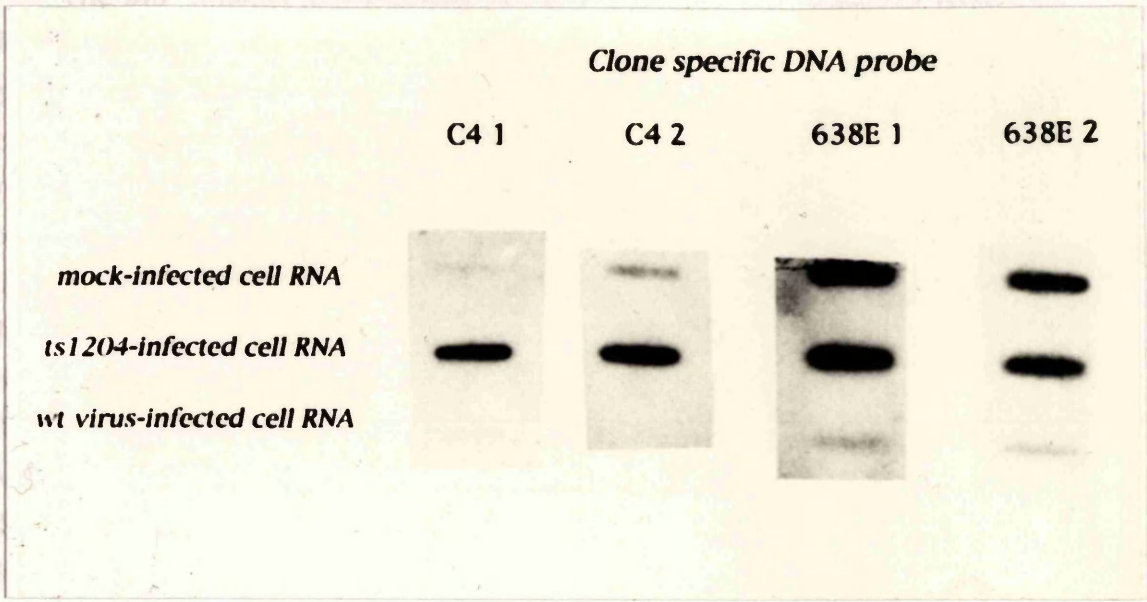


Figure 33. Examination Of HFL Cell Total Cytoplasmic RNA For Sequences Homologous To The C4 cDNA.

HFL cell monolayers were mock-infected or infected with *ts*1204 or *wt*-HSV-1 at a moi of 20pfu per cell at the NPT. At 7 hrs post-infection, the cells were harvested and total cytoplasmic RNA was extracted from the harvested cells as described in section 2B.11.1. Approximately 20μg of each of the total RNA preparations was immobilized onto a nylon membrane using a slot blot manifold. The immobilized RNA was hybridized to a ^{32}P-labelled C4-specific DNA probe prepared by primer extension. Following hybridisation, the membrane was washed thoroughly and the degree of hybridisation was determined by autoradiography. Lanes marked 1 and 2 represent identical experiments performed with independent RNA preparations. Clone 638E demonstrated equal colony hybridisation in differential screening and is presented here as a control.

analysis indicated that the 5' end of clone 84D was homologous to an *alu* repetitive sequence.

The *alu* repeats are a family of short interspersed repeated DNA elements. Nearly 500,000 *alu* repeat copies exist in a haploid genome with an average spacing of approximately 4kbp (Hwu *et al.*, 1986). They are about 300bp in length and are dimeric in structure, comprising a right and a left half region which are separated by an A-rich region (Figure 34 a). Another A-rich region exists at the 3' end of the *alu* repeat which is variable in size. A well defined RNA polymerase III promoter, which directs the transcription of the entire element, is located in the LHS of the repeat. The right and left halves are related with the exception that the right hand side does not contain an active RNA pol III promoter. Although the members of this family can be divided into sub-families based on nucleotide sequence divergence, all of the *alu* sequences identified to date share a recognizable 280bp nucleotide consensus sequence (Jurka and Smith, 1988; Figure 34b). The *alu* repeat sequences contain at least one restriction site for the *Alu* I restriction enzyme.

To confirm the initial finding that the 5' terminus of clone 84D did indeed represent an *alu* repeat element, the degree of sequence homology of the 84D sequence to the *alu* consensus sequence described by Jurka and Smith (1988) was performed using the FASTA program of the GCG sequence analysis software. The results of the comparison are shown in Figure 43.b and illustrate an 80% homology between the 84D putative *alu* repeat sequence and the alu consensus sequence. On this basis it can be concluded that an *alu* repeat lies at the 5' terminal end of the cDNA clone 84D. The significance of this observation will be discussed in section 4.

The initial DNA cross-hybridization studies performed with C4 indicated that the C4 clones shared only a proportion of homologous DNA sequences. It was concluded from DNA cross-hybridization studies that the C4 cDNAs represented the same polyA$^+$ RNA but that the variation in the degree of C4 cross-hybridization was due to each of the cDNA clones being a different size. On the basis of the previous data, however, it is possible that the C4 cDNAs do not represent similar polyA$^+$ RNAs. Instead, the degree of DNA cross-hybridization may have resulted from the presence of a homologous *alu* repetitive sequence.

To address this possibility, experiments were performed to determine whether clones 637H and 634C were homologous to the *alu* repeat or to other

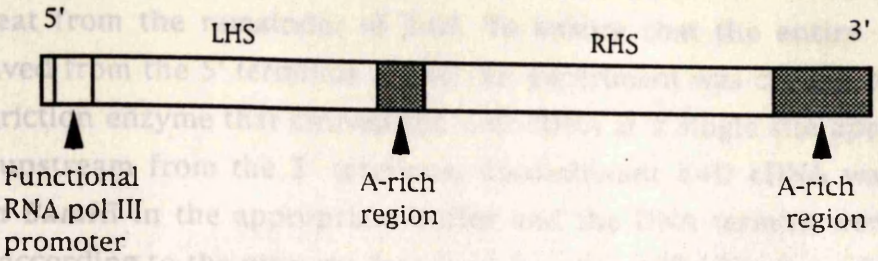
Figure 34. a) A Schematic Representation Of The Alu Repetitive Sequence.

The *alu* repeat, consisting of a left hand side (LHS) and a right hand side (RHS) is shown as a long box with the A-rich sequences represented by the shaded areas. The functional RNA pol III promoter is indicated in the LHS of the repeat and directs the transcription of the entire element. The direction of transcription is from 5' to 3' as indicated in the diagram.

Figure 34.b) Nucleotide Sequence Comparison Between Clone 84D And The Alu Repeat Consensus.

The *alu* consensus sequence defined by Jurka and Smith (1988) and the 5' terminus of clone 84D are aligned to show homologous regions. Homologous regions were identified by the FASTA program of the GCG sequence analysis software. The positions of the A-rich regions and the RNA pol III promoter are shown by arrows. The position at which *Alu* I restriction enzyme cuts the repeat is marked by an asterisk.

a)



b)

5'
Alu consensus
RNA pol III
promoter

53 CGGGCGGATCACCTGAGGTCAGGAGTTCGAGACCAGCCTGGCCAACATGGTGAAACCCC 112

GAGCGCGT CCTGA GTCA GAGTT GAGACCAGCCTAGCC ACAT GTG ACCCT

84D cDNA

A-rich region

113 GTCTCTACTAAAAATACAAAAATTAGCCGGGCGTGGTGGCGCGCGCCTGTAATCCCAGC 171

GTCTCTACT AAATTC AAATTGGGCAGGCATGGTGGCGGGTGCCTGTAGTCCCAGC

172 TACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGCGGAGGTTGCAGTGAG 230

TACTTGGGAGGCTGAGGCACGAGAATCGCTTG ACCCAAGAGATGGAGATTGCAGTGAT

3'

231 CCGAGATCGCGCCACTGCACTCCAGCCTGGGC-GACAGAGCGGGACTCCGTCTCAAAAA 289

CTAAGATCGTGCCACTGCACTCCAGCCTGGGCAAACAGAGCACGACTCCATCTCC

A-rich region

290 AAAAAAA

sequences within clone 84D. This involved the separation of the 5' terminal *alu* repeat from the remainder of 84D. To ensure that the entire *alu* repeat was cleaved from the 5' terminus of 84D, an experiment was carried out to identify a restriction enzyme that cleaved the 84D cDNA at a single site approximately 300 bp upstream from the 5' terminus. Recombinant 84D cDNA was cleaved once with *BamHI* in the appropriate buffer and the DNA termini were labelled with ^{32}P according to the protocol described in section 2B.10.3. One of the ^{32}P-labelled termini was removed by digesting the DNA with *HindIII*. Approximately $1X10^5$ cpm of ^{32}P-labelled 84D recombinant DNA was digested with decreasing concentrations of each of the enzymes marked in Figure 35 and incubated at 37^0 for 15 min. After incubation, the digests were pooled and the partially digested DNA was analyzed on a 5% non-denaturing polyacrylamide gel using as markers ^{32}P end-labelled λ DNA cleaved with *HindIII*. The ^{32}P-labelled cleaved DNA was visualized by autoradiography (Figure 35). The size of the radiolabelled fragments generated by restriction enzyme digestion of the end-labelled 84D cDNA was determined by measuring the relative mobility of each fragment on the polyacrylamide gel and referring to a standard curve that was constructed by plotting the size of each of the marker DNAs (Log_{10}) versus the distance that the DNA fragments migrated through the gel. The results show that *Taq*1 cleaved the 84D cDNA at a single site approximately 400kbp upstream of the 5' terminus of the 84D cDNA. It was therefore assumed that cleavage of 84D with *Taq*1 was sufficient to remove the *alu* repeat from the 5' terminus of the 84D cDNA.

To determine whether the C4 clones shared sequences distinct from the *alu* repetitive sequence, 84D was digested with *Taq*I and the alu repeat was separated from the remainder of the 84D insert DNA. The *alu* repeat and the remainder of 84D were radiolabelled by primer extension and hybridized seperately to 637H, 634C, and 84D cDNAs immobilized onto nylon membranes. Following overnight hybridization the membranes were washed thoroughly and the degree of hybridization analyzed by autoradiography (Figure 36). The *alu* repeat DNA probe failed to hybridize to the 637H and 634C cDNAs suggesting that these clones share sequences with 84D that are distinct from the *alu* repeat element. Thus on this basis, the variation in the degree of DNA cross-hybridization observed between the C4 clones was probably due to each of the insert cDNAs being a different size and not due to the presence of non-specific DNA sequences.

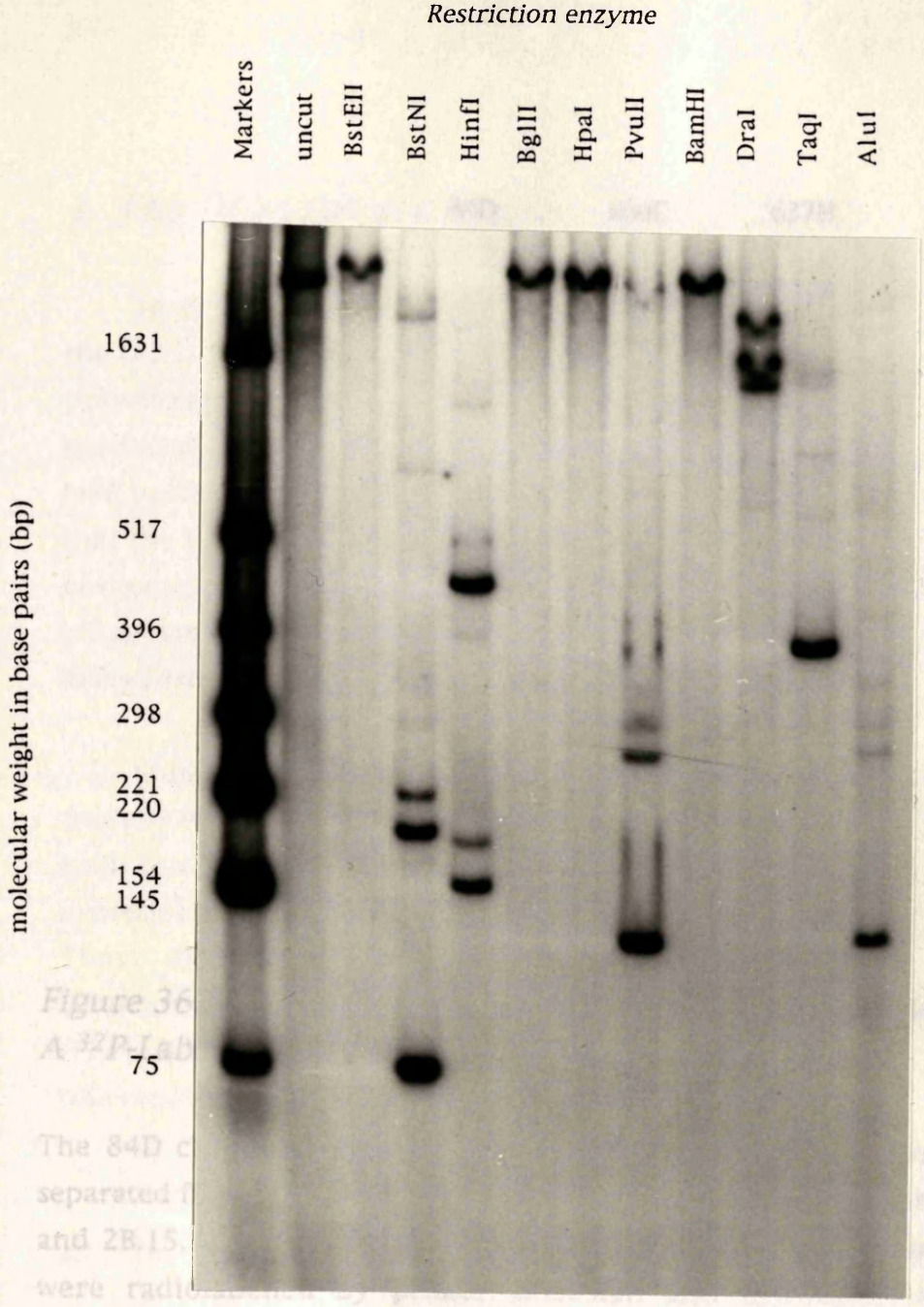


Figure 35. Partial Digestion Of ^{32}P End-Labelled 84D cDNA

Approximately 1x10^5 cpm of ^{32}P-labelled 84D cDNA was digested with increasing concentrations of each of the indicated restriction enzymes (10-0.25 units) and incubated at 37^0 for 15 min. Following incubation, the digests were pooled and analyzed on a 5% non-denaturing polyacrylamide gel. ^{32}P end-labelled *Hind*III fragments of bacteriophage λ DNA were used as markers. The ^{32}P-labelled DNA fragments were visualized by autoradiography.

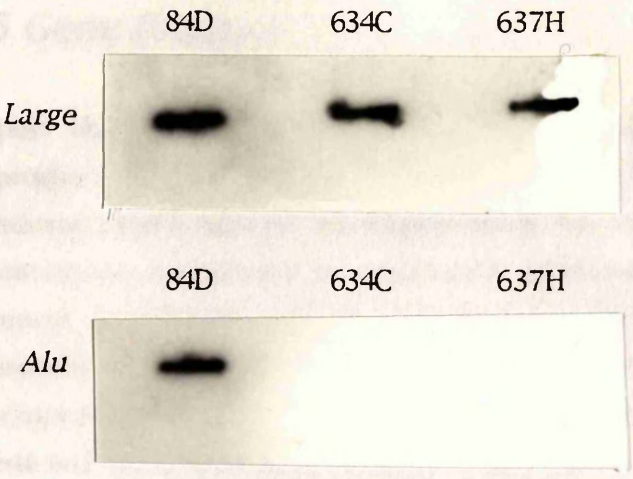


Figure 36. DNA Cross-Hybridization Analysis Of The C4 Clones With A ^{32}P-Labelled Alu Probe.

The 84D cDNA was digested with *TaqI* and the 5' terminal *alu* repeat was separated from the remainder of the 84D cDNA as described in section 2B.13.4 and 2B.15.1. The *alu* repeat (*Alu*) and the remaining 84D sequences (*Large*) were radiolabelled by primer extension and hybridized seperately to duplicate nylon membranes onto which 84D, 634C, and 637H cDNAs (500ng) had been immobilized as indicated. The probes are shown in italics to the left of each membrane. Following overnight incubation at 65^0 the membranes were washed thoroughly and the degree of hybridization determined by autoradiography.

4. Discussion

1. The UL25 Gene Product

In this report the synthesis, processing and intracellular localization of the UL25 gene product have been examined. To study the UL25 protein, a rabbit polyclonal antiserum raised against an oligopeptide for the UL25 protein was produced. The antiserum was shown to specifically immunoprecipitate a 67,000 MW protein present in infected cell extracts and purified virions, suggesting that the UL25 protein is a structural component of the virion. The virus specific polypeptide recognized by the UL25 antiserum was similar in size to the polypeptide predicted from sequence analysis of the HSV-1 genome and so it is likely that this protein was the UL25 gene product.

Studies of the kinetics of UL25 polypeptide synthesis indicated that the protein was initially detectable at early times during infection, reaching its peak rate at 4-5 hrs post-infection. Further studies revealed that the maximal expression of the UL25 gene product was dependent upon viral DNA synthesis. These data suggest that UL25 is regulated as a leaky late viral gene. Furthermore,the UL25 polypeptide was shown to be stably maintained within HSV-infected cells for up to 24 hrs after synthesis, consistent with a structural role required late in viral infection.

2. The UL25 Polypeptide Is Found In L-Particles.

It has been well documented that capsid assembly and packaging of viral DNA occur in the infected cell nucleus. The intracellular location of the UL25 polypeptide has yet to be determined. However, it is conceivable that if the UL25 protein were to function in capsid assembly or DNA packaging then it would be found predominantly within the nucleus. It has been suggested that the assembly of the tegument proteins, either around capsids or as independent condensations, occurs in the cytoplasm (Rixon *et al.*, 1992). However, the location of the UL25 polypeptide in L-particles suggests that L-particles acquire

the UL25 polypeptide in the nucleus rather than the cytoplasm and that the innitiation of tegumentation occurs in the infected cell nucleus.

3. *Ts1204 Has A Defect In Uncoating At The NPT*

Addison *et al.*, (1984), reported that *ts*1204 had a penetration defect at the NPT. This conclusion was based upon two lines of evidence. Firstly, large numbers of membrane bound virions were observed on the surface of *ts*1204-infected cells at the NPT when compared to cells infected with *wt* virus. Secondly, treatment of *ts*1204-infected cells at the NPT with PEG, a compound which promotes membrane fusion, was sufficient to restore viral infectivity. In the present study, PEG reatment of *ts*1204-infected cells at the NPT did not lead to the production of viral antigens. Furthermore, capsids were observed within the cytoplasm of *ts*1204-infected cells at the NPT. Considerable numbers of *ts*1204 virions were also observed bound to the surface of cells at the restrictive temperature, which supports the idea that this mutant is defective in cell penetration. However, enveloped virions were also observed on the surface of cells infected with *wt* virus. On the basis of the present data it is more likely that *ts*1204 has an uncoating defect at the NPT. However, the possibility exists that the *ts*1204 mutation, in addition to preventing uncoating of the viral genome, may affect the rate at which virus enters cells.

The results of EM analysis of thin sections of *ts*1213-infected cells at the NPT support results of previous investigators who showed that the HSV-1 mutant *ts*B7, which like *ts*1213 carries a lesion in the gene encoding the large tegument protein Vmw273, is unable to release the viral genome from the nucleocapsid at the NPT (Batterson *et al.*, 1983).

The discrepancies seen between the present data and previous observations by Addison *et al.* (1984) with regard to the *ts*1204 phenotype are difficult to explain. The idea that *ts*1204 is blocked at a stage following attachment of the virus to the high affinity cellular receptor but prior to membrane fusion, was further supported by the observation that at elevated temperatures, cells treated with a high moi of *ts*1204 were resistant to subsequent infection with *wt* HSV-1 but not HSV-2. Subsequent analysis of HSV-1/HSV-2 recombinants revealed that the region of the genome encoding the

protein important for entry of superinfecting virus was localized within the same region as the gene required for resistance to the drug neomycin (David Smith, unpublished observations). Since the region on the HSV genome responsible for neomycin resistance maps to the amino-terminus of gC (Oyan *et al.*, 1993), it is likely that the type specific effect seen by Addison *et al.* (1984) was probably at the level of the attachment of gC to cell surface HS.

Consistent with the idea that a mutation in the UL25 gene product affects uncoating of the viral genome, is the observation that the UL25 protein is tightly associated with the capsid.

The mechanisms involved in the release of the viral genome from the capsid are poorly understood. It is likely that the uncoating process occurs at the nuclear membrane. Early studies using metabolic inhibitors suggested that *de novo* RNA and protein synthesis were not required for uncoating of the genome, suggesting that a cellular enzyme and/or a virion structural component is required for this event (Hochberg and Becker, 1969). Analysis of the HSV-1 mutant *ts*B7, which carries a lesion in the large tegument protein Vmw273, has implicated this virion polypeptide in the uncoating process (Batterson *et al.*, 1983). In *ts*B7-infected cells at the NPT capsids containing viral DNA, accumulated at the nuclear pores. Upon downshift to the PT, the viral genome was released from the capsid. Although a direct role for Vmw273 in genome uncoating has been suggested, it is possible that it is only indirectly involved in this process, inasmuch as it must be removed from the capsid before release of the viral genome can occur. Therefore, the *ts*B7 mutation may prevent the dissasociation of Vmw273 from the capsid and subsequently block genome uncoating. The observation that the capsid pentamers were frequently absent from preparations of flattened capsid sheets led Vernon *et al.* (1976) to propose that their removal allows the escape of the viral DNA from the capsid shell.

4. Transcriptional Induction Of Cellular Genes During HSV-1 Infection.

Although the expression of viral genes during HSV-infection coexists with a generalized shut off of host protein synthesis, the expression of a minority of

cellular genes is upregulated (Macnab *et al.*, 1985; Kemp *et al.*, 1986; Patel *et al.*, 1986; Kemp and Latchman, 1988; LaThangue and Latchman, 1988). The mechanism by which the activation of these cellular genes occurs against a background of generalized repression is unclear. In the majority of cases, induction of these genes is dependent upon the synthesis of the viral IE proteins in the infected cell.

In the present study, two distinct polyA$^+$ RNAs, C4 and C6, which accumulated in *ts*1204-infected HFL cells at the NPT were isolated. Since *ts*1204 is unable to synthesize viral polypeptides at the restrictive temperature, it is clear that the induction of the C4 and C6 polyA$^+$ RNAs occurs prior to the onset of viral protein synthesis. These observations confirm previous data which showed that the induction of some cellular genes can occur in the absence of viral protein synthesis (Patel *et al.*, 1986; Preston, 1990).

The results of this study indicate that the C6 polyA$^+$ RNA encodes for a cellular polypeptide with a molecular weight of approximately 40 kDa (p40). A number of similar sized proteins have been shown to accumulate during HSV-infection and in transformed cell lines (Macnab *et al.*, 1985, 1992). Immunoprecipitations of p40 with TBS showed that p40 was distinct from the 42 kDa aspartate amino transferase that was immunoprecipitated from HSV-2 infected cells and transformed cells (Lucasson, 1993). A 40 kDa polypeptide was shown to accumulate in HSV-1 infected cells using the monoclonal antibody TG7A (LaThangue and Latchman, 1988). Further studies revealed that the accumulation of this protein was dependent upon the expression of the IE polypeptide Vmw63. It is unlikely, therefore, that p40 is the same as the protein described by LaThangue and Latchman (1988), since the accumulation of p40 occurs in the absence of viral protein synthesis. Sequencing of approximately 150bp downstream of the 3' terminus of the C6 cDNA allowed the prediction of the sequence of 50 amino acids at the putative carboxy terminal end of p40. The predicted amino acid sequence was compared with sequences stored within the Swiss-Prot Database using the FASTA program of the GCG sequence analysis software. The p40 amino acid sequence showed no significant homology to any previously characterized proteins within the database suggesting that p40 is a novel cellular polypeptide. Prediction of the entire amino acid sequence of the polypeptide encoded by the C6 polyA$^+$ RNA would assist in the establishment of the identity and possibly the function of this protein in the viral lytic cycle.

Little information was obtained as to the identity of the protein encoded by the C4 polyA$^+$ RNA. Nonetheless, it is intriguing to speculate that this polyA$^+$ RNA encodes the cellular protein p56, since the C4 polyA$^+$ RNA was barely detectable in mock and *wt* virus-infected cells whilst it accumulated 16-fold during *ts*1204-infection at the NPT. In a second experiment, the C4 polyA$^+$ RNA only accumulated 5-fold in *ts*1204-infected cells. Interestingly the accumulation of the p56 polypeptide was similarly decreased in these cells (results not shown). It would appear that the accumulation of the C4 polyA$^+$ RNA mirrors that of the p56 polypeptide in *ts*1204-infected cells at the NPT. The variable accumulation of the p56 polypeptide in *ts*1204-infected cells was frequently observed. The reduced accumulation of p56 in some cases probably resulted from the high passage number of the cells.

5. Mechanisms Of Induction Of ts1204-Inducible Cellular Genes

Preston (1990) was first to observe the transcriptional induction of a 56 kDa protein (p56) in *ts*1204-infected human foetal lung cells at the NPT. The original proposal that *ts*1204 binds to a high affinity cellular receptor but fails to penetrate the cell membrane at the NPT lead to the idea that the accumulation of p56 in *ts*1204-infected cells results from the prolonged stimulation of the cellular HSV-1 receptor. However, the results of the present study introduce the possibility that the induction of cellular genes during *ts*1204-infection occurs as a result of virus binding, membrane fusion or release of the capsid and tegument proteins into the cytoplasm.

The p56 polypeptide was also induced in cells infected with the mutant *ts*1213 at the NPT, albeit at lower amounts relative to *ts*1204-infected cells (Preston, 1990). Since *ts*1213 and *ts*1204 both appear to have uncoating defects, it is difficult to conceive why there is a greater induction of p56 in *ts*1204-infected cells than those infected with *ts*1213. Interestingly, small amounts of p56 were also detected in *wt* virus-infected cells in the presence of cycloheximide. The accumulation of the p56 polypeptide under these conditions may result from an increase in mRNA stability due to the presence of the cyclohexamide.

Normally, once multivalent attachment between the virus and cell surface components has taken place, the virus is rapidly internalized (DeLuca *et al.*, 1981; Rosenthal *et al.*, 1984). The belief that *ts*1204 attaches to but fails to penetrate the cell membrane at the NPT lead to the idea that the accumulation of p56 in *ts*1204-infected cells was caused by the prolonged stimulation of a cellular receptor to which virus was bound. This idea was supported by the observation that *ts*1204, grown in the presence of 2-deoxy-D-glucose, which prevents glycosylation of viral glycoproteins, failed to induce p56, although attachment of virus to cells was not impaired (Preston, 1990). Furthermore, antibodies specific for gD blocked the induction of p56 by *ts*1204, which suggested that the interaction of gD with the cell surface might be important for the induction of p56 (Preston, unpublished observations). However, these results do not preclude the possibility that p56 induction is triggered by membrane fusion or capsid entry, since inhibition of specific viral interactions with cell surface components would prevent membrane fusion and subsequent release of the capsid into the cytoplasm. The present data suggest that *ts*1204 can enter cells at the NPT, which fail to support the idea that p56 induction occurs by the continued stimulation of a cellular receptor. However, it is possible that the initial stimulation of a cellular receptor by one or more viral glycoproteins can continue after membrane fusion has taken place. Indeed there is evidence to suggest that gD remains associated with the high affinity cellular receptor following membrane fusion, in asmuch as gD present in the infecting virion is the target of gD mediated restriction to superinfection (Campadelli-Fiume *et al.*, 1990). It is unlikely that gC plays an important role in the induction of p56, since p56 accumulation still occurs in HFL cells infected with mutant virus lacking gC.

A number of cell proteins with a molecular weight of 56 kDa have been shown to be induced in cells treated with interferons. Interferons are produced in response to viral infection and it is possible that p56 is a member of a family of interferon induced cell proteins. Rubin *et al.* (1988), used a monoclonal antibody to identify a 56 kDa polypeptide that was induced in cells treated with γ-interferon. This antibody also recognized a 42 kDa cellular polypeptide which also accumulated in γ-interferon treated cells, although its accumulation was not as marked as that of the 56 kDa polypeptide. Further results indicated that the expression of these polypeptides resulted from the interaction of γ-interferon with the γ-interferon receptor on the cell surface. Clearly, it would be interesting to determine whether p56 and p42, which accumulate in *ts*1204-infected cells at the NPT, are the same as the interferon induced polypeptides

identified by Rubin *et al.* (1988). However, p56 was shown to accumulate in the presence of cycloheximide suggesting that prior protein synthesis is not required for the induction of p56 in infected cells. Nonetheless, the possibility exists that the expression of some cellular proteins normally induced by interferons could be mediated by other factors directly related to viral infection In support of this idea is the observation that binding of murine hepatitis virus to astrocytes in tissue culture induces the expression of Ia antigen, a surface antigen which is also expressed on cells following exposure to γ-interferon (Wong *et al.*, 1984; Massa *et al.*, 1986). It is possible that the triggering of expression of the Ia antigen by virus binding results from the stimulation of the γ-interferon receptor on astrocytes by components of the virus.

The finding that p56 does not accumulate in cells infected with *ts*1204 L-particles at the NPT suggests that interactions between viral membrane proteins and cell surface components are not important for the induction of cellular genes during *ts*1204-infection. Alternatively, L-particles may not completely mimic virions in the way they enter cells. McLauchlan *et al.* (1992) investigated the biological properties of L-particles by assaying the functions of two tegument proteins which are present in both types of particle, Vmw65 and the *vhs* protein. Their results showed that L-particles were as efficient as virions in supplying both of these proteins in a biological state, suggesting that the attachment, fusion and release of tegument proteins was the same for both virions and L-particles. These data suggest that the presence of the capsid within the cytoplasm is an important factor in the induction of p56 by *ts*1204.

6. The UL25 Gene Product Is Required At Late Times In Infection.

It has been concluded that the UL25 gene product performs an essential function at late times in infection (Addison *et al.*, 1984), inasmuch as the mutants *ts*1204 and *ts*1208, produce very few capsids at the NPT which lack DNA. The *ts*1204 and *ts*1208 mutations map to different complementation groups than the previously identified DNA positive mutants which fail to encapsidate viral DNA at the NPT. According to these findings it was concluded that the UL25 gene product may be required for capsid stability rather than the cleavage and

packaging of concatemeric DNA. Many similarities have been observed between the mechanisms employed by HSV-1 and the double stranded DNA bacteriophages in capsid assembly and DNA packaging. In recent studies, the large tegument protein Vmw273 has been suggested to function in the packaging of viral DNA in a manner analogous to the well characterized terminases of the bacteriophages (Macnabb and Courtney, 1992c). Another T4 gene product, gpD, has been shown to stabilize the capsid during DNA packaging and it is appealing to speculate that gpD is analogous to the UL25 protein. However, in mature capsids, gpD is present in a 1:1 ratio with the capsid shell protein and forms trimers on the capsid surface suggesting that it is more homologous to the HSV capsid protein VP26 (Imber *et al.*, 1980; Newcomb *et al.*, 1993). Nonetheless, this does not preclude a similar function for the UL25 gene product. Another explanation for the low numbers of capsids found in the nuclei of *ts*1204 and *ts*1208 infected cells is that the UL25 protein is required for the initiation of capsid assembly, such that a *ts* defect in this polypeptide affects the rate of initiation, which in turn results in few capsid being formed at the NPT. Such a function is performed by the T4 bacteriophage gene product gp20, which is involved in the rate-limiting initiation of capsid assembly by anchoring the developing capsid to the bacterial membrane (Onorato *et al.*, 1978; Showe and Onorato, 1978; Black and Showe, 1983). Although a role for the UL25 protein in the initiation of capsid assembly is appealing, it has recently been shown that coexpression of the capsid proteins in a baculovirus system lead to the formation of capsids, suggesting that none of the tegument proteins, including the UL25 polypeptide, are required for the initiation of capsid assembly (Rixon, personal communication). Thus, all of the signals that are involved in the comencement of capsid assembly are represented by the capsid polypeptides.

7. Secondary Structure Analysis Of The UL25 Gene Product.

The predicted secondary structure of the HSV-1 UL25 polypeptide was determined using the Garnier and Peptidestructure programs (Garnier *et al.*, 1978). These programs determine the probability of a residue type adopting one of the structural states, α-helices (characterised by intra-molecular hydrogen bonding between peptides on the same polypeptide chain), β-sheet structures (inter-molecular hydrogen bonding), and reverse or β-turns, which cause the

protein to fold on itself by approximately 180^0. The predictions are approximately 40% accurate for small globular proteins but of questionable application to larger proteins if no structural information is known. The output of the Garnier prediction for the UL25 gene product is shown in Figure 37. Few interesting features were observed by these analyses. β-turns were predicted in several regions of the protein. These structures have been shown to occur at protein surfaces where they assume important roles in molecular function (Richardson, 1981; Leszczyaski and Rose, 1986). A single region of primary sequence which was predicted to have a β-turn structure (residues 397-412) was also observed to be a relatively hydrophilic part of the protein (Figure 37), suggesting that this region may lie on the surface of the UL25 polypeptide. Furthermore, this region of the sequence is highly conserved within the alphaherpesviruses (Figure 38). Although speculative, these data imply that this region may be an important determinant for the possible interaction of the UL25 protein with other virion structural components.

Recently a region of homology between the UL25 protein of HSV-1 and the reovirus μ1 protein was identified as shown in Figure 38 (Jayasuriya, personal communication). Although the region of homology between the reovirus m1 and HSV-1 UL25 polypeptides is relatively short (approximately 26 amino acids), the location of the similarity is interesting since the site of μ1 to μ1C cleavage lies between amino acids 42 and 43 in the μ1 protein sequence (Jayasuriya *et al.*, 1988). The μ1 protein is cleaved during virion assembly to form the cleavage product, μC1, which is the major outer capsid protein of the reovirus. A similar if not identical cleavage of the μ1C, yielding the δ protein, has been suggested to be of importance in the early stages of reovirus entry into host cells (Sturzenbecker *et al.*, 1987). The observed homology between the reovirus μ1 to μ1C cleavage site and the UL25 polypeptide lead to speculation that the UL25 polypeptide may undergo a similar cleavage event. However, pulse-chase analysis of UL25 polypeptide synthesis performed in this study failed to show that it undergoes cleavage. Furthermore the proposed cleavage site was located in a region of the protein that is not highly conserved throughout the alphaherpesviruses as would be expected if the cleavage site were important for the function of the UL25 protein in virus growth.

In conclusion of the data presented here and elsewhere, the function of the UL25 protein in the virion life cycle remains unknown. Clearly, the UL25 gene product is not required for the initiation of capsid assembly as was originally suggested by Addison *et al.* (1984). Since a role for UL25 protein in

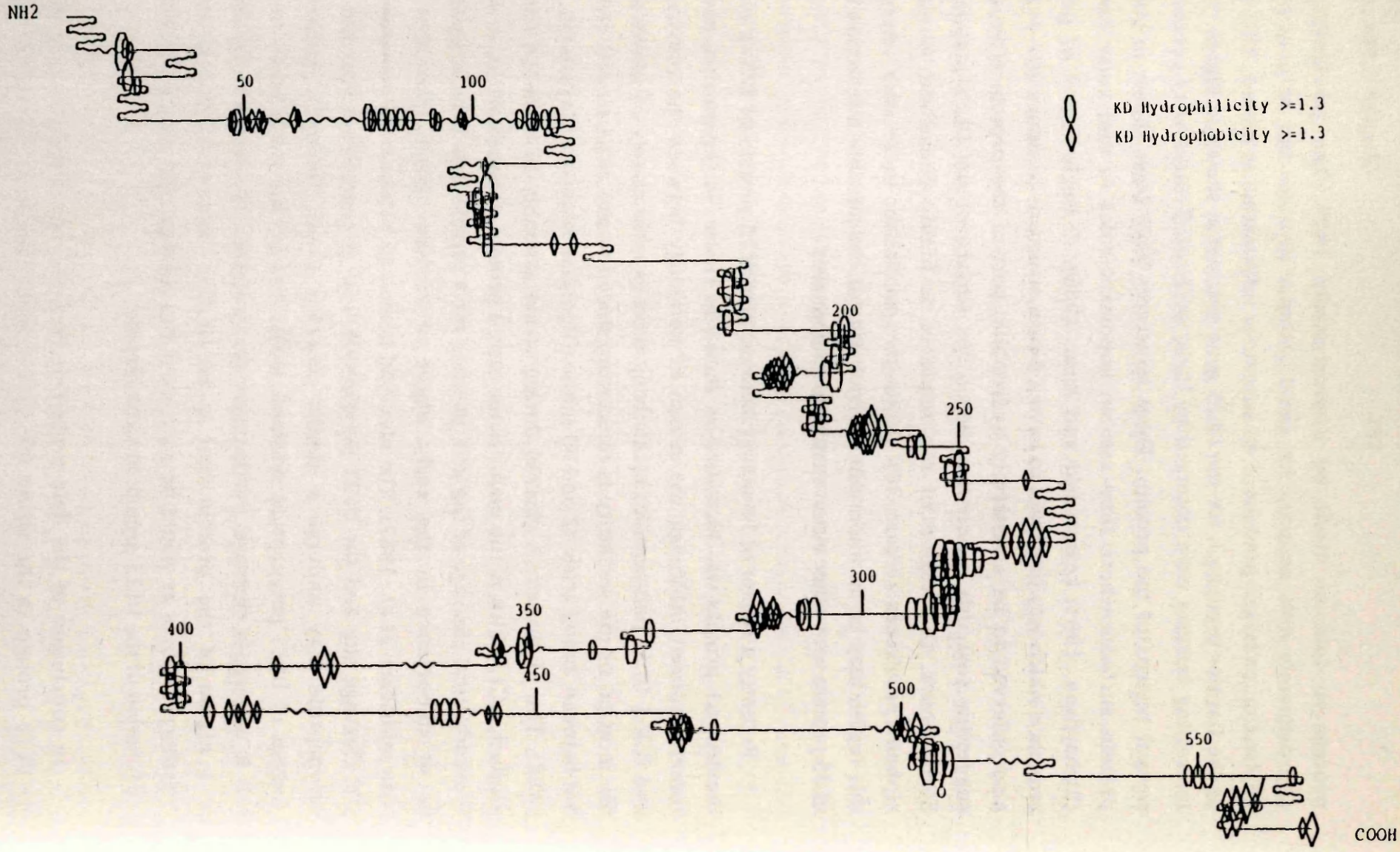

NH2
50
100
200
250
300
350
400
450
500
550
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
COOH

Figure 37. Predicted Secondary Structure Of The UL25 Polypeptide

Secondary structure predictions were made using the GCG Peptidestructure programme, according to Garnier *et al.* (1978). Helices are shown with a sine wave (∿∿), β-sheets with a small saw tooth wave (⁓⁓) and turns with 180° turns. Hydrophilicity (0) and hydrophobicity (◊) are superimposed over the wave.

Figure 38. Predicted Primary Structure Of The HSV-1 UL25 Polypeptide

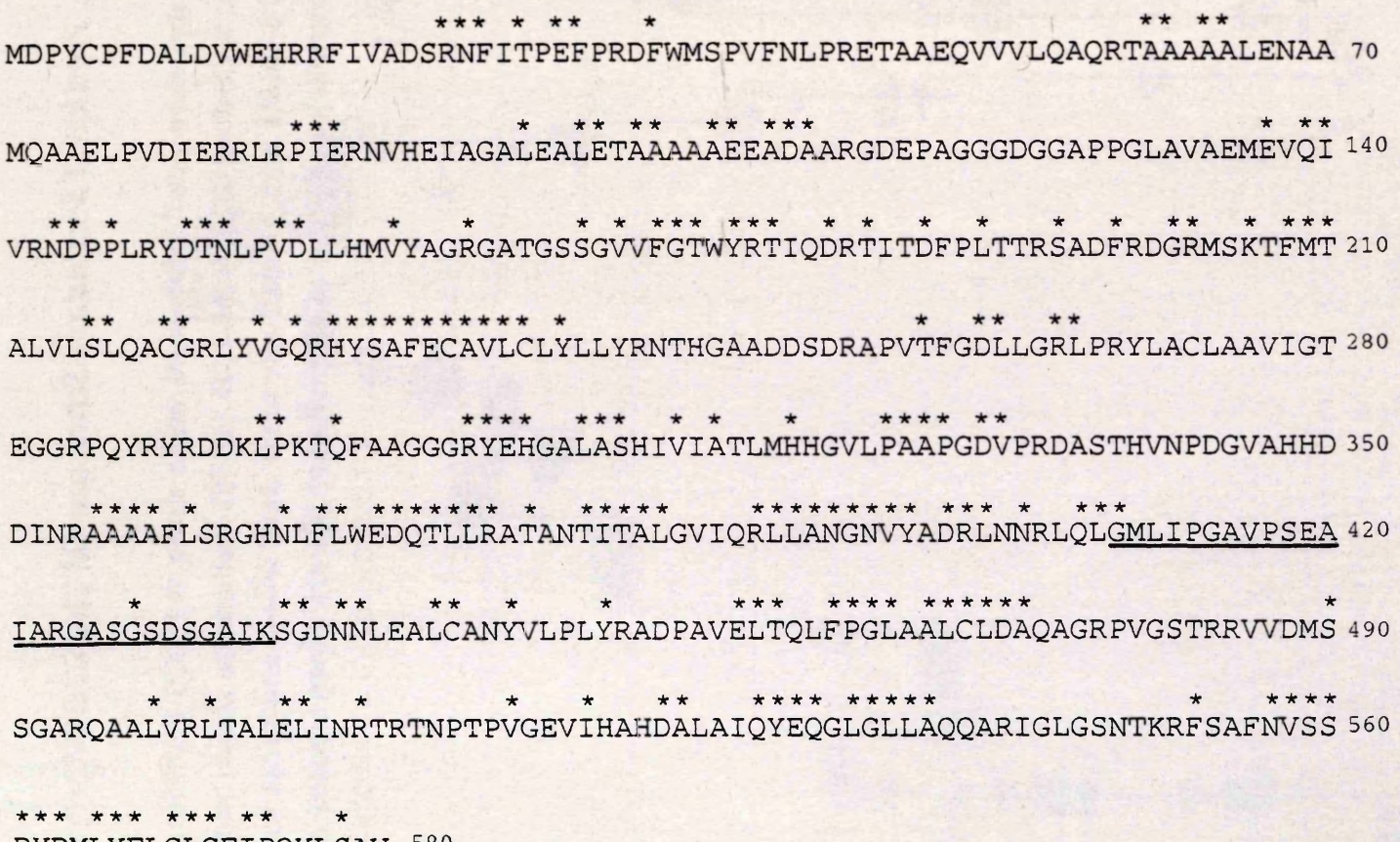


Figure 38. Primary Structure Of The UL25 Gene Product

The predicted amino-acid sequence (1-580) of the HSV-1 UL25 polypeptide is shown in the single letter amino acid code (McGeoch *et al.*, 1988b). Sequence analysis has identified proteins homologous to the HSV-1 UL25 gene product in VZV and EHV-1 (Davison and Scott, 1986; Telford *et al.*, 1992). Amino acid residues that are highly conserved within this family of homologous alphaherpesviruses proteins are asterisked. The putative region of homology with the reovirus μ1 protein is indicated by the solid line.

capsid stability has been suggested, it would be interesting to determine whether the UL25 protein interacts with one or more of the known capsid proteins. From analysis of the secondary structure predictions of the UL25 polypeptide it is likely that some regions of the protein are involved in interacting with other viral polypeptides the identity of which remain to be elucidated.

8. Future Work

There are still several unanswered questions as to the function of the UL25 polypeptide in the virion replicative cycle. As discussed earlier, preliminary EM analysis of a relatively small number of virus infected cells illustrated that *ts*1204 can penetrate the cell membrane, but fails to uncoat the viral genome at the NPT. The analysis of a larger number of virus-infected cells at the NPT would confirm the preliminary findings.

Marker rescue experiments have localized the *ts*1204 mutation to a 400bp region in DNA fragment *BamHIu*. Cloning and sequencing of this fragment will allow the precise bp change of the *ts*1204 mutation to be determined.

The location of the UL25 gene product in the infected cell remains to be elucidated. These studies have been hindered by the lack of an anti-UL25 antibody able to recognise the protein on western blots or immunofluorescence assays. The production of a suitable anti-UL25 antibody which recognises the protein on both western blots and immunofluorescence assays will facilitate further analysis of the cellular location of the UL25 gene product.

Expression of the UL25 protein in bacterial and baculovirus expression systems is currently under way and it is hoped that the expressed proteins will be useful in generating antisera for localisation studies.

Finally, due to the proposed role of the UL25 gene product in capsid stability, it would be interesting to investigate whether the UL25 gene product interacts with other viral proteins. These studies could be carried out using a pull-down assay, with a GST-UL25 fusion protein.

The studies presented in this thesis identified two cDNA clones representing polyA$^+$ RNA which accumulate in *ts*1204 infected HFL cells at the NPT. The identity of the protein products encoded by these polyA$^+$ RNAs remains to be elucidated. It is likely that the C6 cDNA represents a full length polyA+ RNA. Sequencing of the entire C6 cDNA would lead to the identification of the putative C6 ORF. Comparative analysis of the C6 sequence data with sequences stored in the GEN-EMBL databases could also aid in the identification of the C6 polypeptide.

The isolation of a full length C4 cDNA would be useful in determining the size of the polypeptide encoded by the corresponding polyA+ RNA using an *in vitro* transcription and translation system. A full length C4 cDNA could be isolated by screening the cDNA library with a C4-specific probe.

There is a possibility that the C4 cDNA encodes the p56 protein (see previous discussion). This could be investigated using hybrid arrested translation, in which the C4 cDNA is annealled to polyA$^+$ RNA isolated from cells infected with *ts*1204 at the NPT prior to *in vitro* translation of the RNA. If the C4 cDNA contains a portion of the p56 ORF, this will result in the inhibition of translation of the p56 polyA$^+$ RNA.

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