IDENTIFICATION AND CHARACTERISATION OF HERPES SIMPLEX VIRUS GENES REQUIRED FOR ENCAPSIDATION OF DNA

by

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To my:

PARENTS

WIFE WARKA

DAUGHTER HALLA

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SUMMARY

The aim of the study presented in this thesis was to characterise two herpes simplex virus type 1 (HSV-1) DNA positive temperature sensitive (*ts*) mutants, *ts*1233 and *ts*1201, and the genes in which their mutations lie.

Electron microscopic examination of thin section preparations of *ts*1233-infected cells revealed that at the non-permissive temperature (NPT) the nuclei contained large numbers of partially-cored capsids. In contrast to *wt* virus-infected cells, no dense capsids or empty capsids were detected in the nuclei of *ts*1233-infected cells at the NPT. This result suggests that the mutant has a block in the assembly of full nucleocapsids. The effect of the *ts*1233 mutation could not be reversed when mutant virus-infected cells were shifted from the NPT to the permissive temperature (PT) in the presence of a protein synthesis inhibitor. Southern blot analysis of total and encapsidated DNA confirmed that *ts*1233 failed to encapsidate DNA at the NPT, and showed that the DNA synthesized by *ts*1233 at the NPT was in an endless state. This information suggested that most of the mutant DNA was in the form of high moleculer weight (mw) concatemers at the NPT. Previous work had located the *ts* mutation of *ts*1233 within EcoRI *o*. Complementation experiments between *ts*1233 and another HSV-1 mutant *ts*N20, which also had a lesion in HSV EcoRI *o*, showed that *ts*1233, belonged to a different cistron from *ts*N20.

The polypeptide profile of *ts*1233-infected cells was

similar to that of *wt* virus-infected cells. In contrast to the mutant *ts*1201, *ts*1233 processed the structural protein UL26 gene product normally and therefore, the gene in which *ts*1233 maps is not required for the processing of UL26 gene product.

Marker rescue experiments localised the lesion in *ts*1233 within a 150bp fragment which contains the 5' ends of two genes, UL32 and UL33 oriented in opposite directions. UL32 encodes a 64,000 mw polypeptide and UL33 encodes a 14,000 mw polypeptide. The nucleotide sequence of a 392 base pair (bp) fragment from *ts*1233 and two *ts*$^+$ revertants for growth, isolated during this study, was determined. Sequence analysis revealed that *ts*1233 had a single bp change at residue 69210 of HSV-1 DNA nucleotide sequence within gene UL33. The alteration resulted in the substitution of an isoleucine by an asparagine codon. The nucleotide sequence of the revertants in this region was identical to that of *wt* virus DNA. The nature of the mutation in *ts*1233 is consistent with the use of UV-light as a mutagen.

Two oligopeptides, one representing a portion of the amino-terminus and the other representing a portion of the carboxy-terminus of UL33 amino acid sequence were synthesised and coupled either to bovine serum albumin (BSA) or to β-galactosidase and injected into rabbits. Antibodies against the peptides were detected by radio-immunoassays. No virus specific bands were detected when the antisera were reacted with virus infected cell extracts on western blots, however, immunoprecipitation experiments

with virus-infected cell extracts and the antisera gave a very weak specific reaction with a polypeptide of the apparent mw predicted for UL33 gene product.

Attempts to express the UL33 gene product in bacterial expression vectors were unsuccessful. The UL33 gene product was also placed under immediate-early (IE) gene regulation. The IE promoter and upstream regulatory sequence of Vmw175 were inserted in front of the UL33 gene and the UL33 gene containing IE promoter recombined into TK gene of *ts*K virus, which has a defect in Vmw175. Although novel bands were detected in cells infected with *ts*K recombinant virus at the NPT, further work is required to determine whether any of these bands are the UL33 gene product.

*Ts*1201, like *ts*1233 fails to encapsidate DNA at the NPT. Sequence analysis of the 673bp fragment in which the *ts*1201 lesion mapped revealed that the mutation lies 89bp upstream from the amino terminus of UL26. A single bp change was found at a position corresponding to residue 50897 of HSV-1 17*syn*+ nucleotide sequence. This resulted in the substitution of tyrosine with phenyl alanine codon. Both the *ts*+ revertants analys ed retained the *ts*1201 mutation and had second site reversions elsewhere within UL26 gene.

Three oligopeptides, one representing 9 amino acids at the amino terminus of UL26, one representing 12 amino acids from the second potential AUG, and one representing 14 amino acids of the carboxy terminus were synthesised, coupled to β-galactosidase and injected into rabbits. The

antisera all contained oligopeptide antibodies that recognised the peptides which they were raised against. However, in western blot experiments only antibodies against the carboxy terminus of UL26 gene product reacted with a specific virus band in virus infected cell extracts. In immunoprecipitation reactions, only antisera raised against the oligopeptide specific for the carboxy terminus of UL26 gene product gave a strong reaction with a virus specific band with an apparant mw of about 40,000. The ability of the oligopeptide to competitively inhibit immunoprecipitation of this band strongly suggested that antibodies raised against the carboxy terminus were specific to UL26 gene product. The processing of UL26 gene product was further investigated in *ts*1201 and *wt* virus-infected cells at the PT and the NPT using the antibody raised against the carboxy terminus. The oligopeptide antisera reacted only with the high mw forms of UL26 gene products suggesting that the conversion of the UL26 gene product to its lower mw forms was due to processing, probably proteolytic cleavage at the carboxy terminal end of the protein.

A	adenine
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
BCIG	(X-Gal) 5-bromo-4-chloro-3-indolyl-β-D-galactoside
BCdR	5-bromo-2'-deoxycytidine
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
BPB	bromophenol blue
BUdR	5-bromo-2'-deoxyuridine
C	cytosine
Ci	Curies
cm	centimeters
CMV	cytomegalovirus
CO_2	carbon dioxide
CPE	cytopathic effect
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

ds	double strand
DDT	dithiothreitol
dTTP	deoxythymidine triphosphate
E	early
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	sodium ethylen-diamine tetra-acetic acid
EHV	equine herpesvirus
em	electron microscope
eop	efficiency of plating
EtBr	ethidium bromide
G	guanine
%GC	moles percent deoxyguanosine and deoxycytidine
h	hour
HCl	hydrochloric acid
HFL	human foetal lung
hpi	hours post infection
HSV	herpes simplex virus
HU	human serum
HVS	herpesvirus saimiri
ICP	infected cell polypeptides
IE	immediate early
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl-beta-D-thio-galactopyranoside
Kb	kilobase
l	litre
L	late
M	molar

MI	mock-infected
min	minute
ml	millilitre
mm	millimetre
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
mu	map units
MW	molecular weight
NA	nitrous acid
ng	nanograms
nm	nanometer
NP40	nonidet p40
NPC	nucleoprotein complex
NPT	non permissive temperature
OD	optical density
ori	origin of replication
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
pfu	particle forming unit
pi	post infection
PMSF	phenylmethylsulphonyl fluoride.
PRV	pseudorabies virus
PT	permissive temperature
RNA	ribonucleic acid
RNase	ribonuclease
rev	revertant
rpm	revolutions per minute
RT	room temperature

SDS	sodium dodecyl sulphate
sec	second
syn	syncytial
syn$^+$	non-syncytial
T	thymidine
TEMED	N,N,N'-N'-tetramethylethylenediamine
TK	thymidine kinase
TK$^+$	thymidine kinase-positive
TK$^-$	thymidine kinase-negative
ts	temperature sensitive
ts$^+$	wild-type for temperature sensitivity
Tween 20	polyoxyethylene sorbitan monolaurate
UV	ultra violet
V	volts
v/v	volume per volume
V_{MW}	molecular weight of virus-induced protein
VP	virion protein
VZV	varicella zoster virus
wt	wild type
w/v	weight per volume
w/w	weight per weight
µCi	microcuries
µg	microgram
µl	microlitre
unit	unit
%	percentage

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CHAPTER 4

INTRODUCTION

The project presented in this thesis concerns the characterisation of two herpes simplex virus (HSV) temperature sensitive (*ts*) mutants, *ts*1233 and *ts*1201, and the genes in which the mutations lie. *Ts*1233 and *ts*1201 both have structural defects and fail to package virus DNA at the non permissive temperature (NPT). The aim of the following chapter is to provide a review on the biology of HSV, with particular emphasis on the assembly of the virion and encapsidation of HSV DNA.

1.1 DEFINITION AND CLASSIFICATION OF HERPESVIRUSES

Members of the family herpesviridae have been isolated from a wide variety of vertebrates and invertebrates (Roizman, 1982). The virion is 150-200nm in diameter and contains a double stranded (ds) linear DNA genome which is enclosed within an icosahedral capsid. A lipid envelope surrounds the capsid (Wildy *et al.*, 1960; Furlong *et al.*, 1972). Viruses of this family replicate in the nucleus, and acquire their envelopes by budding through the nuclear membrane (Morgan *et al.*, 1954; Wildy *et al*,.1960; Darlington *et al*,. 1968).

The members of herpesviridae family have been classified into three sub-families, according to their biological and pathogenic properties which include host range, duration of the lytic cycle, cytopathology, and characteristics of the latent infection (Roizman *et al.*,

1981; 1982; Matthews, 1982).

1.1.1 Sub-family alphaherpesvirinae

Although members of this sub-family have a narrow host range in nature some, for example HSV, can infect a variety of experimental animals and tissue culture cells. *In vitro*, the reproductive cycle is short, usually less than 24h, and results in destruction of susceptible cells. Latency frequently occurs in the ganglia (Stevens and Cook, 1971; 1972). HSV type-1 (HSV-1) is the prototype virus of this group. Primary virus infection can be inapparent, but is sometimes manifested as acute gingivo-stomatitis. Occasionally the virus causes ocular keratitis and, in very rare cases, acute necrotising encephalitis. HSV-1 is normally spread by direct contact or by droplets from an infected person. HSV type 2 (HSV-2), another member of this sub-family, which is closely related to HSV-1 causes genital lesions in man and is venerally transmitted. Exposure of neonates to this virus results in a disseminated, frequently fatal infection. Although HSV-1 normally causes oral lesions and HSV-2 genital lesions, the distinction is not absolute, since HSV-1 can cause genital lesions, and likewise HSV-2 can infect other parts of the body. Another human alphaherpesvirus is varicella zoster virus (VZV) which causes varicella or chicken pox usually in childhood and zoster or shingles after latent virus reactivation in adults. Other members include pseudorabies virus (PRV) which causes Aujeszky's disease in pigs and B virus, a monkey virus which causes a fatal illness in

humans.

1.1.2 Sub-family betaherpesvirinae

Members of this sub-family are known as cytomegaloviruses and are characterised by restricted host range *in vivo* and *in vitro*. In tissue culture the infection progresses slowly and infected cells become enlarged. Cell types responsible for harbouring latent viruses are unknown. The prototype virus is the human cytomegalovirus (HCMV). Although most infections with HCMV are asymptomatic, HCMV is a major cause of congenital disease. The virus can also cause severe problems in immunocompromised individuals, principally those undergoing transplant surgery (Alford and Britt, 1985), and more recently those with AIDS.

1.1.3 Sub-family gammaherpesvirinae

Members of this group are lymphoproliferative viruses. They normally exhibit a narrow host range *in vivo*. *In vitro*, viruses can infect lymphoblastoid cells, which are usually non-permissive or semi-permissive for virus replication. Viruses are generally specific for either T or B lymphocytes but some, for example herpes virus sylvilagis can infect both types. Although viruses usually have restricted growth in lymphoblastoid cells, many viruses in this sub-family, will productively infect fibroblasts. Epstein-Barr Virus (EBV) is the prototype of this group and has been associated with Burkitt's lymphoma. This virus is the causative agent of infectious

mononucleosis. Other members of this group include Gallid herpesvirus 1 (MDV) which causes Marek's disease in chickens, and herpesvirus saimiri (HVS) which infects primates.

Classification into various families is somewhat arbitrary and subjective, and as a consequence some herpesviruses have been incorrectly assigned, for example MDV. In general, however, the classification system has proved to be reasonably satisfactory.

1.2 THE STRUCTURE OF HERPESVIRUS GENOME

The HSV-1 genome is a linear ds DNA molecule (Becker, *et al.*,1968; Graham *et al.*,1972) with a molecular weight (mw) of about 95-100X10^6 (Kieff, *et al.*, 1971). It has a high overall guanine and cytosine (G+C) content of 68.3%. Different regions of the genome, however, vary in their G+C content, most notably the short repeat region which has a very high G+C value of 79.5% (Davison and Wilkie, 1981; Murchie and McGeoch, 1982; McGeoch *et al.*, 1986).

The HSV genome can be divided into two covalently linked regions, designated L (long), and S (short), representing 77% and 23% of the virus DNA respectively. Each component consists of unique sequences (as shown in fig 1A), flanked by inverted repeat sequences (Sheldrick and Berthelot, 1974; Wadsworth *et al.*, 1975). A direct repeat known as the *a* sequence is present at the genomic termini and also in an inverted orientation at the L-S junction (Grafstrom *et al.*, 1974; 1975; Wadsworth *et al.*,

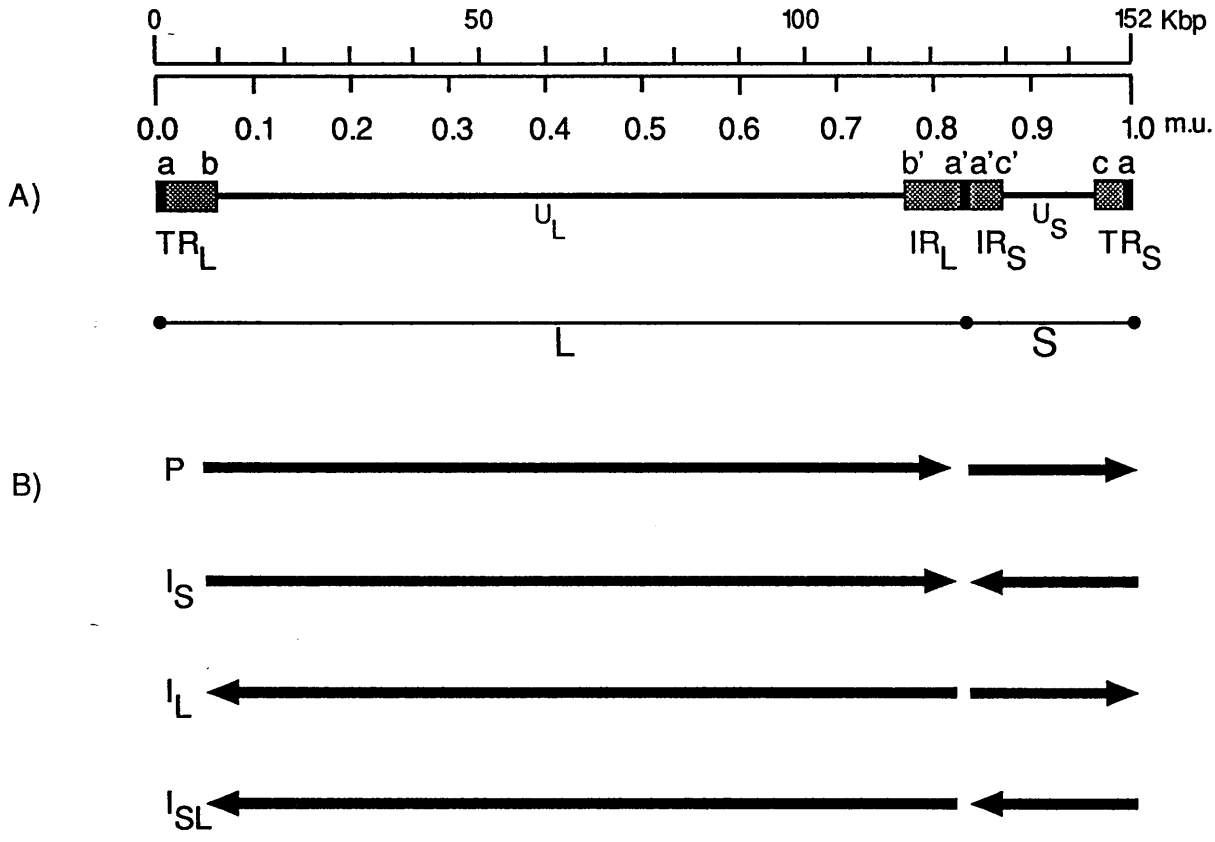

0
50
100
152 Kbp
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0 m.u.
A)
a b
b' a'a'c'
c a
TR_L
U_L
IR_L
IR_S
U_S
TR_S
L
S
B)
P
I_S
I_L
I_SL

Figure 1

A) HSV-1 genome arrangement.

The genome is divided into two regions. The long region (L) consists of a long unique sequence (U_L) flanked by a terminal sequence (TR_L) which is repeated internally in an inverted orientation (IR_L). The short (S) region consists of a short unique sequence (U_S) flanked by a terminal sequence (TR_S) which is repeated internally (IR_S) in an inverted orientation. The direct repeat at the genomic termini is known as the *a* sequence. The remaining sequence within TR_L/IR_L and TR_S/IR_S are referred to as b/b' and c/c' respectively.

B) Genome isomerisation

P : prototype orientation

I_S : inversion of the short region

I_L : inversion of the long region

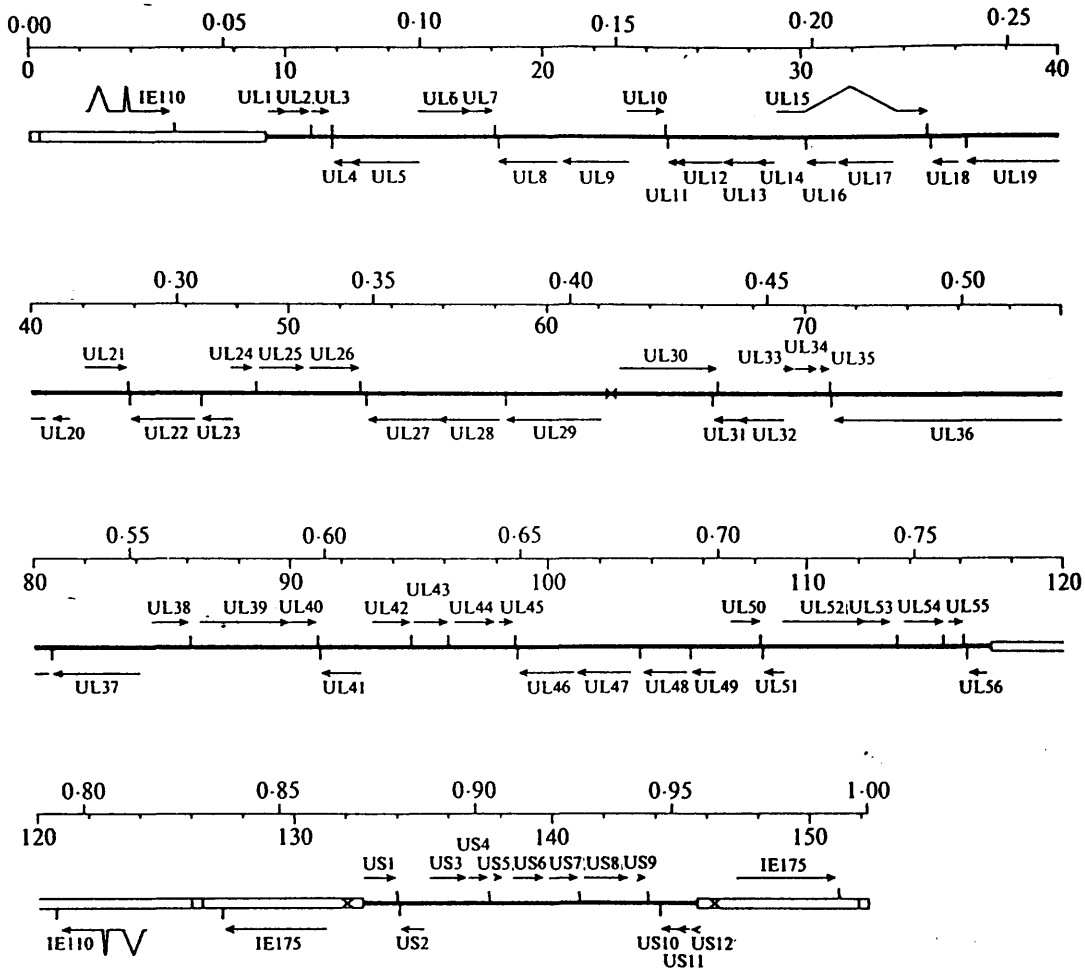
I_{SL} : inversion of the short and long regions

1976; Wagner and Summer, 1978; Davison and Wilkie, 1981; Mocarski and Roizman, 1981). As a consequence of the genome arrangement of HSV, the L and S components can invert relative to each other (Fig. 1B). Thus, DNA extracted from virions or from cells infected with *wt* virus consists of four equimolar populations differing with respect to the orientation of L and S unique regions (Sheldrick and Berthelot, 1974; Hayward *et al.*, 1975; Clements *et al.*, 1976; Delius and Clements, 1976).

1.2.1 Gene organisation in HSV-1 genome

The complete sequence of HSV-1 strain 17 genome contains 152,260 residues in each strand and specifies at least 72 genes encoding 70 distinct proteins (McGeoch *et al.*, 1985; 1986; 1988a; 1988b). From the sequence analysis of U_L it was concluded that 89% of the sequence, representing 56 genes, encoded for proteins (fig. 2). Thus, genes are compactly arranged within U_L. Overlaps of coding sequences in UL occur in two major clusters at genes UL5 to UL14, and UL30 to UL33. In many cases the transcriptional control elements overlap the polypeptide coding regions of the adjacent gene. The largest non-protein coding region of 754bp lies between genes UL29 and UL 30 and contains ori_L. The overall G+C content of U_L is 67% (McGeoch *et al.*, 1988).

The U_S region, which is 12,979 bp in length, contains 12 genes compactly arranged (McGeoch *et al.*, 1985; Rixon and McGeoch, 1985). Although all the genes have separate promoters, most of the genes share 3' termination


0·00
0·05
0·10
0·15
0·20
0·25
0
10
20
30
40
IE110
UL1 UL2 UL3
UL6 UL7
UL10
UL15
UL4 UL5
UL8 UL9
UL12 UL14 UL17
UL11 UL13 UL16
UL18 UL19
0·30
0·35
0·40
0·45
0·50
40
50
60
70
UL21
UL24 UL25 UL26
UL30
UL33 UL34 UL35
UL20
UL22 UL23
UL27 UL28 UL29
UL31 UL32
UL36
0·55
0·60
0·65
0·70
0·75
80
90
100
110
120
UL43
UL38 UL39 UL40
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UL52 UL53 UL54 UL55
UL37
UL41
UL46 UL47 UL48 UL49 UL51
UL56
0·80
0·85
0·90
0·95
1·00
120
130
140
150
US4
US1
US3 US5 US6 US7 US8 US9
IE175
IE110
IE175
US2
US10 US12
US11

Figure 2

Layout of genes in the genome of HSV-1.

The HSV-1 genome is shown in four successive lines, with unique regions represented by solid lines and major repeat elements as open boxes. The lower scale represents kilobases, numbered from the left terminus, and the upper scale represents fractional map units. The sizes and orientations of proposed functional ORFs are shown by arrows. Overlaps of adjacent, similarly oriented ORFs are not shown explicitly. Locations of proposed transcription polyadenylation sites are indicated as short vertical bars. Locations of origins of DNA replication are shown as X. In the U_L region, on the first three lines, genes UL1 to UL56 are labelled. In the U_S region, on the bottom line, genes US1 to US12 are labelled. The locations of introns in the coding regions of gene UL15 and the two copies (TR_L and IR_L) of the IE110 gene are indicated (taken from McGeoch *et al.*, 1988).

sites. In fact all but 2 mRNAs belong to one of four 3' coterminal families. Only genes U_S10 and U_S11 have overlapping coding sequences but in different reading frames. The promoter of U_S10, however, lies within U_S11 coding sequences. The overall G+C is 64%, which is considerably lower than the G+C content of adjacent DNA (IR_S/TR_S) (Rixon and McGeoch, 1984; McGeoch *et al.*, 1985; McGeoch *et al.*, 1988).

The short repeat sequence (TR_S/IR_S), which is 6,633 bp in length, contains only one gene, the immediate early (IE) gene 3 which encodes IE175. The *a* sequence is present at the 3' end of this gene, while at 5' end of IE175 map sequences representing an origin of replication (*ori*$_S$) and the promoter of IE68 (gene U_S1) and IE12 (gene U_S12). The coding sequences of the latter two genes lie within U_S (Murchie and McGeoch, 1982; McGeoch *et al.*, 1985; 1986).

The long repeat region in HSV-1 17*syn*$^+$, which has a G+C content of 71.6% and spans some 9,215 bp, contains a spliced gene encoding IE110 (Perry *et al.*, 1986; Perry and McGeoch, 1988). In addition, the IR_L/TR_L sequence contains six families of tandemly reiterated sequences, ranging from 3 to 55bp, present in different regions of the repeat, and the *a* sequence. Two open reading frames (ORF) have also been described in the long repeat of other HSV-1 strains. The first ORF, which is proposed to encode ICP 34.5, is situated upstream of 5' end of IE110 in strain F (Chou and Roizman, 1986; Ackermann *et al.*, 1986). In HSV-1 strain 17 *syn*$^+$ this region contains multiple stop codons. The other ORF lies at the 3' end of IE110 gene and overlaps IE110

coding sequences. Recently it has been shown that this region is transcribed in latently infected neurons. It has not been shown, however, that these latency-associated transcripts (LAT), encode proteins (Stevens *et al.*, 1987; Rock *et al.*, 1987; Spivack and Fraser, 1987; Wagner *et al.*,1988).

1.3 THE STRUCTURE OF HERPESVIRION

1.3.1 Morphology

The herpesvirion is generally considered to be composed of four complex concentric substructures, referred to as the core, capsid, tegument, and envelope (fig. 3).

1.3.1.a The core

This is the central component of the virion and is thought to consist of a cylindrical protein plug around which the viral DNA is tightly spooled in the form of a torus (Furlong *et al.*, 1972). Studies on infectious rhinotracheitis virus, MDV, and HCMV suggest that the core is attached to the inner surface of the capsid (Nazerian, 1974; Haguenau and Michelson-Fiske 1975).

1.3.1.b The capsid

This structure has a diameter of approximately 120nm, and it is calculated to be composed of 150 hexameric and 12 pentameric units in the form of an icosahedron (Wildy *et al.*, 1960; Furlong, 1978). Until recently it was thought that HSV had a single icosahedral capsid shell, however, data obtained from computer analysis of low dose

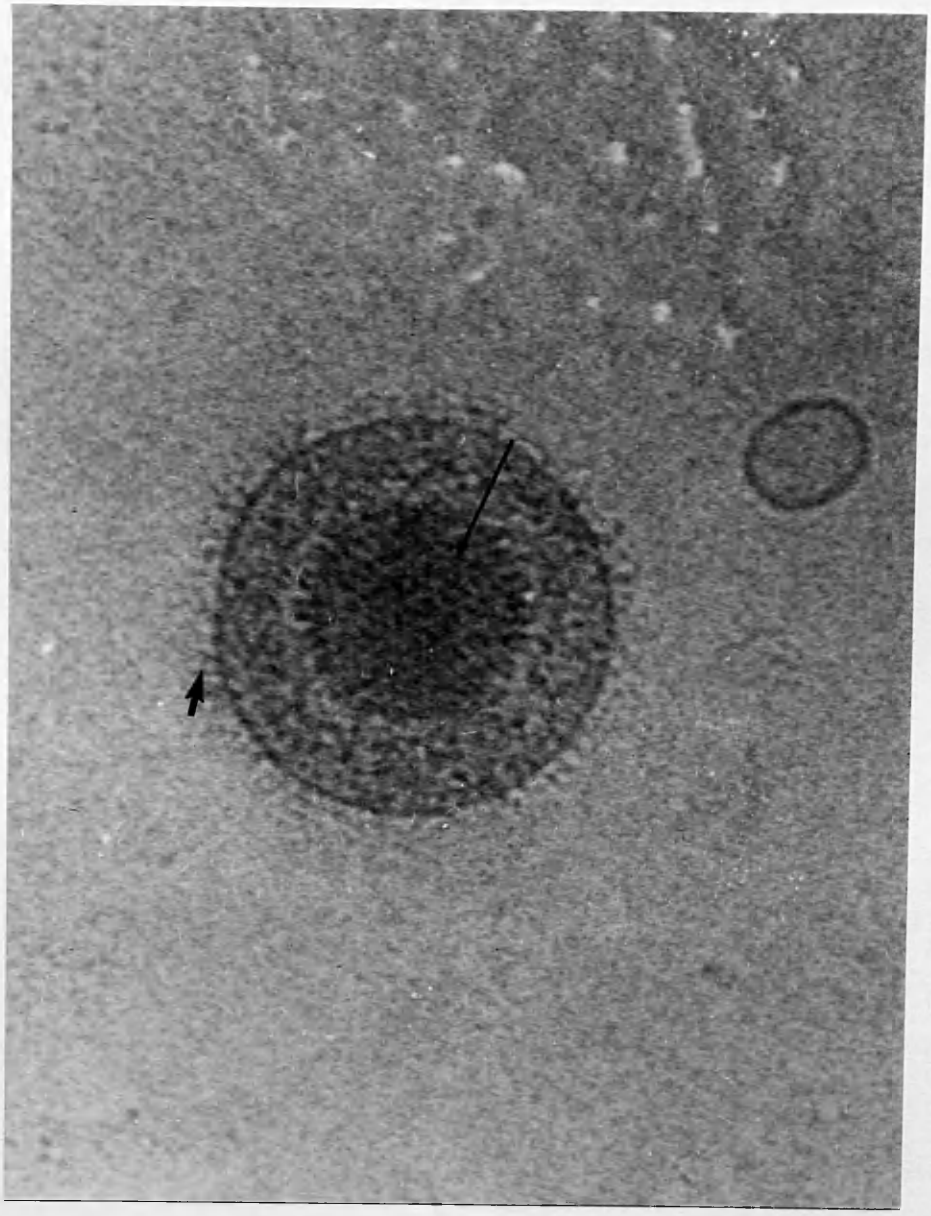


Figure 3

Cryo-electron micrograph of HSV-1 virion.

The virion is suspended in vitreous ice. Visible in the enveloped particle is the nucleocapsid (long arrow), tegument, and envelope with associated glycoprotein spikes (short arrow) . The micrograph was kindly provided by Dr. Frazer J. Rixon.

cryo-electron images of ice embedded capsids revealed that full capsids structure are organised into an outer, intermediate and inner structural layers. The outer layer is arranged according to T=16 icosahedral symmetry. This structure has a diameter of approximately 120nm and is composed of 150 hexameric and 12 pentameric units (Wildy *et al.*, 1960). Adajacent capsomeres are connected by a mass density (Schrag *et al.*, 1989), previously referred to as intercapsomeric fibrils (Palmer *et al.*, 1975; Vernon *et al.*, 1974). The intermediate layer lies on a T=4 icosahedral lattice (Schrag *et al.*, 1989). The inner most layer contains the genomic DNA, which is connected to the external environment by channels through the outer and intermediate layers which coincide along their icosahedral two-fold axis.

1.3.1.c The tegument

This is defined as an amorphous structure which lies between the capsid and the envelope (Wildy *et al.*, 1960; Morgan *et al.*, 1968; Roizman and Furlong, 1974). The thickness of the tegument varies between different herpesviruses and is genetically determined by the virus (McCombs *et al.*, 1971). According to Vernon *et al.* (1982), the tegument is attached to the vertices of the capsid and the inner surface of the envelope, and may have a function in envelopment of the virus particle. It has been suggested that the tegument of EHV-1 virions is composed of granular particles of varying size which form a shell surrounding the capsid. This observation implies that the

tegument might possess some form of structural integrity. In addition it has been concluded that this structure probably has a high water content (Vernon *et al.*, 1982).

1.3.1.d The envelope

This is a trilaminar membrane which is thought to be derived from the inner nuclear membrane, acquired during budding of the virus particles through the nuclear membrane (Darlington and Moss, 1968). The envelope tightly adheres to the tegument and contain spikes 8-10nm long spaced 5nm apart over the surface (Wildy *et al.*, 1960). In a recent electron microscopic study of disrupted virus particles, using monoclonal antibodies to identify individual glycoproteins, it was shown that glycoprotein gB, gC, and gD each form separate spikes that are different in size, morphology and distribution in the envelope (Stannard *et al.*, 1987).

1.4 EARLY STAGES OF HSV INFECTION

The initial stages of the lytic cycle can be divided into three parts: adsorption of the virus particle to the cell, penetration of the capsid into the cytoplasm, and release of viral DNA from the capsid (uncoating).

1.4.1 Adsorption

Attachment of the HSV particle to the cell surface is a rapid process (Hochberg and Becker,1968), and occurs over a wide range of temperatures (Farnam and Newton, 1959). The results of early studies by Hochberg and Becker

(1968) showed that heparin, a negatively charged polysaccharide, prevented the adsorption of the virus to cells and released the virus already attached to the cell surface. On the basis of these experiments it was suggested that the first step in virus adsorption involved an electrostatic interaction. These results were supported and extended by the recent findings of WuDunn and Spear (1989), who demonstrated that virions could bind to heparin in affinity chromatography experiments, and that removal of heparan sulphate from cells reduced the ability of the cell to bind virus. It was concluded that both HSV-1, and HSV-2 normally initiate infection by binding first to heparan sulfate on the cell surface, which serves as a receptor for HSV (WuDunn and Spear, 1989).

The viral proteins responsible for the attachment of the virions to receptors on cell surfaces have not yet been identified, but several envelope viral glycoproteins have been implicated in the process of virus attachment. It is thought that gB, an essential glycoprotein (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Deluca *et al.*, 1982), may play a role in adsorption since virosomes, which are lipid vesicles enriched with HSV-1 glycoproteins, bind to cells more efficiently when gB is present than when it is absent (Johnson *et al.*, 1984). However, the finding that gB deficient virions were able to bind to cells, has clearly shown that gB is not essential for attachment of virus to cells (Cai *et al.*, 1987; 1988a), but is required in a later step in virus entry. Polyclonal and monoclonal antibodies directed against gC and gD

inhibit adsorption of HSV-1 to cells, while high concentration of of IgG and Fc fragment can partially inhibit attachment of the virus to cells, suggesting that gC, gD, and possibly gE, which binds the Fc portion of IgG (Bauke and Spear,1979), may also play a role in virus adsorption (Para *et al.*, 1982; Fuller and Spear, 1985). Since gC and gE are not essential for virus infectivity in tissue culture, those glycoprotens like gB, are not absolutely required for virus attachment (Ruychan *et al.*, 1979; Holland *et al.*, 1983; 1984; Longnecker and Roizman, 1986). A recent study revealed that gD deficient mutants were able to bind to cells, but unable to initiate synthesis of viral early polypeptides (Ligas and Johnson, 1988). These results suggest that gD is also not essential for virus adsorption. From the the work mentioned above together with the finding that gD, gC, and gB form separate spikes on the envelope (Stannard *et al.*, 1987), it seems likely that envelope glycoproteins may individually interact with cell receptors for HSV. Recent results obtained by Campadelli-Fiume *et al.* (1988) and Johnson and Spear (1989), showing that HSV adsorbed to gD expressing cell lines but was unable to be internalised by fusion of virion envelope with plasma membrane, has led to the suggestion that gD may recognise a cell receptor, different from cell surface heparan sulphate. Other studies on cell surface receptors have suggested that HSV-1 and HSV-2 recognise different cell surface receptors (Vahlne *et al.*, 1979; Addison *et al.*,1984).

1.4.2 Penetration

Penetration of nucleocapsids into cell occurs rapidly after the virus particle binds to the receptors on the surface of the cell. Electron microscopic studies indicate that the entry of the virus into the cell occurs either by viropexis (phagocytosis) (Dales and Silverberg, 1969; Hummeler *et al.*, 1969), or by fusion of the viral envelope with cell membrane (Morgan *et al.*, 1968; Abodeely *et al.*, 1970). Evidence presented so far supports the fusion model of virus penetration. In particular, the work by Para *et al.* (1980; 1982), showing that gE from the infecting virions was incorporated into the surface membrane of infected cells immediately after viral penetration, is indicative of fusion between the virion envelope and cell surface membrane.

Several lines of research have implicated gB in virus penetration. Studies using gB-null mutants or *ts* mutants of gB have shown that mutants lacking gB or containing abberrant gB bound as well as *wt* virus to the plasma membrane but were unable to penetrate the cell surface (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Cai *et al.*, 1988). Other work has shown that monoclonal antibodies, which recognise two major antigenic sites within the external domain of gB, neutralized virus adsorbed to cell monolayers (Highlander *et al.*, 1988). These results suggest that gB is important at a stage after virus attachment. Glycoprotein B has also been shown to affect the rate of entry, since mutants with lesions in gB penetrated cells more rapidly than *wt* virus. Furthermore a

syn lesion has been identified within gB (Deluca *et al.*, 1981; 1982; Bond *et al.*, 1982; Bzik *et al.*, 1984; Cai *et al.*, 1988). All of these results indicate that gB is directly involved in virus penetration.

Although gC is non essential, Deluca *et al.* (1982) reported that recombinants of KOSX*ts*B5, which made little or no gC, entered cells at an accelerated rate at the PT in comparison to viruses which contained gC. It has also been demonstrated that virus strains which lacked gC were able to penetrate restrictive cell line XC, whereas strains which had a high gC content failed to do so. This led to the suggestion that gC may influence penetration of virus by negatively modulating the gB-promoted fusion between virion and host cell membranes, or by interacting with other glycoproteins such as gD or gH involved in fusion of membranes (Epstein *et al.*,1984; Machuca *et al.*, 1987).

Recent work by Fuller and Spear (1987) and Highlander *et al*, (1987) suggested that gD has a role in virus penetration since gD-specific monoclonal antibodies (mAB) neutralised in the absence of complement HSV-1 virus bound to cells. The subsequent finding that gD deficient virions were able to bind to tissue culture cells as well as virion containing gD supports this idea (Ligas and Johnson, 1988).

The gene product of UL25, which does not appear to be a glycoprotein, is also important for virus penetration, since a mutant of HSV-1, *ts*1204, which has a lesion in gene UL25, binds to cells at the NPT, but fails to penetrate the cell membrane (Addison *et al.*, 1984).

1.4.3 Uncoating of the viral DNA

The step which follows penetration of herpesvirions into the cell is the release of the viral DNA from the capsids. Studies have indicated that viral DNA is rapidly transported to the sites of replication in the nucleus within 15-20 min post infection (Hummeler *et al.*, 1969; Miyamoto and Morgan, 1971; Wahren *et al.*, 1984). Early work showed that capsids undergo disintegration (Morgan *et al.*, 1968). The transport of viral DNA from the cytoplasm to the nuclei of infected cells was unaffected by RNA and protein synthesis inhibitors, suggesting that the viral DNA is released from the capsid by a pre-existing cellular enzyme or virion protein (Hochberg and Becker, 1968). Analysis of the HSV-1 *ts* mutant, *ts*B7 which failed to uncoat at the NPT, has provided further clues about the release of DNA from the capsid (Knipe *et al.*, 1981; Batterson *et al.*, 1983). The proximity of the λ mutant in the cytoplasm to nuclear pores suggested that the viral DNA enters the nucleus by an active cellular mechanism which probably involves host cell microfilaments (Lycke *et al.*, 1984).

1.5 EFFECT OF HSV INFECTION ON CELLULAR MACROMOLECULAR SYNTHESIS

Infection of cells with HSV results in the inhibition of host DNA, RNA and protein synthesis. Inhibition of protein synthesis is probably a multistep process, resulting from the reduction in mRNA levels and disaggregation of polyribosomes (Sydiskis and Roizman,

1967; Wagner and Roizman, 1969; Nishioka and Silverstein, 1977; 1978; Silverstein and Engelhardt, 1979; Bastow *et al.*, 1986; Strom and Frenkel, 1987). Inhibition of cellular protein synthesis during HSV infection has been divided into two stages referred to as "early" and "delayed" shut-off.

Early shut-off of protein synthesis is thought to be mediated by the virion protein UL41 (Fenwick and Walker, 1978; Peirera *et al.*, 1977; Fenwick *et al.*, 1979; Fenwick and McMenamin, 1984; Schek and Bachenheimer, 1985; Kwong *et al.*, 1988). The isolation of viable mutants in tissue culture, defective in virion host shut-off (vhs), has provided evidence that this function is not absolutely essential for virus replication. It is, nevertheless, important for optimal growth since the mutant virus did not replicate as well as *wt* virus (Read and Frenkel, 1983; Kwong *et al.*, 1988). Recently it has been shown that vhs function decreases the half lives of both host and viral mRNA, and therefore may have a dual role in viral infection, inhibiting host gene expression and enabling the rapid transition of viral gene expression (Strom and Frenkel, 1987; Oroskar and Read, 1989). Evidence obtained by Kwong and Frenkel (1988) suggests that a single virion protein, is responsible for early shut-off of protein synthesis since a population of viruses which had the virion host shut-off (vhs) mutation in UL41 replaced by *wt* sequences regained levels of shut-off similar to *wt* virus. This finding supports the idea that disaggregation of polyribosomes which occures in the absence of viral protein

synthesis and mRNA degradation (Nishioka and Silverstein, 1977; 1978; Hill *et al.*, 1983), are carried out by the same protein. The available evidence favours the idea that UL41 gene product (vhs) either activates a preexisting host nuclease or increases susceptibility of mRNAs to nuclease attack (Kwong *et al.*, 1988). The delayed shut-off of host protein synthesis requires the expression of an early or late viral gene and reduces further the level of host protein synthesis (Fenwick and Clarke, 1982; Hill *et al.*, 1983; Read and Frenkel, 1983; Schek and Bachenheimer, 1985). Although it has been suggested that an additional viral function is required for complete host protein inhibition it is possible that the vhs protein is involved in delayed or complete host shut-off, since the findings of Kwong and Frenkel (1987) and Oroskar and Read (1987; 1989) indicate that mutations in UL41 protein cause an increase in the stability of not only IE mRNA, but also early and late RNAs in absence of protein synthesis inhibitors. There is, however, in cells infected with vhs mutants some inhibition of host proteins although it is delayed and incomplete suggesting another protein may be involved in delayed host shut-off (Read and Frankel, 1983). Stenberg and Pizer (1982) have evidence that an IE gene function is responsible for the decrease in cellular RNA synthesis which follows HSV infection, and this result could account for the findings observed by Read and Frenkel (1983).

1.6 HSV TRANSCRIPTION

HSV DNA is transcribed in the infected cell nucleus

(Wagner and Roizman, 1969). The observation that all stages of HSV RNA synthesis are sensitive to α-amanitin inhibition has led to the suggestion that HSV transcription is carried out by RNA polymerase II (Alwine *et al.*, 1974; Ben Zeev *et al.*, 1976; Costanzo *et al.*, 1977). This conclusion is supported by the finding that naked DNA is infectious (Graham *et al.*, 1973). Since, however, only IE mRNA is synthesised in the absence of *de novo* viral protein synthesis (Honess and Roizman, 1973; 1974; Clements *et al.*, 1977; Watson *et al.*, 1979), it is clear that viral proteins are important for transcription of HSV mRNA .

Since HSV utilizes much of the cellular transcription machinery it is therefore not surprising that many of the transcriptional signals are identical to those of eukaryotic genes and that transcripts are processed in the same way as most cellular mRNAs. A polyadenylation consensus signal "AATAAA" in the DNA, specifying the 3'-termini of transcripts, is essential for the correct processing of viral mRNAs (McKnight, 1980; Cole and Santangelo, 1983), and a consensus "G-T" rich signal "5-YGTGTTYY-3'" is thought to be necessary for efficient formation of the mRNA 3'-terminus. This signal is usually located approximately 30bp downstream from the polyadenylation signal (Taya *et al.*, 1982; McLauchlan *et al.*, 1983; Whitton *et al.*, 1983; McLauchlan *et al.*, 1985; Cole and Stacy, 1985). The "TATA-box" homology, which is thought to be important for accurate initiation of transcription and is located close to the 5'-termini of most eukaryotic genes (Gannon *et al.*, 1979), is also

present close to the 5' end of HSV genes (McGeoch *et al.*, 1985; 1986). Like most cellular mRNAs, HSV transcripts are capped at their 5' end. This modification is thought to be necessary both for efficient translation (Shatkin, 1976), and protection of the mRNA from nucleases and phosphatases (Moss *et al.*, 1977). Methylation of HSV transcripts at internal adenine residues has been reported (Bartkoski and Roizman, 1976; Moss *et al.*, 1977).

In comparison to eukaryotic transcripts, spliced mRNAs are not a common feature in HSV. Only five of the 72 open reading frames have spliced mRNAs. IE 68 (U_S1) and IE 12 (U_S12) genes, which share a common 5'-non-coding region in IR_S but have unique polypeptide coding sequences within U_S, have an intron within the common 5'-non-coding region (Watson *et al.*, 1981; Murchie and McGeoch, 1982; Rixon and Clements, 1982; Watson and Vaude-Woude, 1982). In contrast to IE 68, and IE 12 genes, IE 110, which is a diploid gene, contains two introns in the coding sequence of the gene (Perry *et al.*, 1986). The only other gene in which introns have been identified is UL15. This gene is thought to encode a late viral protein (Costa *et al.*, 1985; McGeoch *et al.*, 1988).

1.6.1 The regulation of HSV transcription

Viral mRNAs and proteins are temporally regulated and expressed as a sequential cascade which is differentia ted into three broad classes defined as IE or α, early (E or β), and late (L or γ) (Honess and Roizman, 1974; 1975; Swanstrom and Wagner, 1974; Clements *et al.*, 1977). Recent

work on IE gene regulation has provided evidence that the cascade pattern of HSV protein expression is more complex than originally proposed by Honess and Roizman (1974; 1975), who based their work on kinetic studies and the effect of metabolic inhibitors on viral protein synthesis.

1.6.2 IE genes, their expression and regulation

IE mRNA species are the first class of transcripts to be detected during a normal viral infection. These mRNAs are made even in the presence of a protein synthesis inhibitor, such as cycloheximide or anisomycin, added at the time of infection, and therefore do not require *de novo* viral protein synthesis for abundant expression (Honess and Roizman, 1974; Clements *et al.*, 1977; 1979; Anderson *et al.*, 1980; Harris-Hamilton and Bachenheimer, 1985). Five major IE polypeptides, V_{MW}175, 110, 68, 63, 12 are synthesised when the protein synthesis block is removed from viral-infected cells (Clements *et al.*, 1977; Preston, 1979a; 1979b; Watson *et al.*, 1979; 1981; Anderson *et al.*, 1980). All IE polypeptides except V_{MW}12 (IE5) are phosphorylated, and transported into the nucleus (Marsden *et al.*, 1976; 1978; Pereira *et al.*, 1977), and with the exception of IE12, all bind to DNA *in vitro* (Hay and Hay, 1980).

Analyses of IE gene promoter and regulatory regions have revealed a number of different upstream elements. Important transcriptional elements were identified in IE genes 1 (V_{MW}110), 2 (V_{MW}63), 3 (V_{MW}175), 4 (V_{MW}68), 5 (V_{MW}12). These included: (I) TATA box homology located

approximately 30 bp upstream from the mRNA start site, (II) A proximal promoter region, located between -37 to -108bp, which is important for transcription initiation since the deletion of this region abolishes transcription of IE genes (Mackem and Roizman, 1982a; 1982b; Cordingley *et al.*, 1983). Within these sequences is a GC-rich region which may be involved in interactions with the cellular transcription factor Sp1 (Jones and Tjian, 1985). (III) Elements containing regulatory signals resembling enhancer-like sequence. These elements can function in either orientation from the promoter and at a distance up to 1300 bp (Cordingley *et al.*, 1983; Lang *et al.*, 1984; Preston and Tannahill, 1984; Bzik and Preston, 1986). (IV) A consensus sequence TAATGARAT (R is a purine), located far upstream within all IE gene promoters (Mackem and Roizman, 1982b; Cordingley *et al.*, 1983; Whitton *et al.*, 1983; Whiton and Clements, 1984; Gaffney *et al.*, 1985).

1.6.3 IE transactivation

IE transcription is stimulated by UL48 gene product ($V_{MW}65$, VP16, also known as trans-inducing factor or "TIF"), present as a component of the HSV-1 infecting virion (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell *et al.*, 1984; Preston *et al.*, 1984; Pellett *et al.*, 1985). Transactivation of IE transcription by $V_{MW}65$ depends on a cis-acting regulatory element which contains the consensus TAATGARAT sequence (Kristie and Roizman, 1984; Preston *et al.*, 1984; Gaffney *et al.*, 1985; Bzik and

Preston, 1986; O'Hare and Hayward, 1987). Although $V_{MW}65$ itself does not bind to DNA (Marsden *et al.*, 1987), it has been recently shown that $V_{MW}65$ associates with cellular proteins, including nuclear factor III octamer-binding protein, to form an IE complex which binds to the regulatory TAATGARAT sequence (McKnight *et al.*, 1987; Kristie and Roizman, 1987; Ace *et al.*, 1988; Gerster and Roeder, 1988; O'Hare and Goding, 1988; Preston *et al.*, 1988). The analysis of deletion and insertion mutants has revealed the existence of at least two functional separable domains in $V_{MW}65$. A domain, localised within the amino-terminal 411 amino acids, is sufficient for binding to the cellular transcription factor (Triezenberg *et al.*, 1988a; 1988b), and a carboxy terminal region, unusually rich in acidic amino acids, also known as the "acid tail" is required for stimulation of transcription (Sadow ski *et al.*, 1988). This acid tail is probably analogous to the activating domains of the yeast gene activator protein GAL4, GCN4 (Ptashne, 1986; Sadowski *et al.*, 1988; Triezenberg *et al.*, 1988a). Characterisation of the HSV-1 $V_{MW}65$ insertional mutant, *in*1814, defective in transinduction of IE gene transcription, revealed that transinduction of IE transcription by $V_{MW}65$ was not essential for virus growth at high moi, but was important at low moi (Ace *et al.*, 1989). Although the precise mechanism of IE transactivation in HSV is still obscure, it has been suggested that the interaction between the yeast activator (GAL4) or the mammalian activator protein (ATF) and the mammalian TATA factor (TFIID) with the adenovirus E4

promoter facilitate promoter recognition by the RNA polymerase II and other initation factors (Horikoshi *et al.*, 1988a; 1988b).

1.6.4 Negative regulation of IE gene expression

It is well known that the synthesis of IE polypeptides decreases as the virus infection proceeds. Observations obtained from the analysis of *ts* and deletion mutants of HSV-1 in Vmw175 suggest that IE synthesis is autoregulated by Vmw175 at the level of transcription (Preston, 1979a; 1979b; Dixon and Schaffer, 1980; Deluca *et al.*, 1985) since the mutants overproduced IE transcripts, and failed to synthesize E and L transcription. This conclusion was supported by results of transient-expression assays with cloned *wt* Vmw175 gene. These experiments showed that E and L viral genes transcription was stimulated by Vmw175 whereas the expression of a gene under the control of Vmw175 promoter was repressed (Everett, 1984; Gelman and Silverstein, 1985; Deluca and Schaffer, 1985; O'Hare and Hayward, 1985a; 1985b; 1987; Mavromara-Nazos *et al.*, 1986a). There is good evidence that binding of Vmw175 to the consensus sequence 5' ATCGTCNNNNYCGRC 3' at the capsite of Vmw175 is important for autoregulation of Vmw175 (Beard *et al.*, 1986; Gelman and Silverstein, 1987a; Deluca and Schaffer, 1988; Paterson and Everett, 1988; Smith *et al.*, 1989). This consensus sequence is also present in Vmw110 promoter, but further downstream of the mRNA start site (Faber and Wilcox, 1986; Kristie and Roizman, 1986a; 1986b; Muller, 1987; Michael *et al.*, 1988)

and it is not known whether this sequence is required for repression of $V_{MW}110$ gene expression. Other IE genes do not contain this binding site and thus it is unclear how those IE genes are repressed.

1.6.5 Early gene expression and regulation

Early genes are transcribed after IE genes, and are not dependent on viral synthesis for maximal expression (Honess and Roizman,1974; 1975). Early proteins include enzymes required for viral DNA synthesis and some structural proteins.

Early genes have been divided into two classes β_1, and β_2, on the basis of their synthesis in the presence of different amino acid analogues and the analysis of polypeptides induced by *ts*K at the NPT (Pereira *et al.*, 1977; Preston, 1979). IE proteins are required for the transcription of early mRNA (Wagner *et al.*, 1972; Swanstrom *et al.*, 1975; Clements *et al.*, 1977).

1.6.6 Early gene transactivation

A varity of approaches, including mutant analysis and transient expression assays, have been used to identify the transactivating IE genes. It has been shown that $V_{MW}175$ is essential for the regulation and transcription of E and L genes. The first experiments implicating $V_{MW}175$ in transactivation were based on the characterisation of the HSV-1 mutant *ts*K with a lesion in $V_{MW}175$ (Stow *et al.*, 1978; Davison *et al.*, 1984). This mutant over produced IE transcripts, and failed to synthesize E gene transcripts at

the NPT. The *ts* phenotype could be reversed by downshift of *ts*K infected-cells from the NPT to the PT. When cells were transferred back to the NPT, IE transcripts accumulated and the amount of E mRNA declined. These experiments suggested that functional $V_{MW}175$ was required throughout infection for the transition from IE to E transcription (Preston, 1979a; 1979b; Watson and Clements, 1980). Similar results were obtained with deletion and other *ts* mutants in $V_{MW}175$ (Dixon and Schaffer, 1980; Deluca *et al.*, 1985). Experiments using cells transformed with either IE or E genes confirmed that $V_{MW}175$ is important for transactivation of E genes (Kit *et al.*, 1978; Sandri-Goldin *et al.*, 1983; Davidson and Stow, 1985). Transient expression systems have been useful for identifying other IE genes, in particular $V_{MW}110$, that affect gene expression (Everett, 1983; 1984a; 1984b; O'Hare and Hayward, 1985a; 1985b; Gelman and Silverstein, 1985; 1986; Quinlan and Knipe, 1985; Mavromara-Nazos *et al.*, 1986b). Using this approach, it was shown that $V_{MW}110$ transactivates all three classes of viral genes and interacts synergistically with $V_{MW}175$ (Everett, 1984; 1985; 1986; Gelman and Silverstein, 1985; 1986; 1987b; Shapira *et al.*, 1987). Despite the finding that $V_{MW}110$ is a strong transactivator, virus mutants lacking a functional $V_{MW}110$ are viable (Stow and Stow, 1986; 1989; Sacks and Schaffer, 1987). Although these mutants have similar phenotypic properties to *wt* virus at high moi, at low moi their growth is impaired and they synthesize reduced amounts of some E and most L proteins (Stow and Stow, 1986; 1989). It is

thought that at low moi where Vmw175 is present in low amounts, Vmw110 is important for boosting viral gene expression.

Results from the analysis of *ts* or deletion mutants and transient expression assays have implicated Vmw63 in regulation of HSV gene expression (Sacks *et al.*, 1985; Everett, 1986; Gelman and Silverstein, 1987b). In transient expression assays, Vmw63 either enhanced or repressed expression of genes activated by Vmw110 and Vmw175 (Sekulovich *et al.*, 1988). It has also been reported that Vmw63 can stimulate expression of a gene containing the gB promoter (Rice and Knipe, 1988).

The two IE polypeptides Vmw68 and Vmw12 did not appear to affect gene expression either alone or in the presence of other IE proteins in transient expression assays (Everett, 1984; DeLuca and Schaffer, 1985), and both genes could be deleted without markedly affecting virus growth in most cells (Post and Roizman, 1981; Mavromara-Nazos *et al.*, 1986a).

1.6.7 Cis-acting and Promoter regulatory sequences of E genes

The control elements responsible for constitutive transcription of early genes have been identified using the viral thymidine kinase (TK) gene, since basal levels of promoter activity could be detected in cells where the TK gene has been integrated into their chromosomes (Minson *et al.*, 1978), and functional TK produced when HSV TK gene is microinjected into *Xenopus laevis* oocytes (Cordingley and

Preston, 1981). Analysis of the TK gene promoter region has been investigated by both insertion and deletion of clustered sets of mutations at random locations of the promoter (McKnight and Kingsbury 1982). The results identified three promoter regulatory elements required for efficient transcription of the TK gene: (I) A TATA box or a proximal signal (II) An upstream distal signal, dsI (III) A second upstream distal signal, dsII (McKnight, 1980, 1982). The two distal signals exhibited sequence homology to one another and were required for quantitative transcriptional control, each distal signal has a GC-rich region which includes the hexanucleotide sequence GGGCGG (McKnight *et al.*, 1984). These GC rich elements bind the Sp1 cellular transcription factor which can stimulate the rate of gene transcription by 10 to 50 fold. DsI has a weaker affinity to Sp1 than dsII, which is explained on the basis of a single nucleotide change from G to C in the GC-consensus sequence 5'GGGGCGGGGC 3' (Jones *et al.*, 1985; Jones and Tjian, 1985). The distal signal dsII also contains an inverted CCAAT-box, (Graves *et al.*, 1986). Two transcriptional factors, CTF isolated from HeLa cells (Jones *et al.*, 1985) and CBP isolated from rat liver nuclei (Graves *et al.*, 1986), have been shown to bind to this pentanucleotide. These two proteins appear to be different gene products with a related ability to bind to specific sequences (McKnight and Tjian, 1986). Although several workers have been unable to identify any specific promoter region essential for transactivation of gD and TK in trans-inducing assays, Coen *et al.* (1986) showed that sequences

which were sensitive to base substitution upstream of the TK gene were important for recognition by cellular transcription factors, therefore suggesting that E gene trans-activation by IE proteins possibly occurs by an interaction with cellular factors which recognise promoter elements.

1.6.8 Late gene expression and regulation

Genes whose expression is significantly reduced in the presence of viral DNA synthesis inhibitors such as ara-C and PAA are defined as late (L) genes (Honess and Roizman, 1974; 1975; Clements *et al.*, 1977). Late transcripts are divided into two classes leaky late (γ_1 or $\beta\gamma$) and true-late (γ_2 or γ). Leaky-late transcripts are easily detectable in the absence of virus DNA replication, but only reach maximum abundance after DNA synthesis commences (Clements *et al.*, 1977). True-late mRNA synthesis is also dependent on viral DNA replication for high level of expression. Unlike leaky-late mRNAs, however, true-late transcripts are present in very small amounts early in infection (Powell *et al.*, 1975; Holland *et al.*, 1980; Johnson and Everett, 1986a; Homa *et al.*, 1986). The promoter region of Us11, a true-late gene, has been investigated in detail using a transient expression system to assay the expression of L genes (Johnson and Everett, 1986a). No detectable elements upstream of the TATA box were required for fully regulated expression of Us11 promoter. This result suggested that only the TATA-box homology and capsite were required for late gene expression

(Johnson and Everett, 1986a). The finding that gD could be converted from an E gene to a L gene by removal of promoter sequence upstream the TATA-box confirmed these results (Johnson and Everett, 1986b). Similar results were obtained for another L gene, UL44 (gC) (Homa *et al.* 1986; Shapria *et al.*, 1987).

It is thought that prior to DNA synthesis, L genes are repressed in their expression by the major DNA binding protein (MDBP, $V_{MW}136$), since *ts* mutants with lesions in MDBP overproduced late gene products at the NPT (Godowski and Knipe, 1985).

Both $V_{MW}175$ and $V_{MW}110$ have been shown to transactivate L genes in transient expression assays (Deluca and Schaffer, 1985; Mavromara-Nazos *et al.*, 1986a). The recent findings obtained from the analysis of $V_{MW}63$ deletion mutants, which failed to synthesize $V_{MW}63$ transcripts (McCarthy *et al.*, 1989), suggest that $V_{MW}63$ down-regulates transcription of certain E genes, and stimulates transcription of some L genes, since $V_{MW}63$ mutants overexpressed E proteins, and did not make true late proteins.

1.7 Herpesvirus DNA Replication

1.7.1 Viral DNA synthesis

Semi-conservative HSV DNA synthesis takes place in the nuclei of infected cells (Roizman, 1969). Replication can be detected in BHK cells by 3 hpi and reaches a maximum between 9-11 hpi (Wilkie, 1973; Rixon, 1977). The amount of input HSV DNA entering the replicative pool is low,

estimates vary from 5-10% (Jacob and Roizman, 1977). By contrast, more than 60% of input PRV DNA participates in DNA synthesis (Ben-Porat *et al.*, 1976). Electron microscopic analysis of viral DNA present in the nuclei of infected cells revealed that the linear genome circularises early in infection (Jacob and Roizman, 1977; Ben-Porat and Veach, 1980). Restriction endonuclease analysis of non-inverting mutants, lacking most of the internal inverted repeat sequences including the *a* sequence (Poffenberger *et al.*, 1983; Poffenberger and Roizman, 1985), revealed that circularisation is rapid, and does not require *de novo* protein synthesis. These results suggest that fusion of the termini is mediated by a host or virion protein (Poffenberger and Roizman, 1985). Sequence analysis of the joint and terminal region indicates that circularisation of linear HSV genome probably occurs by direct ligation of the termini (Davison and Wilkie, 1983).

DNA has been extracted from cells replicating virus at early and late times in infection and examined under the electron microscope. Early in infection circular molecules with linear tails larger than unit length viral genome, Y-shaped molecules, and a variety of linear DNA molecules differing from mature viral DNA in structural features such as lariats, loops (eye and D loops) have been observed (Ben-Porat *et al.*, 1976; Shlomai *et al.*, 1976; Ben-Porat and Tokazewiski, 1977; Friedmann *et al.*, 1977; Jacob and Roizman, 1977; Jean *et al.*, 1977, Becker *et al.*, 1978). Evidence, particularly from PRV, favours the idea that the genome may replicate early in infection as a theta

molecule. Late in infection, large tangles of DNA, many of which are greater than unit length viral DNA molecules are present. Sedimentation properties of HSV replicating DNA at late times of infection are consistent with electron microscopic observations that the viral DNA is of high molecular weight. Data obtained from restriction endonuclease analysis revealed that the proportion of terminal fragments relative to the joint spanning fragments was low in replicating virus DNA (Ben-Porat and Rixon, 1979; Jacob *et al.*, 1979), suggesting that replicating HSV DNA is endless. On the basis of electron-microscopic observations of replicative intermediates, sedimentation properties of newly synthesised viral DNA late in infection, and results obtained from restriction endonuclease analysis of replicating viral DNA, Jacob *et al.* (1979) proposed that HSV replicates via a rolling circle mechanism, whereby head-to-tail concatemers of DNA are formed by the continuous synthesis of one strand and discontinuous synthesis of the other strand. During replication virus DNA contains nicks and gaps which are repaired at later stages (Wilkie, 1973; Wadsworth *et al.*, 1976). Short stretches of ribonucleotides along the viral genome have also been reported (Biswal *et al.*, 1974). The role of these ribonucleotides is not clear. It is possible that these stretches may act as a primer for discontinuous DNA synthesis.

1.7.2 Cis-acting elements involved in DNA replication

The first evidence for multiple origins of

replication was obtained from electron microscopic observations on replicating HSV-1 DNA (Shlomai *et al.*, 1976; Friedman *et al.*, 1977). Similar observations were obtained for PRV (Jean *et al.*, 1977; Ben-Porat and Veach, 1980). Studies on HSV defective viruses, which are generated by serially passaging virus stocks at high moi, identified two classes of defective viruses, both of which consist of tandem reiterations of portions of the virus genome. Class I contain tandem reiterations of U_S linked to the entire S repeat region, while class II has DNA sequences from U_L fused to a small part of the S repeat (Frenkel *et al.*, 1976; Graham *et al.*, 1978; Kaerner *et al.*, 1979; Locker and Frankel, 1979), Subsequent studies provided direct evidence that the repeat units of class I and II of HSV defective genomes contains origins of replication (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982; 1985). The experiments showed that monomeric units of both defective classes were amplified to generate tandemly reiterated DNA when cotransfected into cells with *wt* HSV DNA which provided essential helper functions *in trans*. Using a plasmid replication assay the oris sequence in the short repeat was located within a 90bp region in the intervening sequences between the 5' transcribed region of $V_{MW}175$ genes and $V_{MW}68$ or $V_{MW}12$ genes (Mocarski and Roizman, 1982; Stow, 1982; Stow and McMonagle, 1983). The ori_L sequence, which lies within the noncoding sequences at 5' ends of DNA polymerase and the MDBP genes, was difficult to determine because deletions occurred at high frequency in ori_L when plasmids carrying the sequence were propagated

in bacterial vectors. Cloning of ori_L was, however, successfully achieved using a yeast vector which allowed the precise localisation of *ori*$_L$ in HSV-1 DNA (Weller *et al.*, 1985). The sequence of *ori*$_L$ was also determined by directly sequencing *ori*$_L$ in HSV-1 strain 17 virion DNA (Quinn and McGeoch, 1985) and in defective DNA of HSV-1 strain angelotti (Gray and Kaerner, 1984). Sequence analysis revealed that both HSV-1 strain 17*syn*$^+$ *ori*$_S$ and *ori*$_L$ contain a palindromic sequence with an A-T rich central region (Murchie and McGeoch, 1982; Quinn and McGeoch, 1985).

A question which has been raised is whether *ori*$_L$ and both copies of *ori*$_S$ are essential for viral DNA synthesis ? Clearly both *ori*$_S$ are not essential for DNA synthesis, since a mutant of HSV-1 lacking one copy of *ori*$_S$ has been isolated in tissue culture (Longnecker and Roizman, 1986). Furthermore, the isolation of the HSV-1 mutant *dl*61, which is able to replicate despite the deletion in *ori*$_L$, suggests that a functional *ori*$_L$ is not essential for virus replication in tissue culture (Polvino-Bodnar *et al.*, 1987). On the basis of these results it was thought that one origin of replication was sufficient for virus replication. Recent findings of Smith *et al.* (1989), however, suggest that the presence of at least one copy of *ori*$_S$ is essential for virus replication since viable deletion mutants lacking all copies of *ori*$_S$ could not be generated in HSV-2. The reason for three origins of replication in the virus genome is still unclear.

1.7.3 Identification of proteins involved in DNA metabolism

A variety of approaches have been employed to identify viral gene products which are important for DNA replication. In early studies, virus-infected cell extracts were screened for the presence of enzymes with altered properties from uninfected cell enzymes. Using this approach the TK (Dubbs and Kit, 1964), a DNA exonuclease (Morrison and Keir, 1968), a DNA polymerase (Hay *et al.*, 1971), ribonucleotide reductase (Cohn, 1972), dUTPase (Wohrab and Francke, 1980; Preston and Fisher, 1984), and the DNA repair enzyme uracil-DNA glycosylase (Caradonna and Cheng, 1981; Caradonna *et al.*, 1987) were detected. DNA binding properties of certain proteins have been used to identify gene products which might be important for DNA replication such as the MDBP (Bayliss, *et al.*, 1975; Purifory and Powell, 1976), origin specific DNA binding protein (Elias *et al.*, 1986), and more recently the non-specific ds DNA binding protein (Marsden *et al.*, 1987; Parris *et al.*, 1988). The characterisation of mutants has been another approach used to identify genes involved in DNA replication. Analysis of *ts* and host range (*hr*) mutants which fail to synthesize DNA under restrictive conditions identified seven genes as candidates involved in DNA replication (Conley *et al.*, 1981; Coen *et al.*, 1984; Weller *et al.*, 1983; Carmichael *et al.*, 1988; Goldstein and Weller, 1988; Marchetti *et al.*, 1988; Carmichael and Weller, 1989).

A new approach has been used by Challberg (1986),

to identify genes required for HSV replication. Cloned restriction fragments of HSV-1 DNA were screened for the ability to supply the functions required for the replication of plasmids containing *ori*$_S$ or *ori*$_L$ when transfected into tissue culture cells. Using this complementation assay, Wu *et al.* (1988), identified seven HSV genes that were necessary for transient expression of plasmids containing *ori*$_S$ or *ori*$_L$. These were UL30 which encodes DNA polymerase (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985), UL29 which encodes the MDBP (Conley *et al.*, 1981; Quinn and McGeoch, 1985), a 65,000 M_{MW} non-specific ds DNA-binding protein encoded by UL42 (Marsden *et al.*, 1987; Parris *et al.*, 1988), and four less well characterised genes UL5, UL8, UL9, and UL52. Results obtained by Olivo *et al.* (1988), suggested that the product of gene UL9 binds to a viral origin of replication. Similar results were obtained by Weir *et al.* (1989), using a gel retardation assay. An HSV-1 specific DNA helicase activity has been identified in virus-infected Vero cells (Crute *et al.*, 1988), and shown to consist of three subunits. Immunochemical analysis suggested that these three subunits are the products of HSV-1 UL5, UL8, UL52. In addition, it was reported that a DNA primase activity was tightly associated with the three subunits (Crute *et al.*, 1989). It is thought that the helicase-primase complex primes lagging-strand synthesis as the complex unwinds DNA at the replication fork.

1.7.4 Viral DNA maturation and cis-acting signals

Cleavage of high mw concatemeric DNA to unit-length linear genomes is closely associated with encapsidation of viral DNA. The characterisation of *ts* mutants of PRV and HSV has revealed clear evidence that these two processes are linked together (Ladin *et al.*, 1980; 1982; Preston *et al.*, 1983; Addison, 1986; Sherman and Bachenheimer, 1987; 1988). To date, most studies have been directed towards determinating the cis-acting sequences required for cleavage and packaging. All defective virus genomes contain sequences from the terminus of the S region of HSV genome, suggesting that these sequences encode the signal(s) for cleavage and packaging of DNA (Kaerner *et al.*, 1981; Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). Studies on defective viral genomes generated by serial passage of HSV-1 virus stocks at high moi have also shown that only full-length defective genomes were present in cytoplasmic virions, suggesting that only capsids containing unit length DNA were enveloped (Vlazny *et al.*, 1982). Subsequent work by Stow *et al.* (1983), demonstrated that all the signals required for HSV-1 DNA encapsidation were located within the *a* sequence, since only plasmids contain ing HSV-1 *ori*s and the *a* sequence were packaged into capsids when transfected into cells with helper virus DNA. The *a* sequence, which is present as a direct repeat at both the termini, and in an inverted orientation at the L-S junction, is composed of unique elements (U_b, U_c), separated by direct repeated (DR) elements. It has been shown that DR1 elements lie at each end of a single *a*

sequence, and multiple copies of *a* sequence are separated by a single DR1 element (Figure 4). Heterogeneity in the size of *a* sequences of different HSV strains is due to the variation in the copy number of DR elements (Locker and Frenkel, 1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Mocarski *et al.*, 1985). Sequence analysis of HSV DNA has revealed that the *a* sequence ends at both termini in a partial copy of DR1 (Davison and Wilkie, 1981; Mocarski and Roizman, 1982), such that the ligation of TR_S and TR_L regenerated the complete DR1 between two *a* sequences. It was concluded from this information that the cleavage site is present within the DR1 element (Mocarski and Roizman, 1982). The findings obtained from the analysis of HSV-1 strain Justin defective genomes revealed the presence of a complete copy of DR1 at the end of the *a* sequence adjoining the short repeat sequence but only 4bp of DR1 at the novel U_S-*a* junction. This finding led to the conclusion that the DR1 sequence does not contain the cleavage-packaging signal (Mocarski *et al.*, 1985). Studies by Varmuza and Smiley (1985) supported this prediction. Various sub-fragments from the *a* sequence were inserted into the viral TK gene and assayed for the ability to direct cleavage/packaging when introduced into a plasmid containing an HSV-1 origin of replication. The mutant TK- fragments were also inserted into HSV DNA and the recombinants screened for the ability to produce novel termini. A 250 bp subfragment, which lacked DR1 and part of U_b at one end and contained an incomplete copy of DR1 at the other end, retained cis-acting cleavage-packaging

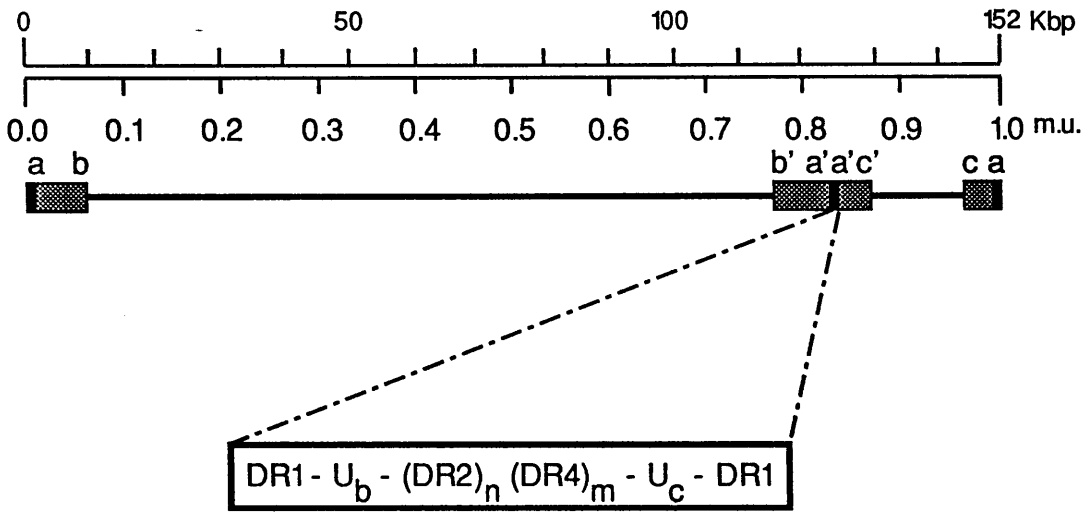

0
50
100
152 Kbp
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0 m.u.
a b
b' a'a'c'
c a
DR1 - U_b - $(DR2)_n$ $(DR4)_m$ - U_c - DR1

Figure 4

The "*a*" sequence of HSV-1 strain F, which is present as a direct repeat at both termini and in an inverted orientation at the L-S junction. The region shown is composed of unique elements (U_b and U_c), separated by direct repeated elements (DR). At the each end of a single "*a*" lies a DR1 element. (taken from Chou and Roizman, 1985).

signals. Furthermore, sequence analysis revealed that the novel termini generated by the TK- recombinant viruses were produced by two distinct cleavage events, one specifying the location of the new S terminus, the other the new L terminus. Both cleavage sites occured in flanking TK DNA sequences at a defined distance from the cleavage signals. On the basis of these results Varmuza and Smiley (1985) proposed that in the standard HSV genome the two cleavage events occurred normally within DR1, but that the cleavage signals mapped elsewhere within the *a* sequence, probably within U_b and U_c. Eventually, it was shown that two separate signals, one located within U_b and the other in U_c element of the *a* sequence, were essential for cleavage and encapsidation of defective virus (Deiss and Frenkel, 1986; Deiss *et al.*, 1986). Comparison of concatemeric junctions of six different herpesviruses revealed two blocks of conserved sequences (referred to as pac1 and pac2) which were homologous to U_b and U_c. It was suggested on the basis of these findings that there was a uniform mechanism for cleavage and packaging of herpesvirus DNA (Deiss *et al.*, 1986). Using a transient cleavage assay it was demonstrated that a 179bp cloned fragment from the junction of two tandem *a* sequences, containing the cis-acting signals for cleavage and encapsidation, was cleaved at the appropriate site even though the fragment was unable to replicate in HSV-infected cells (Nasseri and Mocarski 1988). This result suggested that cleavage is independent of replication or concatemerisation. The same fragment, when linked to an HSV origin of replication, was able to be

replicated and packaged into progeny virus as a defective genome in the presence of helper virus.

1.7.5 Viral DNA packaging models

Several models have been proposed to explain the processing of concatameric DNA into packaged unit length DNA. The simplest of all models is based on a single cleavage event within DR1 to produce L and S termini. However, this model (Fig. 5A) requires junctions bearing two or more tandem *a* sequence for processing (Mocarski and Roizman, 1982). Since 50% of L-S junctions contain only one *a* sequences, there must be a mechanism which discriminates between a single and multiple *a* sequences or, alternatively, a system for regenerating a lost *a* sequence. It is also clear from work on defective genomes that one *a* sequence is sufficient in the monomer unit for cleavage and packaging. These observations led Varmuza and Smiley (1985) to propose two models for cleavage and packaging. In the first model, known as the staggered nick-repair model, the termini are generated by staggered single-stranded nicks followed by repair synthesis rather than ds cleavages proposed by Mocarski and Roizman (1982). S and L recognition complexes bind to signals in U_b and U_c respectively and each complex makes a single-stranded nick in the adjacent DR1 sequence at the end of the *a* sequence (Fig. 5B). Repair synthesis occurs across the staggered cleavages to produce two termini, each containing an *a* sequence. Junctions bearing two or more tandemly reiterated *a* sequences can also be processed by cooperation

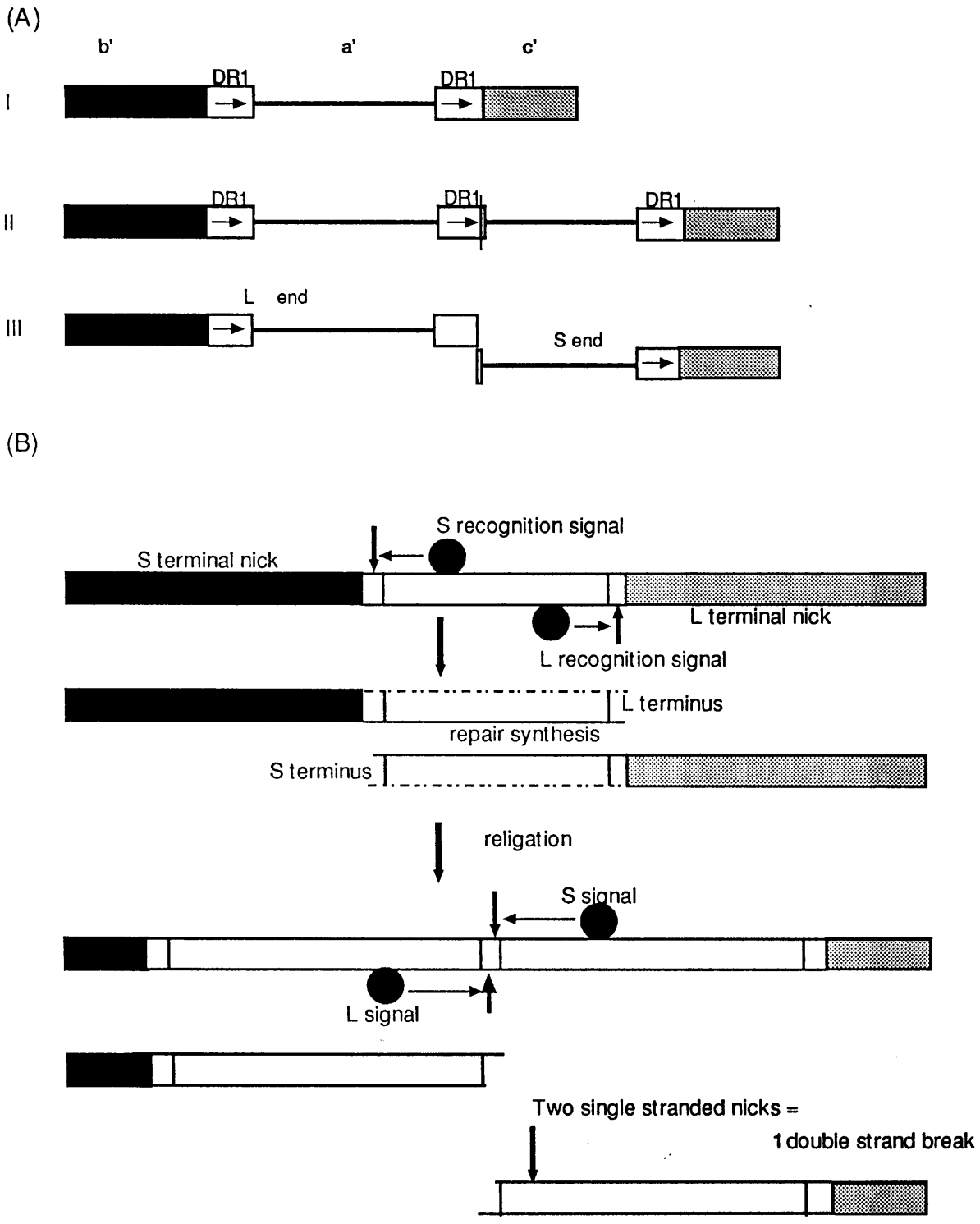

(A)
b'
a'
c'
DR1
DR1
I
DR1
DR1
DR1
II
L end
III
S end
(B)
S recognition signal
S terminal nick
L terminal nick
L recognition signal
L terminus
repair synthesis
S terminus
religation
S signal
L signal
Two single stranded nicks =
1 double strand break

Figure 5

A)

DNA cleavage model of Mocariski and Roizman (1982), A single cleavage event within the DR1 element generates the L and S termini. (I) The single *a* sequence present at the L-S junction is flanked by DR1. (II) Tandemly repeated *a* sequences share a single copy of DR1. (III) A single cleavage event within a copy of DR1 separating two adjacent *a* sequence may give rise to the termini.

B)

The staggered nick-repair model. The L and S termini arise either by staggered single-strand nicks followed by repair synthesis or, alternatively, the junctions bearing two or more reiterated *a* copies are processed by cooperation between L and S recognition complexes in adjacent *a* sequence and produce a double strand break. The circles represent the L and S recognition complexes bound to signals in U_b and U_c respectively. Taken from Varmuza and Smiley (1982).

between L and S recognition complexes in adjacent *a* sequences. It was suggested that the ds break created by this process generates ends which have a protruding 3' nucleotide (Mocarski and Roizman, 1982). Varmuza and Smiley (1985) also proposed a simple mechanism, the theft model, in which two separate ds cleavages events occur (Fig. 6), one creating an L terminus, the other an S terminus. Thus, at any L-S junction containing a single *a* sequence, only one cleavage is made creating (for example) an L terminus with an *a* sequence and an S terminus lacking it. This step is followed by packaging of the genome containing the *a* sequence at the terminus until the next L-S junction in the same orientation is encountered. A second cleavage event (in an opposite fashion) takes place to generate (for example) an S terminus with an *a* sequence and an L terminus lacking one. The theft model was subsequently modified by Deiss *et al.* (1986) to include polarity in cleavage-packaging process. In their model, referred to as the directional cleavage model, the packaging complex binds to the concatameric viral DNA at random and proceeds in a random walk until a junction containing the *a* U_c signal is found (Fig. 7). Cleavage occurs at the first DR1 element producing a terminus with a 3' protruding nucleotide. Packaging begins at the generated L terminus, which contains one or more *a* sequences, and the DNA is scanned for the next direct repeat junction. A second cleavage occur within the DR1 element proximal to the first U_b signal within this junction to give a terminus with 3' single-base overhang

Figure 6

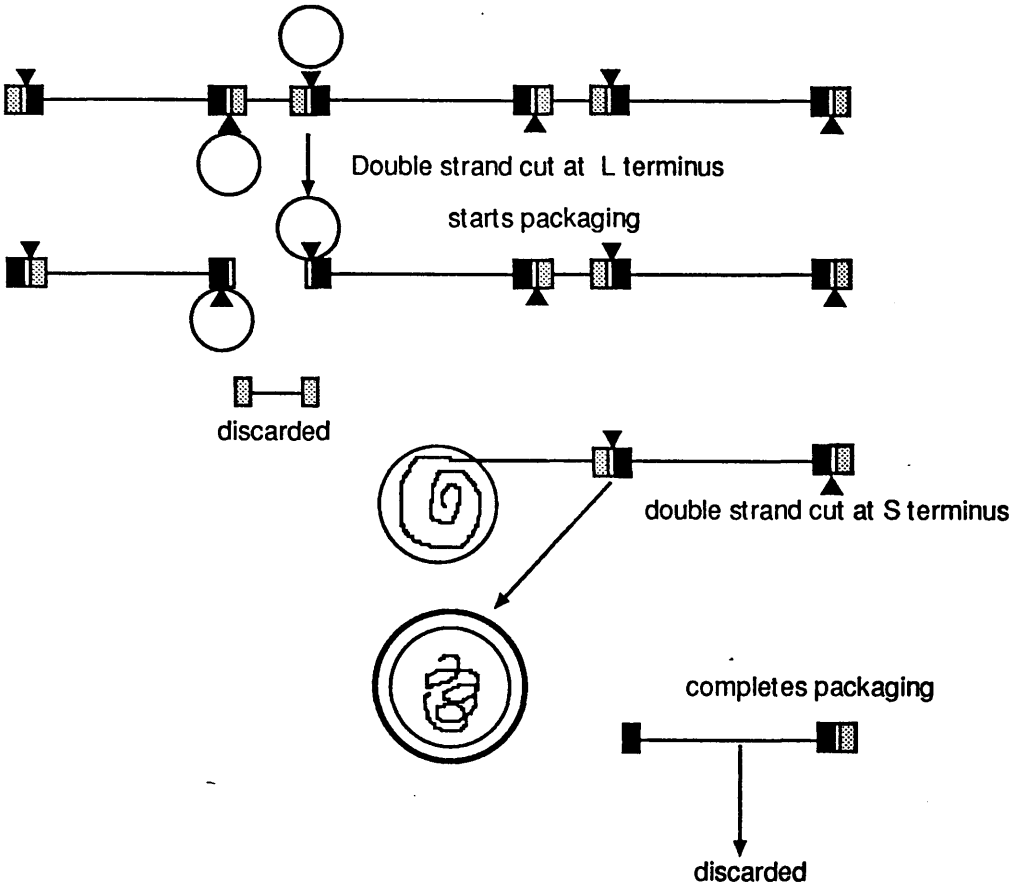

Double strand cut at L terminus
starts packaging
discarded
double strand cut at S terminus
completes packaging
discarded

Figure 6

The Theft model proposed by Varmuza and Smily (1982) for cleavage and packaging of DNA. A ds cleavage at one of two cleavage sites creates an L terminus carrying an *a* sequence and an S terminus lacking an *a* sequence. Packaging of the genome carrying the *a* sequences after the L terminus is initiated and continues until the proper orientated L-S junction is encountered. At this point a cleavage at the S terminal is made generating an S terminus with an *a* sequence and an L terminis lacking the *a* sequence. The L and S ends lacking *a* sequence are thought to be rapidly degraded. Triangles represent packaging signals while circles represent packaging complexes.

Figure 7

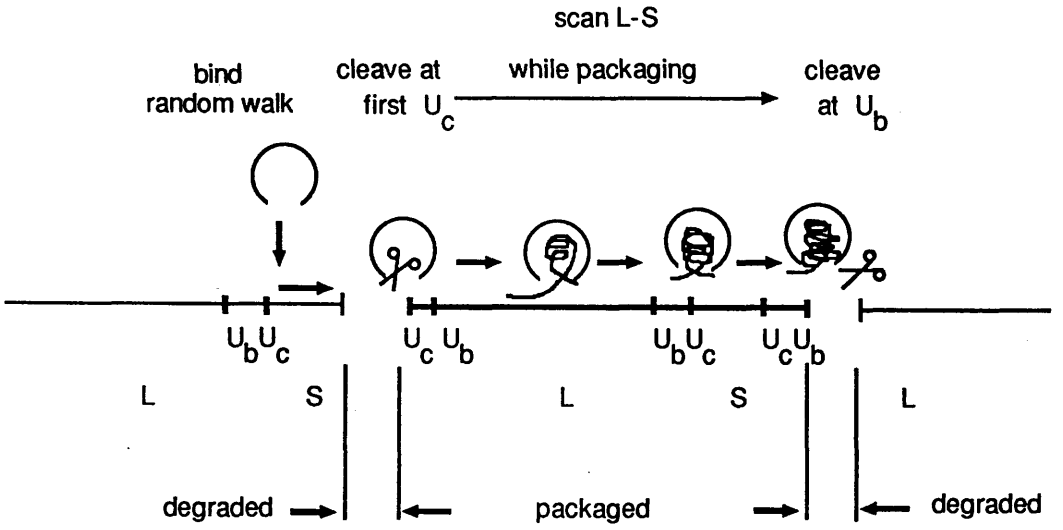

scan L-S
bind
random walk
cleave at
first U_c
while packaging
cleave
at U_b
$U_b U_c$
$U_c U_b$
$U_b U_c$
$U_c U_b$
L
S
L
S
L
degraded
packaged
degraded

Figure 7

Directional cleavage model proposed by Diess *et al.*, (1986) In this model packaging complex binds to concatemeric standard viral DNA and moves randomly until a junction containing a U_c signal is found. Cleavage occurs at the first DR1 element, producing a terminus with 3' single base overhang. The generated L terminus, which contains one or more *a* sequences, is packaged towards the L-S junction. DNA is scanned for the next direct repeat junction and a second cleavage event occurs proximal to the first U_b signal encountered to give rise to a 3' single base overhang. Termini lacking an *a* sequence are suggested to be rapidly degraded. The open circle represent the packaging complex.

(Deiss *et al.*, 1986). In this model and the preceding one unpackaged termini, lacking an *a* sequence, are generated. Since termini lacking *a* sequences have not been detected it has been suggested that they are rapidly degraded (Varmuza and Smiley, 1985; Deiss *et al.*, 1986). To account for absence of termini devoid of *a* sequences Deiss *et al.* (1986) postulate another model based on the double-strand break and gap repair mechanism for gene conversion proposed by Szostak *et al.* (1983) to explain recombination events resulting in gene conversion. In this model a complex binds to and moves randomly along the DNA. Packaging begins without cleavage at the first U_c sequence encountered and proceeds in an L-S direction until a directly repeated junction is encountered. The first and second junctions interact by the ds break-gap repair mechanism resulting in the reciprocal copying of an *a* sequence from one junction to the other. The generated double *a* junctions are cleaved within the DR1 elements flanked by the U_c and U_b and packaged. During this process no termini lacking *a* sequences are generated, and the *a* sequence is amplified. Unlike the other models this model accounts for the structure of defective genomes generated from plasmids, containing an *a* sequence with a deletion in U_b and an origin of replication, cotransfected into cells with helper virus DNA. The possibility that the defective genome progeny acquired the *a* sequence by high frequency recombination with helper virus, however, cannot be ruled out.

1.8 HSV ENCODED PROTEINS

Early studies using one dimensional SDS PAGE, identified about 50 HSV-induced polypeptides in virus-infected cells (Honess and Roizman, 1973; Powell and Courtney, 1975; Marsden *et al.*, 1976). Recently, however, analysis of the complete DNA sequence of HSV-1 17*syn*$^+$ genome has indicated the presence of at least 72 genes specifying 70 distinct proteins (McGeoch *et al.*, 1985; 1986; 1988).

1.8.1 Structural proteins

Over 30 virus polypeptides have been identified in purified virions, suggesting that nearly half of the HSV-induced proteins are structural (Spear and Roizman, 1972; Heine *et al.*, 1974; Cassai *et al.*, 1975; Marsden *et al.*, 1976). It should be noted, however, that although several minor proteins are present as virion components in some preparations, it is not clear whether they represent contaminating non-structural proteins, or whether they are in fact true components of the virion.

1.8.1.a Capsid proteins

The protein composition of capsids has been largely determined by purification of capsids from the nuclei of infected cells. In HSV-1 two types of intranuclear capsids, designated as A and B capsids, were identified using sucrose gradients (Gibson and Roizman, 1972), In comparison to A capsids, B capsids had a faster sedimentation rate, contained DNA and had an internal core

structure. A capsids lacked DNA and contained mainly coreless capsids. Recently it has been suggested that the heavy band containing B capsids was in fact a mixture of DNA containing capsids and capsids lacking DNA but containing some internal structure (partially-cored capsids) (Rixon *et al.*, 1988). These capsids could also be seen in thin section preparations of virus-infected cells under the electron microscope.

Seven polypeptides with apparent MW ranging from 12,000-155,000, have been recognised in HSV-1 full capsids (Heilman *et al.*, 1979; Zweig *et al.*, 1979; Cohen *et al.*, 1980) (See table 1).

The major capsid protein (also referred to as $V_{MW}155$, ICP5, VP5, NC1) is encoded by gene UL19 (Costa *el al.*, 1984; Davison and Scot, 1986; McGeoch *et al.*, 1988), and has been identified as the capsomere protein which is arranged in a six-fold symmetry within the icosahedron T=16 (Wildy *et al.*, 1960; Palmer *et al.*, 1975; Furlong, 1978; Vernon *et al.*, 1981; Stevens *et al.*, 1986; Weller *et al.*, 1987; Schrag *et al.*, 1989). Recent evidence obtained from cryo-electron microscopy of HSV-1 suggests that pentameric capsomeres may also be composed of $V_{MW}155$ (Schrag *et al.*, 1989).

Early electron microscopic studies of disrupted capsids indicated that the capsomeres were linked by intercapsomeric fibrils (Vernon *et al.*, 1974; Palmer *et al.*, 1975), and that NC3 or NC4 or both may represent the intercapsomeric fibrils (Vernon *et al.*, 1981). However, the results obtained by Braun *et al.* (1984a) on surface

iodination labelling of capsids revealed that $V_{MW}33$ (see table 1) and not NC3 or NC4, was located on the outer capsid surface. On the basis of this finding, it was suggested that this protein may function as intercapsomeric connections. The cryo-electron microscopy study by Schrag *et al.* (1989) confirmed that there are capsomeric connections and calculated that the mass of this protein could be accommodated within the outer surface of the capsid. On the assumption that the protein density at each local three-fold position is a trimer, then the capsid should contain 960 copies of $V_{MW}33$ which is similar to estimates of the copy number of this protein within the capsid (Heine *et al.*, 1974; Schrag *et al.*, 1989).

The capsid protein $V_{MW}50$ (table 1), is a DNA binding protein (Braun *et al.*, 1984a). The exact location of this protein within the capsid is uncertain. However, surface iodination studies suggest that this protein is present in an internal position in the capsid (Braun *et al.*, 1984b). Schrag *et al.* (1989) speculated that this protein was located within the outer capsid (at a radius between 500 A° and 425 A°), which is consistent with the observations of Zweig *et al.* (1979) that there are disulfide-linkages between $V_{MW}50$ and $V_{MW}155$. Recent data obtained from the sequencing of the amino-terminus of capsid proteins (F.J. Rixon, personal communication), suggest that UL38 encodes the capsid protein $V_{MW}50$. The HSV-1 *ts* mutant, HSV-1A44*ts*2, which fails to assemble capsids at the NPT, has a *ts* defect within UL38 (Pertuiset *et al.*, 1989). This finding suggests $V_{MW}50$ is required for

TABLE 1

predicted molecular weight (if known)	Apparent MW X 10^3	Proposed nomenclatures (A)	(B)	(C)
149,075	155	NC1	p155	VP5 (ICP5)
50,260	50	NC2	p50	VP19C (ICP32)
62,466	40	NC3/4	p40	VP22/22a(ICP35)
NA	33	NC4	p32	VP23
"	26	NC5	p25	VP24
"	12	NC6	p12	ND

TABLE 1

Capsid polypeptide nomenclature proposed by different workers and the their approximate mw compared with the predicted mw determined from the nucleotide sequence of HSV (McGeoch *et al.*, 1988). NA-not available since no capsid protein have been assigned to a gene.

(A) The nomenclature used by Cohen *et al.*(1980) where NC represents nucleocapsid proteins.

(B) The nomenclature proposed by Heilman *et al.* (1979).

(C) The nomenclature used by Gibson and Roizman (1972) for virion proteins. The virus-infected cell polypeptide nomenclature is given in Brackets (Heine *et al.*, 1974). (ND) not detected.

In this thesis capsid proteins have also been referred to by their approximate mw e.g. $V_{MW}155$.

the formation of capsids.

A family of related polypeptides, designated as VP22a, VP22, the assembly protein (Gibson and Roizman, 1972; 1974), p40 (Heilman *et al.*, 1979; Zweig *et al.*, 1979; 1980), or ICP35 (Braun *et al.*, 1984b), is encoded by gene UL26 (Preston *et al.*, 1983; McGeoch *et al.*, 1988). This assembly protein was thought to be a major component of full DNA-containing capsids, but not empty capsids lacking any internal structure (Gibson and Roizman, 1972; Braun *et al.*, 1984b). Recent work by Rixon *et al.* (1988), however, based on immunoelectron microscopy has provided clear evidence that p40 is present in partially-cored capsids which do not contain DNA and is not a major component of full capsids or mature virions. Since the p40 is only transiently associated with capsids it cannot be an integral capsid protein. Further investigations are needed to determine whether the p40 is required for the formation of partially-cored capsids. VP21, which was thought to be an unprocessed form of the ICP35 family, is only present in type B and C capsids (C capsids are prepared by treating virions with detergents which remove the envelope) (Gibson and Roizman, 1972; Braun *et al.*, 1984a). Schrag *et al.* (1989) proposed that VP21 may be present within the intermediate shell T=4, although the estimated amount of VP21 present in the capsid (Heine *et al.*, 1972) is not sufficient to explain the excess in density found in the cryo-electron microscopic constructions. It is likely that the nucleocapsid contains an additional as yet unidentified component (Schrag *et al.*, 1989).

The location and role of the other two capsid proteins p25, and p12 are not known. It has been suggested that p12, a highly basic protein, may be an internal component of the capsid (Vernon *et al.*, 1981), and involved in the events leading to the packaging of HSV DNA (Cohen *et al.*, 1980). Cryo-electron microscopic studies by Schrag *et al.* (1989) suggested that the mass inside the inner capsid is large enough to accommodate some protein in addition to the genomic DNA and the polyamine spermine detected in earlier studies by Gibson and Roizman (1971), and Roizman and Furlong (1974). It is thought that the function of spermine in the nucleocapsid is to neutralise the phosphate charge on DNA, to allow the DNA to be densely packed into the capsids.

1.8.1.b HSV tegument proteins

The tegument is thought to contain at least 15 or more non-glycosylated proteins. The assignment of structural proteins to the tegument is generally based on their presence in de-enveloped virions, and their absence from capsids purified from infected cell nuclei (Gibson and Roizman, 1972; Lemaster and Roizman, 1980). This criterion, however, excludes non-glycosylated proteins close to the envelope, for example $V_{MW}65$, which are solubilised by detergents (Spear, 1980; Johnson *et al.*, 1984). In practice, therefore, detergent-soluble non-glycosylated structural polypeptides are normally placed in the tegument.

The tegument polypeptide $V_{MW}65$, which is the

product of gene UL48, is responsible for trans-activating IE gene transcription (Batterson and Roizman, 1983; Campbell *et al.*, 1984). The precise structural role of $V_{MW}65$ is not known, although work on the HSV-2 *ts* mutant *ts*2205 (Halliburton and Timbury, 1976) has shown that it is important in stabilizing the capsid (Ramsay, 1987) .

A 10,000 MW phosphoprotein encoded by gene US9 has also been identified as a tegument protein. Oligopeptide antiserum, raised against a portion of US9 amino acid sequence, precipitated a family of polypeptides with MW of around 10,000 from NP40 soluble extracts of HSV-1 virions (Frame *et al.*,1986). The 10,000 MW protein is located on the surface of capsids in the nucleus, and it is thought that this protein becomes associated with the nucleocapsids shortly after their formation.

The gene UL36 encodes a huge tegument protein known as $V_{MW}273$ (Batterson *et al.*, 1983; McGeoch *et al.*, 1988). The lesion in the HSV-1 strain HFEM *ts* mutant *ts*B7 was localised within this protein. Capsids from input virions of *ts*B7 accumulated at the nuclear pores at the NPT and failed to release the viral DNA into the nucleus (Batterson *et al.*, 1983), suggesting that this protein is important for uncoating.

A protein kinase activity has been located in the tegument by Lemaster and Roizman, (1980). It was suggested that the protein kinase is possibly a host casein kinase II, since there are similarities between the tegument protein kinase and the host enzyme (Stevely *et al.*, 1985).

1.8.1c Envelope glycoproteins

At least seven major glycoproteins have been recognised on the virion surface (table 2). These glycoproteins have been intensively studied since they mediate entry of the virus into the infected cell, the spread of virus between cells, and they form targets for antibody-mediated neutralization of virion infectivity and therefore may be potential targets for anti-HSV vaccines (Spear, 1985; Marsden 1987). The genes encoding the glycoproteins are distributed throughout the genome within both U_S, which contains a high concentration of glycoproteins, and U_L (Fig 8). The virion glycoproteins gH, gB, gC, gD, gI, gE all possess N-terminal hydrophobic signal sequences and C-proximal hydrophobic sequences. It should be noted that a minor glycoprotein, which has not been identified as a component of the envelope, is thought to be encoded by gene US5,(McGeoch, 1985; McGeoch *et al.*, 1985; McGeoch *et al.*, 1987; McGeoch *et al.*, 1988a). Finally, sequence analysis of U_L have identified eight other potential membrane-associated genes, UL1, UL3, UL10, UL20, UL34, UL43, UL45 and UL53 (McGeoch *et al.*, 1988).

1.9 VIRION ASSEMBLY

1.9.1 Capsid assembly

The chain of events leading to nucleocapsid assembly in herpesviruses is still unclear. There is, however, general agreement that structural proteins migrate from the cytoplasm to the nucleus where capsids are assembled (Morgan *et al.*, 1954; 1959; Olshevsky *et al.*,

TABLE 2

The function of HSV glycoproteins

Function	glycoproteins gB	gC	gD	gE	gG	gH	gI
Essential genes in tissue culture	yes	no	yes	no	no	yes	no
Adsorption	+?	+?	+?	+?			
Penetration	+	+	+			+	
Cell fusion (*syn*)	+	+					
Virus egression	-	-	-	-		+?	
Neutralisation	+	+	+	+	+	+	
C3b receptor		gC-1 gC-2					
Fc receptor				+			

(+) indicates a positive role
(-) indicates a negative role
(?) either unclear or unknown

For references refer to the text

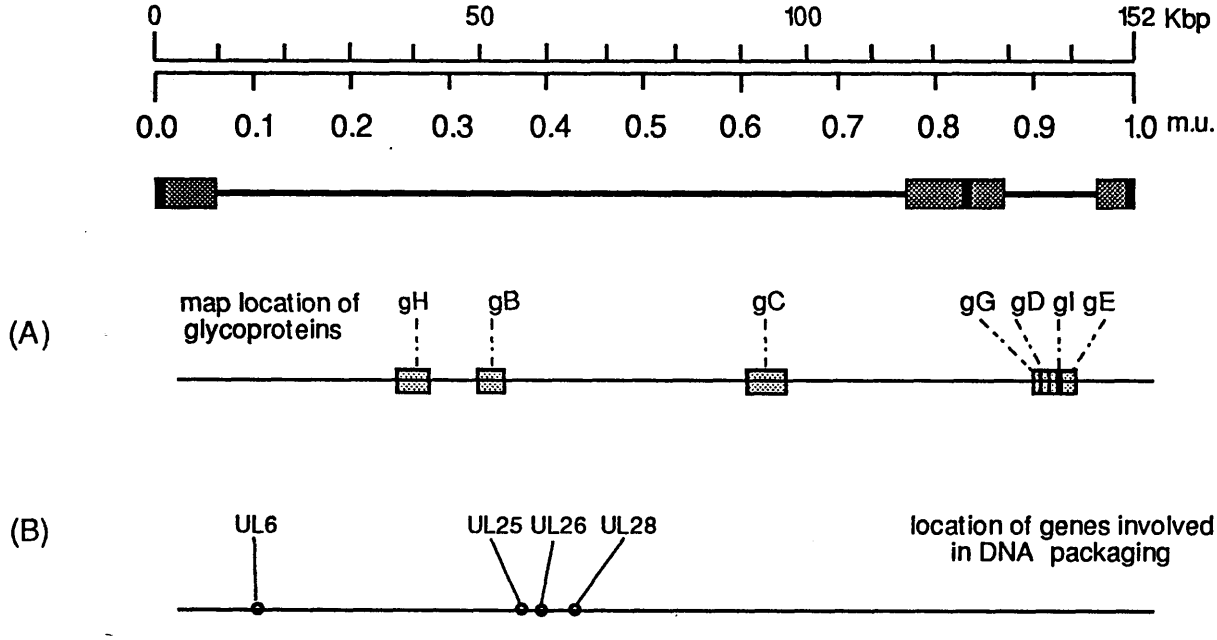

0
50
100
152 Kbp
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0 m.u.
(A)
map location of glycoproteins
gH
gB
gC
gG
gD
gI
gE
(B)
UL6
UL25
UL26
UL28
location of genes involved in DNA packaging

Figure 8

The diagram represents the map location of HSV-1 (A) glycoproteins and (B) the genes which are thought to be involved in DNA encapsidation (refer to the text for details).

1967; Spear and Roizman, 1968; Ben-Porat *et al.*, 1969). Analysis of *ts* mutants of PRV, which failed to assemble capsids but synthesised DNA at the NPT, suggested that capsid assembly is required for the continual movement and accumulation of capsid proteins in the nuclei of infected cells (Ladin *et al.*, 1980; 1982). The nuclear matrix, i.e. the residual nuclear skeletal framework obtained by sequential extraction of purified nuclei with low salt, DNase, and high salt treatment (Berezney and Coffey, 1977), is thought to be the site within the nucleus where herpesviruses capsids are assembled, and viral DNA replication takes place (McCready *et al.*, 1980; Pardoll *et al.*, 1980; Bibor-Hardy *et al.*, 1982a; 1982b; Tsutsui *et al.*, 1983; Ben-Porat *et al.*, 1984; Bibor-Hardy *et al.*, 1985). As mentioned earlier, VMW155 is the major component of T=16. Like VMW155 mutants, the UL38 *ts* mutant HSV-1 A44*ts*2 fails to produce capsids at the NPT (Pertuiset *et al.*, 1989). This finding suggests that UL38 gene product is also an essential component of capsids. Analysis of DNA +ve *ts* mutants of PRV and HSV, together with pulse-chase experiments have shown that empty, coreless capsids are breakdown products of full, DNA-containing capsids (Ladin *et al.*, 1980; 1982). This information suggests that an internal shell must be present for the formation of the outer *T*=16 capsid shell. It is interesting to note that electron translucent core-like structures have been observed in the nuclei of cells infected at the NPT with VMW155 *ts* mutants. The correct processing of UL26 gene product ICP35 is probably important for capsid assembly,

since the mutant *ts*1201, which has a defect in p40 (ICP35), produces capsids at the NPT which have a larger internal core structure than capsids produced by packaging mutants containing defects in other genes (Preston *et al.*, 1983; Addison, 1986). In a recent study on capsids of EHV-1 the only difference in the protein composition between coreless empty capsids and partially-cored capsids was the presence of p40 in partially cored capsids (Newcomb *et al.*, 1989). This work supports the results obtained by Rixon *et al.* (1988) which demonstrated the presence of p40 in partially-cored capsids. Either p40, which is present outside the capsids, is required for the maintainance of the internal core structure or a minor unidentified protein is important. To date no scaffolding proteins have been identified, however, it is likely that viral proteins in addition to VMW155 and VMW50 will be required for the formation of capsids.

1.9.2 Encapsidation of herpesvirus DNA

The available experimental evidence favours a model in which the virus DNA is packaged into a preformed capsid. A model based on this mechanism was first suggested by Perdue *et al.* (1976). It was proposed that DNA enters intermediate capsids (partially-cored capsids) which contain an internal structure and DNA is spooled around a large cylinder core structure. During this process the cylinder is condensed to form a densely staining toroid of the type observed by Furlong *et al.* (1972) in thin sections of HSV-1 infected cells under the electron microscope. The

insertional model was based on work on EHV-1 (Perdue *et al.*, 1975; 1976). Three distinct species of EHV-1 capsids were identified on renografin-76 density gradients. Analysis of the capsid species revealed significant differences in their protein composition and DNA content. Capsids were classified into light or empty, medium or partially-cored and heavy or DNA-containing capsids. Perdue *et al.* (1976) showed that partially-cored capsids were precursors to DNA-containing capsids, since, in "pulse-chase" experiments, during the "chase" the amount of radioactive label in partially-cored capsids decreased and this was accompanied by an increase in label in DNA-containing capsids. Observations of capsids by Schrag *et al.* (1989), using cryo-electron microscopy revealed that there are channels located at the six coordinated positions of the T=4 lattice, which are aligned with the channels that penetrate through T=16 shell. Schrag *et al.* (1989) suggested that the genome might be inserted through these channels. Perdue *et al.* (1976) proposed that the intermediate capsids contained four cylinders which fused to form a large cylinder around which the DNA is spooled. In view of the recent finding that HSV has an inner as well as an outer capsid, this scheme should be viewed with some doubt. Further evidence favouring the entry of DNA into partially-cored capsids has been obtained from the studies of DNA positive *ts* mutants of PRV and HSV-1 which fail to encapsidate DNA at the NPT (Schaffer *et al.*, 1973; 1974; Cabral and Schaffer, 1976; Atkinson *et al.*, 1978; Ben-Porat *et al.*, 1982; Sherman and Bachenheimer, 1987; 1988). Cells

infected with these mutants at the NPT generally contained large amounts of partially-cored capsids in the nuclei. In cases where the effect of the mutation could be reversed upon shiftdown to the PT in the presence of cycloheximide, the number of partially-cored capsids decreased and this accompanied by the appearance of full capsids in the nucleus and virus particles in the cytoplasm (Ladin *et al.*, 1980; Preston *et al.*, 1983). Various photographs have been published from electron microscopic studies on HSV and CMV which claim to show dense filaments, presumed to be viral DNA, extending from the nucleoplasm through the capsids to the cores (Friedmann *et al.*, 1975; Haguenau and Michelson-Fiske, 1975).

An alternative method for the formation of DNA containing capsids was suggested by Pignatti and Cassai (1980). They proposed that newly synthesised DNA is packed and condensed into nucleoprotein complexes (NPC) containing HSV-encoded proteins VP5, VP12, VP15.5, VP19, and VP24. The mature nucleocapsid is formed by the addition of other structural proteins to the NPC. The possibility, however, that NPC detected in virus-infected cells represents degraded nucleocapsids has not been excluded. It should be noted that this model of DNA encapsidation has little support, since the weight of evidence favours insertion of DNA into a preformed capsid.

Results obtained from work on herpesvirus DNA maturation (See section) suggest that replicated viral DNA concatemers are cleaved at specific sites while DNA is packaged into capsids (Vlazny *et al.*, 1982; Varmuza and

Smiley, 1985; Deiss and Frenkel, 1986; Deiss *et al.*, 1986). *Ts* mutants of PRV and HSV which fail to encapsidate viral DNA at the NPT also fail to cleave endless DNA into unit length DNA. This finding suggests that the concatemeric DNA is cleaved while the DNA is being packaged (Ladin *et al.*, 1980; Preston *et al.*, 1983; Addison, 1986). Subsequent work on defective virus genome supports this relationship between cleavage of viral DNA concatemers and encapsidation, since most of the termini of defective genomes were present in DNase-protected structures in the nuclei of virus-infected cells (Deiss and Frenkel, 1986).

1.9.3 Genes involved in DNA encapsidation

Characterisation of HSV *ts* mutants with defects in DNA packaging at the NPT has led to the identification of several genes involved in DNA encapsidation (see fig 8); these include genes UL6, UL25, UL26, UL28 (Preston *et al.*, 1983; Addison *et al.*, 1984; Addison, 1986; Sherman and Bachenheimer, 1987; 1988). Analysis of the HSV-1 *ts* mutant *ts*1201 with a defect in UL26 revealed that the mutant failed to package virus DNA and to process p40 to its lower MW forms at the restrictive temperature. The effect of the mutation could be reversed upon down-shift of the mutant-infected cells from the NPT to the PT. When virus-infected cells were transferred to 31° in the presence of cycloheximide correct processing of p40 was achieved and concatemeric DNA was cleaved and packaged. The partially-cored capsids, which accumulated in the nucleus at the NPT, decreased after mutant-infected cells were shifted to 31°

and this was accompanied by the appearance in the nucleus of capsids containing DNA. The results suggested that the processing of p40 is important for DNA packaging (Preston *et al.*, 1983). The *ts* mutant of HSV-1 with a defect in gene UL25 made low numbers of partially-cored capsids in the nucleus at the NPT, and no full capsids were detected. The precise role of gene UL25 in capsid assembly and DNA packaging is u nclear. It may be important for capsid stability since only low numbers of capsids were present in the nuclei at t he NPT (Addison *et al.*, 1984; Addison, 1986). The analysis of HSV-1 *ts* mutant *ts*1203 with a lesion in gene UL28 has revealed that partially-cored capsids accumulated in the nucleus at the NPT, and concatemeric DNA was not cleaved and packaged (Matz *et al.*, 1983; Addison, 1986; Rixon *et al.*, 1988).

Other polypeptides have also been implicated in DNA encapsidation. The product of gene UL38, VP19C, has been speculated to play a role in DNA encapsidation, since this protein is present as an internal component of full and empty capsids, and binds to DNA. It was suggested that this protein may function in packaging or anchoring the DNA in the capsid (Braun *et al.*, 1984b). However, no evidence has been presented to support this suggestion. The identification of the gene products of U_S11 (McGeoch *et al.*, 1985; Johnson *et al.*, 1986), V_{MW}21 and 22, which interacted specifically with the *a* sequence of HSV-1 DNA, led Dalziel and Marsden (1984) to propose that this polypeptide is involved in packaging and/or genome inversion. This gene, however, is unlikely to be important

in virus DNA packaging because it is not essential for growth of virus in tissue culture and because the products of Us11 localise very strongly within the nucleoli of the infected cell (Longnecker and Roizman, 1986; Brown and Harland, 1987; Maclean *et al.*, 1987).

1.10 Envelopment and Egression of HSV

The nucleocapsids are thought to acquire their envelope by budding through the inner nuclear membrane (Darlington and Moss, 1968; Nii *et al.*, 1968; Watson, 1973; Roizman and Furlong, 1974; O'Callaghan and Randall, 1976). The sites of envelopment in the nuclear membrane appear to lack normal complement of cellular proteins, but are enriched with viral glycoproteins (Asher *et al.*, 1969; Spear and Roizman, 1970). Other sites of envelopment have also been reported, such as golgi membranes and cytoplasmic vacuoles (Haguenau and Michelson, 1975). On the basis of electron microscopic studies on HSV a novel process of envelopment, whereby nucleocapsids acquire envelopes by *de novo* synthesis around DNA containing capsids, was suggested (Atkinson *et al.*, 1978; Dargan and Subak-Sharpe, 1983).

The precise mechanism of envelopment and the viral polypeptides involved in envelopment are not known. It was proposed by Gibson (1981) that exposed hydrophobic regions of tegument proteins present on the nucleocapsid surface promoted capsids envelopment by the interacting with hydrophobic elements, possibly viral glycoproteins located on the nuclear membrane. However, no tegument proteins or glycoproteins have yet been shown to be involved in such an

interaction. It has been suggested that ICP35 may play a role in envelopment, since it was thought that ICP35 coated the surface of full capsids and was absent from empty capsids (Gibson and Roizman, 1972; Braun *et al.*, 1984b). It was proposed that the presence of ICP35 on the capsid surface permitted full capsids to interact with other proteins and to eventually become enveloped. Work, however, by Rixon *et al.* (1988) has shown that ICP35 is in fact present in partially-cored and not full capsids. It is therefore unlikely that ICP35 is directly involved in envelopment. Subsequent cryo-electron microscopic studies of Schrag *et al.* (1989) revealed that both empty and full capsids but no partially-cored capsids were present in their purified virion preparations suggesting that the process of envelopment does not distinguish between full or empty capsids. Schrag *et al.* (1989) proposed that the absence rather than the presence of ICP35 could act as the capsid surface marker for envelopment.

Virions spread to other cells either by fusion of virus-infected cell membranes with adjacent cellular membranes, or by release of virus into the extracellular space. The transport of the virion from the nucleus to the cell surface is thought to be relatively fast and takes about 1-2h after virus maturation. Virus particles are thought to move to the cell surface with streaming of the cytoplasm, since in cells treated with cytochalasin B, an inhibitor of cell movement by microfilaments, HSV particles were retained within the nucleus (Marciano-Cabral *et al.*, 1977). Two mechanisms have been proposed for the transport

of virus from the nucleus to the plasma membrane. The first is reverse phagocytosis (Morgan *et al.*, 1959; Nii *et al.*, 1968; Katsumoto *et al.*, 1981) whereby the virus particle enters a cytoplasmic vacuole, and is transported within the vacuole to the plasma membrane. Fusion of the vacuole with the cytoplasmic membrane releases the enveloped virus into the extracellular space. In the second mechanism the virus particle buds through the inner nuclear membrane into vesicles and moves along the reticulo-endothelial system to the golgi complex, and from there to the cytoplasmic membrane. The results obtained by Johnson and Spear (1982), favour the latter mechanism. They found that enveloped virions accumulated in membrane vesicles when HSV-infected cells were treated with the ionophore monensin, which is thought to inhibit the transfer of vesicles from the golgi apparatus to the cellular membrane.

Very little is known about proteins involved in envelopment. Results obtained from the analysis of mutants deficient in particular glycoproteins, such as gD (Ligas and Johnson, 1988) or gE (Longnecker and Roizman, 1986; 1987; Neidhardt *et al.*, 1987) have revealed that none of these well characterised viral glycoproteins are essential for virus envelopment. It is possible that gH may have a role in envelopment as it has been shown that a monoclonal antibody directed against gH inhibited plaque formation when added in an overlay after virus adsorption (Buckmaster *et al.*, 1984), and efficiently inhibited cell fusion by syncytial virus strains (Gompels and Minson, 1986). A

mutant, *ts*Q26, which has a point mutation in gH, has been isolated. This mutant fails to form plaques at the NPT (Weller *et al.*, 1983 Desai *et al.*, 1988). Intracellular virus retaining gH is infectious while extracellular virus lacking gH is non-infectious (Desai *et al.*, 1988). However, the possibility that the block in infectivity is at a stage of entry not exit cannot be ruled out and that gH may act in a similar manner to gD.

1.11 LATENCY

A common feature of herpesviruses is their ability to establish latent infections (Goodpasture, 1929; Stevens and Cook, 1971). HSV reactivation often leads to recurrent epithelial lesions which can be triggered by UV-irradiation, fever, hormonal changes, stress or other stimuli. Studies have shown that the latent virus genome resides in neuronal cells (Cook *et al.*, 1974; McLennan and Darby, 1980). Latent HSV has also been found in sensory and sympathetic ganglia of man (Bastian *et al.*, 1972). HSV-1, HSV-2 wt viruses and *ts* mutants have been successfully isolated from different regions of latently infected mice by cocultivating the explanted tissue, such as ear skin, foot pad, the dorsal root ganglia as well as the corneas of infected rabbits (Al-Saadi *et al.*, 1983; Cook and Brown, 1987; Cook *et al.*, 1987). Recently it was reported that HSV-2 could also establish latency in infected mouse foot pad, and that treatment with acycloguanosine *in vivo* or *in vitro* did not prevent virus recovery (Al-Saadi *et al.*, 1988; Clements and Subak-Sharpe,

1988).

Although most of HSV-1 virion DNA is linear, it is thought that latent virus DNA exists either as a circle or as a large, unintegrated concatemer since genomic termini could not be detected (Rock and Fraser, 1983; 1985; Efstathiou *et al.*, 1986; Mellerick and Fraser, 1987). The latent HSV-1 virus DNA has a nucleosomal structure similar to that of cellular chromatin, and it has been suggested that this structure is important in the control of HSV-1 gene expression during latency (Deshmane and Fraser, 1989).

Little is known about the mechanism involved in the establishment, maintenance and reactivation of HSV-latency. It has been demonstrated recently that viral DNA replication was not required for the establishment of latency in experimental animals (Coen *et al.*, 1989a), and that viral DNA inhibitors did not prevent the establishment of latency *in vitro* (Wilcox and Johnson, 1988). The role of TK in latency is controversial. The problem with early experiments using TK^- viruses was that mutations in TK affected not only TK but also adjacent genes. Recently a mutant of HSV-1 strainF containing a deletion in TK has been shown to establish latency in rabbits but not in mice, favouring the possibility that the rabbits were able to complement a function important for latency, which was deleted from the virus (Meignier *et al.*, 1988). This is supported by work on TK deletion mutants by other workers (Sears *et al.*, 1985). More recent data, however, have provided strong evidence that TK is not essential for the establishment of latent infection but may be required in

the reactivation process (Coen *et al.*, 1989a; 1989b; Efstathiou *et al.*, 1989; Tenser *et al.*, 1989). In addition, $V_{MW}110$ of HSV-1 has been proposed to play a role in the establishment and reactivation of latency (Leib *et al.*, 1989), along with the host cellular factors such as nerve growth factor (Wilcox and Johnson, 1988).

During acute viral infection most of the genome is actively transcribed (Clements *et al.*, 1977; Wagner, 1985). In latent infections of the peripherial and central nervous systems of humans and experimental animals, however, transcription is limited to a small region of the genome (Dealty *et al.*, 1987; Rock *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987; Steiner *et al.*, 1989). HSV transcripts present in latently infected cells have been termed latency-associated transcripts (LATs). The two major transcripts, which are not polyadenylated, appeared to share their 5' and 3' ends and to be produced by alternative splicing (Wechsler *et al.*, 1988a; 1989). These mRNAs are complementary to $V_{MW}110$ mRNA and partially overlap the 3' end of $V_{MW}110$ gene (Rock *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987). LATs have been shown to be localised in the nucleus of the neuron (Rock *et al.*, 1987; Stevens *et al.*, 1987; Javier *et al.*, 1988; Wechsler *et al.*, 1988b). It is not clear whether these transcripts are translated. Results, however, obtained from S1 nuclease and primer extension mapping suggest that an open reading frame may be a protein-encoding region (Wagner *et al.*, 1988), and that a small amount of the abundant transcript may be translated.

Although the functions of these transcripts have not yet been elucidated, evidence from the analysis of deletion mutants, which do not make detectable amounts of LAT transcripts, clearly demonstrates that LAT transcripts are not required for establishment and maintenance of latency in mouse neurons (Javier *et al.*, 1988). Work on the HSV-1 variant 1704, however, showed that reactivation was significantly delayed in comparison to wt virus, suggesting that these transcripts had a role in reactivation (Steiner *et al.*, 1989). It is also thought that the LAT transcripts may keep $V_{MW}110$ region of the DNA in an open conformation such that during reactivation the $V_{MW}110$ gene can be rapidly transcribed.

1.13 CELL TRANSFORMATION AND ONCOGENESIS BY HSV

The involvment of HSV-2 in the development of cervical carcinoma has long been speculated upon. The higher prevalence of HSV-2 antibodies in woman with cervical neoplasia than in matched controls (Nahmias *et al.*, 1980), and the detection of HSV sequences in certain carcinoma biopsies led to the suggestion that HSV might be a causal agent (McDougall *et al.*, 1980; Eglin *et al.*, 1981; Macnab *et al.*, 1985). However, in a prospective study Vonka *et al.* (1986) failed to find an association between HSV-2 and cervical neoplasia.

Morphological transformation studies have been used to investigate the oncogenic potential of HSV (Macnab, 1987). Duff and Rapp (1973) were the first to demonstrate that UV-inactivated HSV-2 induces morphological

transformation of mouse 3T3 cells. Subsequent studies identified a region from the HSV-1 genome, referred to as MTRI (Morphological transformation region), which maps in XbaI *f* fragment between 0.29 to 0.45mu (Camacho and Spear, 1978; Reyes *et al.*, 1979). Two MTRs have been identified in HSV-2 DNA. MTRII maps in the BglII *n* fragment between 0.58-0.62mu (Reyes *et al.*, 1979; Galloway and McDougall, 1981; Cameron *et al.*,1985; Jones *et al.*, 1986), and MTRIII maps in BglII *c* between 0.54 to 0.58mu (Peden *et al.*, 1982; Jariwalla *et al.*, 1983).

A "hit and run" mechanism of cell transformation has been proposed for HSV (Skinner, 1976; Minson *et al.*, 1976), since the viral DNA was not retained within the transformed cells (Minson *et al.*, 1976; Galloway and McDougall, 1983; Galloway *et al.*, 1984; Cameron *et al.*, 1985), and there did not appear to be any HSV-encoded transforming proteins or oncogenes (Cameron *et al.*, 1985; Macnab *et al.*, 1985). Studies have suggested that HSV DNA can stimulate expression of cellular genes and induce specific mutations (Schlehofer and Zur Hausen, 1982), and cause gene amplification in a similar manner to chemical carcinogens (Lavi, 1981) while the viral DNA polymerase may be involved in cellular gene amplification (Matz *et al.*, 1984), and induce chromosomal rearrangments.

1.14 GENETICS STUDIES OF HSV

Perhaps one of the most important approaches which has enabled individual genes to be identified and their functions elucidated is the isolation and analysis of HSV

mutants. The information obtained on the genomic location of the mutations and virus-encoded polypeptides has helped to expand our knowledge of the functional organisation and regulation of HSV genes.

1.14.1 Temperature sensitive mutants

In HSV the majority of conditional lethal mutants isolated are *ts*. Generally, the temperature sensitivity is produced by a missense substitution of an amino acid in the protein which results in a polypeptide with a temperature-impaired function. As a consequence, *ts* mutants of HSV are able to replicate at the PT (31°-34°) but not at the NPT (38°-39.5°). At the high temperature the affected protein is either unstable or is unable to form a functional conformation. *Ts* mutants can arise spontaneously in *wt* virus stocks, however, most *ts* mutants have been induced by treatment of virions with UV light, nitrous acid, or nitrosoguanidine, by treatment of viral DNA with nitrous acid, hydroxylamine or UV light, or by exposing virus-infected cells to 5-Bromodeoxyuridine (BUdR) or 2-aminopurine (Schaffer *et al.*,1970; Timbury, 1971; Brown *et al.*, 1973; Schaffer *et al.*, 1973; Esparza *et al.*, 1974; Manservigi, 1974, Chu *et al.*, 1979; Machtiger *et al.*, 1980; Jofre *et al.*, 1981). The total number of essential genes encoded by the HSV genome is still unclear. More than 30 cistrons in HSV-1 (Schaffer *et al.*, 1978) and 20 cistrons in HSV-2 (Timbury *et al.*, 1976; Schaffer *et al.*, 1978), have been identified by complementation studies, suggesting that there are at least 30 essential genes. Although *ts*

mutants have been extremely useful in elucidating the functions of essential genes, there are large regions of the genome in which it has not been possible to induce *ts* lesions affecting virus viability. For this reason alternative approaches have been employed to extend the range of genetic material.

1.14.2 Insertion and deletion mutants

Deletion and insertion mutagensis have been used to determine functional domains of both essential and non-essential proteins and to identify non-essential genes. A variety of insertional mutagensis strategies have been adopted, including insertion of TK (Post and Roizman, 1981), mini-Mu-phage-system (Jenkins *et al.*, 1985), transposon Tn5 carrying a kanamycin resistant marker (Weber *et al.*, 1987). Analysis of insertion and deletion mutants showed that, with the exception of gD, all Us genes are dispensable for growth in tissue culture (Longnecker and Roizman, 1986, 1987; Brown and Harland, 1987; Weber *et al.*, 1987).

1.14.3 Drug resistant HSV mutants

Many drugs against HSV are nucleoside analogues which interfere with viral DNA replication, and select mutations which depend in most cases on the HSV-encoded TK for their antiviral effect. The virus-encoded enzyme will phsosphorylate a variety of nucleoside analogues, converting them from harmless compounds to an active drug which inhibits viral DNA polymerase activity by binding

irreversibly to the active site, or by causing chain termination when incorporated into DNA. Most mutants resistant to analogues are deficient in TK activity (Kit and Dubbs, 1963; Dubbs and Kit, 1964; Ellion *et al.*, 1977; Stow *et al.*, 1978; Field *et al.*, 1980), and compensate for their deficiency in exponentially growing cells by using cellular dTMP. Although most acycloguanosines-resistant mutants have altered TK, TK^+ acyclovir resistant mutants have been isolated with lesions in DNA polymerase. The altered polymerase fails to interact with the phosphorylated acyclovir, thus preventing the incorporation of the lethal nucleotide analogue into virus DNA (Crumpacker *et al.*, 1980; Larder and Darby, 1985; Larder *et al.*, 1987). Some drugs act directly on DNA polymerase, for example phosphonoacetic acid (PAA). This drug interacts with the pyrophosphate binding site on the viral DNA polymerase (Hay and Subak-Sharpe, 1976; Leinbach *et al.*, 1976; Purifoy and Powell, 1977). PAA-resistant mutants have been isolated with an altered DNA polymerase.

1.14.4 Immune cytolysis-resistant mutants *(icr)*

Mutants which are resistant to immune cytolysis have either altered conformation of glycoproteins, or aberrant glycosylation or fail to insert viral glycoproteins into the plasma membrane. Hence, mutant-infected cells becomes resistant to antibody and complement mediated immune cytolysis (Machtiger *et al.*, 1980; Glorioso *et al.*, 1980; Pancake *et al.*, 1983). An *icr* locus has been identified within UL32 (Coen *et al.*, 1984).

1.14.5 Host range mutants (hr)

Transformed cell lines which provide essential gene products in trans to complement defects in viral genes have been developed. HSV mutants, with inactive essential genes, are incapable of growth on normal cells, but can be propagated on cell lines which express the required *wt* gene. The isolation of hr mutants has been particularly useful in cases where it has not been possible to isolate *ts* mutations and for detailed functional analysis of essential genes such as $V_{mw}175$ (Davison and Stow, 1985; Deluca *et al.*, 1985), gB (Cai *et al.*, 1987), MDBP (Oberg and Schaffer, 1987), and the UL8 gene product (Carmichael and Weller, 1989).

1.14.6 Syncytial Mutants

Mutants with defects in certain genes of HSV cause extensive fusion of virus-infected cells instead of individual cell rounding. Virus mutants which give rise to syncytial plaque morphology are known as *syn* mutants. At least five loci are thought to influence syncytial morphology (See table 3), of which only UL1 and UL53 appear to specify membrane-associated proteins (McGeoch *et al.*, 1988).

1.14.7 Genetic interactions

1.14.7.a Complementation

Generally complementation refers to enhancement of yield or plaque formation at the NPT in cells mixedly

TABLE 3

Genes associated with syn loci

(*) Gene	Map coordinates of isolated mutants(m.u.)	Locus no.	Ref. No.
UL1	0.04-0.064	Syn 4	1
UL24	around 0.3	Syn 5	2
UL27	0.345-0.355	Syn 3	3
UL53	0.724-0.74	Syn 1/2	4

References
1- Little and Schaffer, 1981.
2- Sanders *et al.*, 1982.
3- Ruyechan *et al.*, 1979; Deluca *et al.*, 1982.
4- Little and Schaffer, 1981; Bond and Person, 1984; Pogue-Geile *et al.*, 1984
*- McGeoch *et al.*, 1988

infected with two different mutants. Complementation occurs as a result of the interaction between the gene products of two *ts* mutants, one of which is defective in one gene product while the other is defective in another. Normally, each mutant can compensate for the defective gene product in the other virus by supplying the active gene product. However, it is possible sometimes that only one mutant can supply a functional gene product for the other mutant and therefore, only the yield of one of the mutants is enhanced in the mixed infection. Two quantitative assays, the progeny yield test and the infectious centre test have been widely used. However, the progeny test is considered to be more reliable since fewer recombinant viruses are generated. A complementation index greater than at least 2.0 is regarded as positive complementation. (Timbury, 1971; Brown *et al.*, 1973).

1.14.7.b Recombination

Results of recombination frequencies obtained from crosses of *ts* mutants led to the construction of linear linkage maps (Brown *et al.*, 1973; Schaffer *et al.*, 1974; Timbury and Calder, 1976). Subsequent work using *ts* mutations and a variety of selected and unselected markers led Honess *et al.* (1980) to conclude that the genetic linkage map was circular and that recombination normally occured between concatemeric or circular DNA. A circular genetic map was also obtained with PRV *ts* mutants. In addition evidence was obtained suggesting that recombination occured mainly between parental DNA rather

than progeny genomes prior to DNA replication (Ben-Porat *et al.*,1982). The finding that recombinants were in an arrangement that minimized the number of crossover events supports the idea that parental DNA participates in the generation of recombinants (Umene, 1985). In contrast to these observations it was shown that recombination frequency increased with time, indicating that both parental and progeny DNA took part in recombination (Ritchie *et al.*, 1977; A. MaClean, 1988), and that multiple rounds of recombination occured.

Recombination can occur between HSV-1 and HSV-2 (Timbury and Subak-Sharpe, 1973; Esparza *et al.*, 1976). Restriction endonuclease analysis has been used to map the crossover points in recombinants (Morse *et al.*, 1977; Preston *et al.*, 1978; Wilkie *et al.*, 1979). Intertypic recombinants have been useful for mapping mutations and polypeptides on to the virus genome (Halliburton *et al.*, 1977; Marsden *et al.*, 1978, Morse *et al.*, 1978; Preston *et al.*, 1978), although more precise localisation of markers has been achieved by intratypic marker rescue technique using cloned restriction endonuclease viral fragments (Stow *et al.*, 1978; Stow and Wilkie, 1978; Chartrand *et al.*, 1979; 1981; Preston, 1981).

Studies on intramolecular recombination have suggested that site-specific recombination between inverted copies of the *a* sequence in a single genome is responsible for inversion of unique sequences (Mocarski and Roizman, 1981; Smiley *et al.*, 1981; Chou and Roizman, 1985). The *b* sequence in the long repeat is thought to be less actively

involved than the *a* sequence in inversion of U_L (Longnecker and Roizman, 1986). However, results obtained recently by Weber *et al.* (1988), has provided evidence that the *a* sequence in HSV-1 is not a target sequence for site specific recombination. Instead sequence inversion in HSV-1 appears to be mediated by the same complex of proteins that replicate the viral DNA, and not by an independent recombinase function acting at a specific site. It is clear, however, that viral DNA replication is not absolutely required for recombination, since recombinants can be formed between two DNA negative mutants with lesions in $V_{MW}175$ (V. Preston, personal communication).

MATERIALS

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 *ts* mutants were characterised. The first mutant, *ts*1201 (Preston *et al.*, 1983), was derived from the multiple mutant 17*ts*JC116 (Coates, 1982). EcoRI *f* from a *syn*$^+$ derivative of 17*ts*JC116 was cloned into the plasmid pACYC184 and recombined into 17*syn*$^+$. *Ts*1201 was isolated from this transfection experiment. The revertants for growth at the NPT, *ts*1201 rev2, and *ts*1201 rev3, were independent isolates from low passage mutant stocks (Preston *et al.*, 1983). The second HSV-1 mutant, *ts*1233, was isolated from a UV mutagenised wt virus stock by Miss I. McDougall. Two independent revertants for growth at the NPT, *ts*1233 rev1 and *ts*1233 rev2, were isolated from low passage stocks of the mutant during this study. Additional viruses used were HSV-17*ts*K*syn*$^+$ (Crombie, 1975; Preston, 1981), HSV-1 KOS*ts*N20 (Schaffer *et al.*, 1974), HSV-1/HSV-2 recombinant BX1(31-1) (Marsden *et al.*, 1976), and the *wt* HSV-2 strain HG52 (Timbury, 1971). Seed stocks of viruses were kindly provided by Mrs M.Murphy with the exception of *ts*K and *ts*1233 which were supplied by Dr. V.G. Preston.

TISSUE CULTURE CELLS

BHK21 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.

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, 100 ug/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 were also used in this study:-

Eagle's medium containing n% calf serum.———— EC_n.

" " " n% foetal calf serum.—— EFC_n.

" " " n% human serum.———— EHu_n.

Agar overlay. This consisted of Eagle's medium lacking phenol red, 10% calf serum, and 0.6% Noble's agar.

BACTERIA

Escherichia coli strain DH1 (F^-,recA1, gyrA96, end A1, supE44)(Hanahan, 1983), was used for propagating plasmids.

Escherichia coli strain JM101 was the host bacterium for M13mp18, M13mp19, and recombinant bacteriophage used in DNA sequencing analysis (Messing *et al.*, 1981).

Escherichia coli strain RB791 (=W3110 $lacI^q$, L_8, Brent and Patashue, 1981) was used for the expression of

UL33 gene.

BACTERIAL CULTURE MEDIA

Bacteria were either grown in L-broth or 2YT medium.

PLASMIDS

The following plasmids were supplied by Dr. V. G. Preston:

PLASMID	INSERT	VECTOR
pGX22	BamHI *k* of *ts*$^+$ 17syn$^+$	pAT153.
pGX24	BamHI *a* " " "	" .
pGX56	BamHI *u* " " "	" .
pGX74	EcoRI *o* " " "	pACYC184
pGX142	KpnI *t* " " "	pAT153.
pGX198	BamHI *u* of *ts*1201	" .
pGX208	BamHI *u* of *ts*1201 rev2	" .

Three other plasmids used in this study were kindly provided by Dr. C. Preston.

pKK233-2 : A vector used for high level expression of an intact cloned gene (Amann and Brosius, 1985). This vector contains a unique NcoI site at the 3' end of the trc promoter for cloning either blunt end or NcoI fragments,

pCI15 : An expression vector containing a deletion of 15 amino acids from the N-terminal portion of the bacteriophage cI repressor (Amann and Brosius, 1985).

p23 : was derived from the plasmid pGX166 and contains IE Vmw175 promoter sequences inserted into HSV-1 TK gene (Details of this plasmid are in chapter 3).

CHEMICALS

Chemicals were obtained from the following suppliers:

BDH chemicals, Poole, England; Bio-Rad Laboratories, California, USA; Koch-Light Laboratories, Suffolk, England; Pharmacia Fine Chemicals, Uppsāla, Sweden; and Sigma(London) Ltd. Reagents for electron microscopy were obtained from two companies, Agar Aids, Stanstead, Essex, and Taab Laboratories, Emmer Green, Reading. Analytical grade reagents were used wherever possible.

ENZYMES

Restriction endonucleases were supplied by Gibco/Bethesda Research Laboratories (U.K.) Ltd., Paisley, Scotland, Northumbrian Biological Ltd., Cramlington, England, and Boehringer Corporation Ltd., Lewes, England. Other enzymes were obtained from the following suppliers:

ENZYME	SUPPLIER
Calf intestinal phosphatase.	Boehringer Corp. Ltd
Proteinase K	"
Lysozyme	Sigma Chemical Ltd.
DNase	"
RNase	"
DNA polymerase holoenzyme	New England Biolabs.
Klenow fragment DNA polymerase	Gibco, Bethesda Lab.

OLIGONUCLEOTIDES

Phosphorylated XhoI oligonucleotide linkers were purchased from New England Biolabs, CP laboratories.

RADIOCHEMICALS

All radiochemicals were obtained from Amersham International plc.

ANTIBODIES

Monoclonal antibody 5010B, specific for HSV-1 P40 (UL26 gene product), was prepared by Dr. A. Cross.

OLIGOPEPTIDES

Oligopeptides specific to part of UL33 gene product were purchased from Peptide and Protein Research, Windson Berks, England. Oligopeptides specific to a portion of UL26 gene product were made in the Institute of Virology by Miss Anna Owsianka, using LKB BioLynx 4170 Automated Peptide Synthesiser.

IMMUNOLOGICAL REAGENTS

Protein A-Sepharose was obtained from Sigma (London) Ltd. BSA coupled to CNB activated Sepharose 4B was prepared by Dr. G. Hope. [^{125}I]-Protein A was made and generously supplied by Mrs. M. Murphy at the Institute of Virology. Bis-diazotized benzidine (DAB) was prepared and supplied by Dr. H. Marsden. A 10% suspension of formalin fixed *Staphylococcus aureus* was provided by Dr. V. G. Preston.

ANIMALS

New Zealand white rabbits were purchased from Hylyne Rabbits Ltd., England. Polyclonal antibodies were raised in these animals.

MISCELLANEOUS

Nitrocellulose membranes were supplied by Schleicher and Schuell, Dassel, West Germany. Noble's agar was obtained from Difco Ltd. Photographic film was supplied by Kodak Ltd., London, England. Plastic petri dishes, and plastic microtiter plates (24-wells and 96-wells) were purchased from Nunclon Ltd. Plastic 850 cm^2 roller bottles for cell culture were supplied by Becton Dickinson Ltd.

STANDARD SOLUTIONS

Tissue Culture Reagents and Buffers

Giemsa stain	1.5% (w/v) Giemsa in glycerol, heated at 56° for 120 min and diluted with an equal volume of methanol.
Phosphate buffered saline (PBS)	170 mM NaCl, 3.4mM KCl. 10mM Na_2HPO_4 and 2mM KH_2PO_4 pH 7.2 (Dulbecco and Vogt, 1954).
Tris-saline	140mM NaCl, 30mM KCl, 28mM Na_2HPO_4, 1mg/ml glucose, 0.0015%(w/v) phenol red, 100 units/ml penicillin, 100 µg/ml streptomycin, 25mM Tris-HCl pH 7.4.
Trypsin	0.25% (w/v) trypsin (Difco) in tris-saline.
Versene	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red.

DNA Gel Electrophoresis Buffers

Dye Ficoll	15% (w/v) ficoll, 0.2% (w/v) bromophenol blue, 100mM EDTA.
Electro-elution buffer (10X)	50mM sodium acetate, 10mM EDTA, 400mM Tris-HCl pH 7.8.
Elution buffer	500mM ammonium acetate, 1mM EDTA and 0.1% SDS.
TBE buffer (10X)	89mM tris-HCl pH 8.3, 89mM boric acid, 2mM EDTA.

Southern Blot Buffers

Denhardt's buffer (5X)	0.1% (w/v) Ficoll, 0.1 (w/v) polyvinylpyrollidone, 0.1% (w/v) BSA.
Gel Soak I	200mM NaOH, 600mM NaCl.
Gel Soak II	1000mM Tris-HCl pH 8.0, 600mM NaCl.
Hybridization buffer	5X Denhardt's buffer, 0.1%(w/v) SDS 6X SSC, 30µg/ml denatured calf thymus DNA.
Nick translation buffer	50mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 50ug/ml BSA, 1mM DTT.
SSC buffer (10X)	3000mM NaCl, 300mM tri-sodium citrate, pH with 300mM citric acid to pH 7.5.

DNA Sequencing Reagents and Buffers

Gel bottom mix (6%) (2.5X TBE)	150ml 40% acrylamide:N,N' methylene bis acrylamide (20:1), 540g urea, 50g sucrose, 20ml 1% bromophenol blue (BPB), dissolved in H_2O, de-ionised, filtered and made up to 1000ml with 62.5ml of 40X TBE. For every 14ml of bottom mix 30µl of 25% ammonium persulphate (APS) and 30µl TEMED were added.
Chase mix	dATP, dTTP, dCTP, dGTP, each at a concentration of 0.5mM in H_2O.
Formyl dye	0.1% (w/v) BPB, 0.1% (w/v) XC, 20mM Na_2EDTA in deionised formamide.
Labelled mix	6µl of [^{32}P] dATP (1mCi/100µl), and 18µl of 11.8µM dATP.

Sequencing buffer (10X) 100mM Tris-HCl pH 8.5, 100mM $MgCl_2$.

TE buffer 10mM Tris-HCl pH 7.4, 0.1mM EDTA.

Gel top mix 150ml 40% acrylamide:N,N' methylene bis acrylamide (20:1), 540g urea dissolved in H_2O and made up to 1000ml with 50ml 10X TBE. For 100ml of top mix add 160µl 25% APS and 160µl TEMED were added.

Sequencing solutions:

\µl	dA-O	dT-O	dC-O	dG-O
5mM 7-deaza dGTP	20	20	20	1
5mM dCTP	20	20	1	20
5mM dTTP	20	1	20	20
TE (10X)	50	50	50	50
H_2O	540	370	370	370

Sequencing mixes:

\µl	dN-O	ddNTP	H_2O
T seq	500	500 (600µM ddTTP)	0
C seq	"	105 (140µM ddCTP)	395
G seq	"	155 (200µM ddGTP)	345
A seq	"	125 (140µM ddATP)	375

Protein Gel Electrophoresis Buffers

Destain 5% Methanol, 7% acetic acid.

Gel fixative 50% Methanol, 7% acetic acid, 0.2% Commassie brilliant blue.

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.

Tank buffer 53mM Tris, 53mM glycine, 0.1%(w/v) SDS

Western Blot Buffers

Antibody solution	1X NT, 0.005% Tween 20, 1% BSA.
Blocking buffer	5X TBS, 3% gelatin, mixture left at 37° for 1h to allow gelatin to dissolve.
Borate buffer	160mM borate, 140mM NaCl pH 9.
Blotting buffer	25mM Tris, 192mM glycine, 20% (v/v) methanol (pH 8.3).
Bis-diazotized benzidine (DAB)	0.23g benzidine hydrochloride, 180mM HCl, 50mM sodium nitrate.
KI solution	1X NT, 0.05% Tween 20, 1M KI.
NT buffer	0.09% NaCl, 1mM Tris (pH 7.4).
Protein A solution	1X NT, 3% BSA.
Tris buffered saline (TBS) (5X)	20mM Tris, 500mM NaCl (pH 7.5).
Wash buffer	1X NT, 0.05% Tween 20.

Immunopreciptation Buffers

Disruption buffer	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.
Glycine elution buffer	200mM Glycine pH 2.5.
RIPA buffer	0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) NP40, 1mM EDTA, 150mM NaCl, 10mM Tris-HCl pH pH 7.4.
Washing buffer	600mM LiCl, 100mM Tris-HCl pH 7.4, 1% (v/v) β-mercaptoethanol.

Zweig's buffer	0.1M Tris-HCl pH 8, 10% glycerol,0.5% NP40, 0.5% sodium deoxycholate, 0.2mM phenylmethylsulphonyl fluoride (PMSF).

Bacterial Growth Medium and Cloning Solutions

L-broth	170mM NaCl, 5 g/l yeast extract 10 g/l Difco Bactotryptone, supplemented with 25-50µg/ml of appropriate antibiotic.
Ligation buffer (10X)	200mM Tris-HCl pH 7.5, 100mM MgCl, 100mM dithiotheritol (DTT) and 5mM ATP.
STET buffer	8% (w/v) sucrose, 5% (v/v) NP40, 50mM EDTA, 50mM Tris-HCl pH 8.
2YT	292mM NaCl, 16 g/l bactotryptone,10 g/l yeast extract.

Miscellaneous

Cell lysis buffer	0.6% SDS, 10mM EDTA, 500ng/ml pronase, 10mM Tris-HCl pH 7.4.
E.M. fixative	2.5% gluteraldehyde in PBS.
HEPES buffered saline (HEBS)	130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5 mM D-glucose, 21mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) pH 7.05.
NTE buffer	10mM Tris-HCl pH 7.5, 100mM NaCl, 1mM EDTA.
RSB	10mM Tris-HCl pH 7.5, 10mM KCl, 1.5mM $MgCl_2$.

METHODS

2.1 CELL CULTURE

BHK cells were grown in rotating 850cm² culture bottles containing 100ml tissue culture medium in an atmosphere of 95% air, 5% CO_2 at 37°. One bottle gave a confluent monolayer of approximately $3X10^8$ cells which was used to seed five further 850cm² bottles. Cells were harvested by washing the monolayers first with 20ml of versene, then with 40ml trypsin:versene (1:1). Cells were shaken off the bottles and resuspended in culture medium. HFL cells were routinely grown in flat flasks. A confluent monolayer from a 175 cm² flask was sufficient to seed two flasks of the same size. To obtain a subconfluent monolayer of BHK or HFL cells after an overnight incubation at 31°, cells were seeded at a density of $2X10^6$ per 50mm petri dish and $1X10^6$ per 30mm dish.

2.2 VIRUS CULTURE

2.2.1 Production of virus stocks

Virus stocks were prepared from confluent BHK cells grown in 850cm² glass roller bottles. Growth medium was removed and the cells were infected with a moi of 0.003 pfu per cell in 60ml EC_5. Virus-infected cells were incubated at 31° for 3-4 days until extensive CPE had developed. Cells were shaken off the roller bottles into the medium and pelleted at 1500 rpm at 4° for 15 min The virus-infected cell pellet was resuspended in a small

volume of supernatant medium, and sonicated to release cell-associated virus. Cell debris was removed by centrifugation at 3000 rpm for 15 min at 4°. The sonication step was repeated if necessary and the cell-associated virus stocks were pooled and stored at -70°. Cell-released virus from the clarified virus-infected cell medium was concentrated by centrifugation at 12,000 rpm at 4° for 2h. The pellet was resuspended in virus-infected medium by sonication, and virus stored at -70°.

2.2.2 Sterility checks

Medium from virus-infected cells was streaked onto blood agar plates and plates incubated at 31° for 3-5 days. Any virus stocks containing bacteria which grew on blood agar were discarded.

2.2.3 Titration of virus stock

Serial ten-fold dilutions of virus stocks were made in PBS containing 5% calf serum. Growth medium was removed from 80% confluent BHK cell monolayers in 50mm plates and 100ul of diluted virus added to each dish. After virus absorption for 1h at either 37° (for plates to be incubated at the PT) or 38.5° (for plates to be incubated at the NPT), cells were overlaid with EHu$_5$ to prevent formation of secondary plaques. Plates were incubated either for 2 days at a NPT of 38.5° or 39°, depending on the mutant, or for 3 days at the PT of 31°, after which the medium was replaced with Giemsa stain for 15 min at RT. The plates were washed with water to remove excess stain, and virus plaques

counted under a dissecting microscope.

2.2.4 *Ts* mutant infections

Virus infections were carried out at a NPT of 38.5° or 39° depending on the mutant, and a PT of 31°. Cell monolayers were absorbed at the required temperature for a maximium of 1h. The medium for washing and overlaying virus-infected cells grown at the NPT was prewarmed to 42°. All manipulations at NPT were carried out as rapidly as possible to prevent any drop in the NPT.

2.2.5 Isolation of spontaneous *ts*$^+$ revertants of *ts*1233

Single plaques were isolated from a low passage stock of *ts*1233 titrated on BHK cells at 31°. BHK cell monolayers from five 50mm petri dishes were infected with the progeny from a single plaque and a small seed stock prepared. The seed stocks were titrated at the NPT and cell monolayers showing CPE after incubation for 2 days were harvested. Progeny virus which formed plaques at the NPT were plaque purified once at the NPT and twice at the PT. All plaques were isolated from cells overlaid with agar. 50mm plates of BHK cells were infected with *ts*$^+$ virus from two independent seed stocks of *ts*1233, and virus from each plate harvest was used to grow up a high titre virus stock.

2.3 PREPARATION OF VIRION DNA

2.3.1 Large scale preparation of DNA

BHK cells were grown in 5-10 850cm^2 roller bottles.

These were infected with virus as described for the production of stocks. When extensive CPE had developed, the virus-infected cells were shaken into the medium and pelleted by low speed centrifugation for 15 min at 4°. The infected cell pellet was resuspended in 20ml RSB containing 0.5% NP40. After incubation on ice for 10 min, the sample was centrifuged at 2,500 rpm for 10 min at 4°. The pellet of nuclei and cell debris was resuspended in 20ml RSB-NP40 buffer, and the sample left on ice for 10 min prior to centrifugation. The two cytoplasmic supernatant fractions were pooled with the clarified virus-infected cell medium and the virus concentrated by centrifugation at 12,000 rpm for 2-3h at 4°. The virus pellet was resuspended in 5ml NTE buffer and lysed by the addition of SDS at a final concentration of 2% (w/v). The virus DNA was extracted three times with phenol saturated with TE, followed by a chloroform extraction. The DNA was precipitated by the addition of two volumes of ethanol, concentrated by low speed centrifugation and resuspended in 10ml 10mM Tris-HCl. RNA was removed by incubating the sample with 10µg/ml RNaseI overnight at RT. The DNA solution was deproteinised with proteinase K at 31° for 2-3h, then extracted once with phenol-chloroform (1:1), once with chloroform, made 200mM with NaCl, and precipitated with two volumes ethanol. The DNA was pelleted by low speed centrifugation, lyophilised and resuspended in 10mM Tris-HCl pH 7.6.

2.3.2 Small scale preparation of virus-infected cell DNA

HFL cells ($4X10^6$) were infected with a moi of 5 pfu

virus per cell and incubated at the appropriate temperature for 18h before extraction of DNA. Total virus-infected cell DNA or DNase-resistant (encapsidated) DNA was prepared from the virus-infected cells as described by Stow *et al.* (1983).

2.3.2.a Total virus-infected cell DNA

Cell-released virus in the growth medium was pelleted by centrifugation at 18,000 rpm for 1h at 4° in an SS21 rotor. The virus pellet was resuspended in a small volume of lysis buffer containing 500µg/ml pronase and combined with virus-infected cells harvested in 2ml of the same buffer. After incubation for 4h at 37°, NaCl was added to a final concentration of 200mM, and the DNA solution was extracted twice with phenol:chloroform (1:1), once with chloroform and precipitated with ethanol. DNA was pelleted, lyophilised and resuspended in 400µl H_2O containing 10µg/ml RNase I.

2.3.2.b Encapsidated DNA (DNase-resistant DNA)

Virus-infected cells were harvested into 0.5ml RSB, containing 0.5% (v/v) NP40 and 100µg/ml DNase I, combined with cell-released virus, and incubated for 2h at 37°. SDS, EDTA, and pronase, at the same concentration present in lysis buffer, were added and incubation continued for a further 2h at 37°. The DNA was extracted twice with phenol:chloroform (1:1), once with chloroform and precipitated in ethanol. DNA was pelleted, lyophilised and resuspended in 200µl H_2O containing 10µg/ml RNase I.

2.4 ANALYSIS OF DNA

2.4.1 Restriction endonuclease digestion of DNA

DNA was digested with restriction endonucleases according to the conditions recommended by the suppliers for each enzyme.

2.4.2 Agarose gel electrophoresis

DNA fragments were separated on horizontal agarose gels containing agarose at a concentration of 0.8-1% (w/v), (depending on the size of DNA fragments to be resolved) in 1X TBE buffer containing 0.5µg/ml ethidium bromide. DNA samples in 10% (v/v) dye ficoll were loaded into wells formed with plastic combs, and the gel was electrophoresed at 2-10 v/cm² in 1X TBE buffer at RT for 8-18h. The separated fragments were visualised under UV light.

2.4.3 Purification of DNA fragments from agarose gels

Separated DNA fragments were eluted from gel slices by electrophoresis at 100v for 3h using electroelution buffer. DNA fragments were further purified by extraction twice with phenol:chloroform (1:1) and once with chloroform. Sodium acetate (pH 5.5) was added to a final concentration of 0.3M and DNA fragments precipitated with an equal volume of isopropanol at RT. DNA fragments were pelleted, lyophilised and resuspended in 10mM Tris-HCl (pH 7.5).

2.4.4 Polyacrylamide gel electrophoresis (PAGE)

PAGE was used for separating small DNA fragments

ranging from 30-900 bp in size. Vertical slab gels consisted of 10% polyacrylamide (prepared from a stock solution of acrylamide/N-N'-methylene bisacrylamide in a ratio of 29:1 w/v) in 0.55X TBE. A final concentration of 0.006% (w/v) APS, and 0.004% (v/v) TEMED were added to the polyacrylamide solution to polymerise the gels. Teflon combs were used to form wells. Electrophoresis was carried out at 3v/cm² for 12-16h in 0.55X TBE. Gels were stained in 0.55X TBE containing 0.5µg/ml EtBr for 30 min, and the DNA bands were visualized under UV light.

2.4.5 Purification of DNA fragments from polyacrylamide gels

The polyacrylamide gel slice containing separated DNA fragment was placed in a small eppendorf tube pierced with holes at the bottom and centrifuged in a large eppendorf tube. The crushed gel slice was incubated in 400ul of DNA elution buffer overnight at 42°. DNA samples were filtered through a GF/C glass fiber disc fitted in a syringe, and precipitated with two volumes of ethanol. The DNA pellet was dissolved in 0.3M sodium acetate and reprecipitated with ethanol. DNA fragments were concentrated by centrifugation, lyophilised and resuspended in H_2O.

2.5 MARKER RESCUE TECHNIQUE

Marker rescue experiments were carried out as described by Stow *et al.* (1978) with the modification of Preston (1981). Calcium chloride was added to a final

concentration of 130mM to a solution containing 0.8µg of intact mutant virion DNA, 10µg/ml calf thymus carrier DNA, HSV-1 DNA fragment (usually between 5-10 fold molar excess relative to virion DNA) in 1X HEBS. The samples were left for 5 min at RT to allow a fine suspension to develop. The suspension was added to 50mm dishes containing BHK cell monolayers from which growth medium had been removed. The cells were incubated at 37° for 45 min, then overlaid with EC_5. At 4h pi, the cells were washed once with EC_5, and treated with 25% (w/v) DMSO in 1X HEBS for 4 min at RT. DMSO was removed from the monolayers by washing the cells twice with EC_5, and cells were incubated in 4ml of EC_{10} at 31° for 3-4 days until extensive CPE had developed. Cells were scraped into the growth medium, disrupted by sonication, and the virus yield was determined at the PT and NPT.

2.6 COMPLEMENTATION YIELD TEST

Cells were infected at a moi of 10 pfu per cell (5 pfu per cell of each of the *ts* mutants in the mixed infections). After 1h absorption at the NPT, cells were washed three times with warm EC_5 to remove unadsorbed virus, and incubated in 2ml of EC_5 for 24h at the NPT. After this time cells were scraped into growth medium, sonicated and virus titrated at the PT and NPT to determine the virus yield. Complementation indices (CI) were calculated as described by Brown *et al.*, (1973) using the following formula:

$$CI = \frac{(X + Y)^{PT} - (X + Y)^{NPT}}{1/2\{(X^{PT} + Y^{PT}) - (X^{NPT} + Y^{NPT})\}}$$

where $(X+Y)^{PT}$ and $(X+Y)^{NPT}$ represent the titre of progeny virus from the mixed infection of *tsX* and *tsY* at PT and NPT respectively. Usually CI greater than five were considered positive.

2.7 SOUTHERN BLOT ANALYSIS

2.7.1 Transfer of DNA fragments to nitrocellulose

The method used was essentially the same as that described by Southern (1975). The agarose gel, containing separated restriction endonuclease fragments, was shaken gently in 200mM HCl for 30 min at RT to depurinate the DNA. The gel was then washed in distilled water, treated with gel soak I for 1h to denature the DNA, and neutralized with gel soak II for 1h. The gel was then placed on two sheets of Whatman 3mm filter paper, the ends of which were in contact with a reservoir of 10X SSC buffer. A nitrocellulose sheet, moistened with distilled water, was placed on top of the gel, and any air bubbles were removed. Four sheets of 3mm filter paper, cut slightly smaller than the gel size, were placed over the nitrocellulose sheet, and a weighted stack of paper towels was laid on the Whatman filter paper. The nitrocellulose sheet was removed the next day, rinsed in 2X SSC buffer, air dried and baked in a vacuum oven at 80° for 2h. The blotted gel was soaked with 1X TBE containing 0.5µg/ml EtBr for 30 min and

visualised under shortwave UV light to ensure that efficient transfer of DNA fragments from the gel had occured.

2.7.2 In vitro [^{32}P]-labelling of DNA by nick translation

DNA fragments were labelled with [^{32}P] *in vitro* as described by Rigby *et al.* (1977). Plasmid DNA (0.5µg) in 50µl reaction mixture containing, 40µM dATP, 40µM dTTP, 1X nick translation buffer, and 1X10^{-6} mg/ml DNase I, was incubated in a 37° water bath for 2-3 min. The mixture was then placed on ice, and 30µCi each of α-[^{32}P]-dGTP, α-[^{32}P]-dCTP together with 1 unit of *E.coli* DNA polymerase I were added. Incubation was carried out at 15° for 2h. The isotope incorporation into DNA was determined by spotting 2µl of the reaction mix on to a filter disc, precipitating the DNA with 5% TCA (w/v), and measuring the amount of radioactivity by Cherenkov counting. The reaction was terminated by the addition of 100mM EDTA, and the samples extracted once with phenol:chloroform (1:1). A Sephadex G50 column (10ml) equilibrated with TE buffer was used to separate unincorporated deoxyribonucleoside triphosphates from [^{32}P]-labelled DNA. The first peak of radioactivity, which contained the nick-translated DNA, was eluted, denatured in 0.2N NaOH for 10 min at RT, and then neutralised with 0.2N HCl. A specific activity of 1X10^{7}-1X10^{8} cpm per ug plasmid DNA was usually obtained.

2.7.3 DNA blot hybridisation

High stringency conditions of DNA hybridisation in

aqueous solution were based on those described by Southern, (1975) and Denhardt, (1966). Vacuum-dried nitrocellulose sheets were prehybridised in 20ml solution, containing 6X SSC, 5X Denhardt's buffer, 0.1% (w/v) SDS and 20µg/ml denatured calf thymus carrier DNA, at 72° in a sealed plastic bag submerged in a shaking water bath. After 2-3h the prehybridisation solution was replaced by hybridisation solution which contained, in addition to the above reagents, $1X10^7$ cpm of denatured [^{32}P]-labelled DNA probe per ml of hybridisation mix. Incubation was continued overnight at 72°. After incubation the nitrocellulose sheets were washed four times in 2X SSC, once in 1X SSC, and once in 0.5X SSC. All washes contained 0.36% SDS and 10mM sodium pyrophosphate. Blots were air-dried and placed in contact with Kodak X-Omat XS-1 film and a Dupont phosphotungstate intensifying screen at -70° for 24h or lo nger.

2.8 RECOMBINANT DNA TECHNIQUES

2.8.1 Construction of chimeric plasmids

Vector DNA was linearised with the appropriate enzyme, treated with calf intestinal phosphatase at a concentration of 5 units per µg of plasmid DNA in the presence of 20mM Tris-HCl (pH 8) and 40mM NaCl. The sample was incubated at 37° for 1h, after which the DNA was extracted twice with phenol:chloroform (1:1,v/v), once with chloroform, and precipitated with ethanol. A 4-10X molar excess of the purified HSV-1 DNA fragment relative to phosphatase-treated vector (40ng) was incubated overnight

at 15° in a 20µl ligation reaction containing 1 unit of T4 DNA ligase in ligation buffer.

2.8.2 Preparation of competent bacteria and plasmid DNA transfection

The procedure used for DNA transfection was based on the method described by Cohen *et al.*,(1972). *E.coli* K12 strain DH1 was grown in 50ml of L-broth to an OD_{590nm} of approximately 0.25. Bacteria were pelleted at 3000rpm for 15 min at 4°, resuspended in 25ml of ice-cold sterile 100mM $CaCl_2$ and incubated on ice for 1h. The cells were pelleted and resuspended in 0.5ml 100mM $CaCl_2$. At this stage the competence of the bacteria for transfection was tested using uncleaved plasmid. A sample containing 0.1ml of $CaCl_2$-treated cells and 20-40ng of plasmid DNA was incubated on ice for 1h. The cells were heat shocked at 42° for 2 min, and 1.5ml of L-broth was added to the bacteria. After 1h incubation at 37° in a shaker, the bacteria were plated onto L-broth agar containing either 50µg per ml ampicillin or tetracycline depending on the plasmids used. Plates were incubated overnight at 37°. Routinely $>1X10^6$ colonies per µg plasmid vector DNA were obtained. Competent bacteria were left at 4° for 16-18h prior to transfection since treatment for this period of time had been shown to increase the competence by 5 fold.

2.8.3 Small scale preparation of plasmid DNA (STET preps)

Small amounts of plasmid DNA were prepared using a modified method of Holmes and Quigley (1981) described by

Maniatis *et al.*,(1982). Single colonies from a bacterial plate were grown in 1.5ml L-broth containing 35-50µg/ml ampicillin or tetracycline (depending on the vector used) in an orbital shaker at 37° for 16-18h. The bacteria were pelleted at 10,000 rpm for 40 sec, and resuspended in 75µl STET buffer. After the addition of 6µl of 10mg/ml lysozyme, the sample was boiled for 1-2 min and centrifuged at 10,000 rpm for 10 min. The supernatant was extracted once with phenol:chloroform (1:1), once with chloroform, and the plasmid DNA precipitated with an equal volume of isopropanol at RT in the presence of 300mM sodium acetate. The DNA was pelleted at 10,000 rpm for 10 min, lyophilised and resuspended in 40µl Tris-HCl (pH 7.4).

2.8.4 Large scale preparation of plasmid DNA

The method used was based on the procedure described by Godson and Vapnek (1973). A flask, containing 500ml L-broth supplemented with 25-50µg/ml ampicillin or tetracycline, was inoculated with 2ml of an overnight bacterial culture. After incubation at 37° in an orbital shaker for 6-7h, a final concentration of 25µg/ml chloramphenicol was added and incubation continued for a further 16h. Bacteria were pelleted by centrifugation at 4,000 rpm for 15 min at 4°. The pellet was resuspended in 5ml of 25% (w/v) sucrose in 50mM Tris-HCl pH 8. After the addition of 2.5ml of 5mg/ml lysozyme in 50mM Tris-HCl pH 8, the mixture was incubated for 30 min on ice. EDTA (pH 7.9) was added to a final concentration of 50mM, and 5 min later, NaCl and SDS were added to a final concentration of

750mM and 2.5% (w/v) respectively. The sample was left at 4° for 2-3h, then centrifuged at 18,000 for 1h at 0° to remove debris and high molecular weight bacterial DNA. The resulting supernatant was extracted twice with phenol:chloroform (1:1), and once with chloroform. Ethanol (2.5 volumes) was added and samples left at -20° for 2h. DNA was pelleted by centrifugation at 2,500 rpm for 15 min. The pellet was resuspended in 2-4ml of 10mM Tris-HCl, 0.1mM EDTA containing 100µg/ml RNase and incubated at 37° for 2-3h. Proteinase k, at a final concentration of 200µg/ml, was added and the sample incubated for 2h at 31°. The DNA was extracted twice with phenol:chloroform (1:1) and once with chloroform and precipitated at RT with 0.5 vol isopropanol in the presence of 300mM sodium acetate. The plasmid DNA was pelleted at 2,500 rpm for 30 min at RT, washed with 70% ethanol, lyophilised, and resuspended in 1ml 10mM Tris-HCl (pH 7.4).

2.8.5 Bacterial stocks

For long term storage, bacteria were concentrated by centrifugation at 4,000 rpm for 10 min and stored in 40% (v/v) glycerol, 1% (w/v) bactopeptone at -70°.

2.8.6 Construction of recombinant bacteriophage DNA

Two bacteriophage vectors M13mp18, and M13mp19 (Norrander *et al.*, 1983) were used. The double stranded RF DNA was linearised with the appropriate restriction endonuclease. A 4-10X molar excess of the purified HSV-1 DNA fragment relative to the linearised vector (50ng) was

incubated at 15° for 12-24h in 20µl ligation mix containing 1 unit of T4 DNA ligase in ligation buffer.

2.8.7 Transfection of bacteriophage DNA into E.Coli JM101

The procedure for preparing competent *E.coli* strain JM101 (Messing, 1979) was essentially the same as the one used to make competent DH1 except that the bacteria were grown in 2YT medium instead of L-broth. Cells (200µl) were incubated with 10µl of ligation mix on ice for 1h in glass tubes. Cells were heat treated at 42° for 5 min, then 200µl of fresh bacterial stock was added together with 25µl 2.5% isopropyl-D-thiogalactoside (IPTG), 25µl 2% 5-chloro-4-bromo-3-indolyl-β-D-galactoside (BCIG or X-gal) in dimethylformamide and 3ml of top agar warmed to 42°. The sample was plated on to L-broth agar plates and incubated overnight at 37°.

2.9 Preparation of single stranded DNA for sequencing

Bacteriophage which contained inserts gave white plaques, while uninterrupted vectors gave blue plaques. Bacteriophage from a white plaque were transferred to 1.5ml of 2YT containing 1/100 dilution of an overnight culture of JM101, and the sample incubated in an orbital shaker at 37° for 5-6h. Bacteria were pelleted by centrifugation and the supernatant transferred to a tube containing 200µl of 20% polyethelene glycol in 2500mM NaCl. After at least 30 min at RT, the sample was centrifuged at 13,000 rpm for 5 min, and the bacteriophage suspended in 100µl of TE. The bacteriophage DNA was extracted with 50µl of phenol

saturated with TE. The DNA was precipitated in three volumes of ethanol in the presence of 100mM sodium acetate, concentrated by centrifugation, washed with 70% ethanol and lyophilised. Finally the single stranded (ss) DNA was resuspended in 50µl of 0.2% TE.

2.9.1 Sequencing single stranded DNA

The dideoxy chain termination/M13 vector method of DNA sequencing (Sanger *et al.*, 1977; 1980; Messing and Vieira, 1982) was used during this study, the steps are:

A) Annealing primer to template DNA:

2µl of bacteriophage ssDNA was mixed with 2.5ng of M13 17 base oligonucleotide primer in 10µl reaction mix containing 1X sequencing buffer. The sample was incubated at 37° for 30 min.

B) Sequencing reaction:

Two units of Klenow polymerase were added to 10µl of annealed DNA, and 2µl of the mix were dispensed in each of 4 wells which contained 2µl of either dT, dC, dG, and dA squencing mix in a 96-well microtitre plate. 2µl of labelled mix was then added to each well. The plate was covered, centrifuged at 1000 rpm briefly and incubated at 31°. After 15 min 2µl of chase mix was added to each well and incubation continued for a further 30 min. Prior to loading on a gel, 2µl of formyl dye was added to each sample, and the microtitre plates boiled for 2 min in a water bath.

C) Sequence fractionation:

The boiled sequencing reaction (2-4µl) was loaded

into a well of gradient sequencing gel and the gel electrophoresed at 70 watts for 4h. The notched glass plate was removed and the bonded gel washed with 10% acetic acid for 30 min, rinsed with tap water, and dried for 2-3h at 75°-80°. The gels were placed in contact with X-Omat XS-1 film, and the film exposed overnight before being developed.

2.9.2 Preparation of gradient sequencing gels

The unnotched glass plate was treated with 10ml solution of ethanol containing 300µl of 10% acetic acid and 50µl of waker silane GF38, and left to dry for 3 min. The plate was washed three times with ethanol and polished well. The notched glass plate was treated with 2ml of repelcote and subsequently washed with ethanol. The glass plates were taped together, and 7ml of bottom gel mix taken up in a syringe with 7ml top mix, after adding APS and TEMED, were poured into the glass plate sandwich. Followed by the remaining top gel mix. Gels were left to polymerize for 1h before being used. Wells were formed with a plastikard comb.

2.10 LABELLING OF VIRUS DNA WITH [^{32}P] ORTHOPHOSPHATE

The method used was based on the procedure described by Lonsdale (1979). BHK cells in phosphate-free Eagle's medium containing 1% calf serum (P.I.C. medium) were seeded at a density of 5X10^{5} cells per linbro well. The following day, cells were infected with a moi of 5-10 pfu per cell, and virus absorbed at 37°. One hour later,

cells were washed twice with P.I.C. medium, overlaid with 450µl of the same medium and incubated at 31°. At 3hpi, 50µCi of [^{32}P]-orthophosphate (carrier free) in 50µl was added to each well. After incubation for 48h at 37°, the cells were lysed by the addition of 0.5ml of 5% SDS (w/v), and the samples left for 10 min at 37°. DNA was extracted once with phenol saturated in TE buffer and precipitated with 2 volumes of ethanol. The DNA was subsequently concentrated by centrifugation at 2,000 rpm for 10 min at 4°, lyophilised and resuspended in 150µl H_2O. Samples were digested with restriction endonuclease, and the fragments separated on an 0.8% agarose gel. After electrophoresis, the gel was dried onto the glass plate at 80°, and placed in contact with X-Omat XS-1 film. The film was exposed for a minmium of 18h.

2.11 ANALYSIS OF VIRUS INDUCED PROTEINS

2.11.1 Preparation of radiolabelled cell extracts

Sub-confluent HFL cells or BHK cells were infected at a moi of 20 pfu per cell. After 1h absorption at the PT or NPT, cells were washed once with growth medium, overlaid with the appropriate medium, and incubated at either PT or NPT. Immediately prior to addition of [^{35}S]-methionine, cells were washed twice with prewarmed PBS, and incubated in prewarmed PBS containing 100µCi/ml [^{35}S]-methionine for 15-30 min. The monolayers were washed twice with ice cold water and harvested. Alternatively, samples were washed with prewarmed medium and incubation continued for a further 5h before the samples were harvested. Virus-

infected cell extracts were harvested in 300µl disruption buffer, and were boiled for 5 min before SDS PAGE. All radiolabelled infected-cell polypeptide samples were stored at -20° prior to analysis.

2.11.2 Immunoprecipitation using monoclonal antibodies

Radiolabelled virus-infected cells were lysed by the addition of RIPA buffer, transferred to glass vials and sonicated. Cell debris was removed from the samples by centrifugation at 10,000 rpm for 15 min. The procedure used for immunoprecipitation was based on the method described by Kessler (1975). $1X10^6$ cpm [^{35}S]-labelled virus-infected cell extracts were incubated with 20ul of diluted ascitic fluid overnight at 4°. Sheep anti-mouse immunoglobulin (10µl) was added and incubation continued at 4°. After 4h, 100µl of 10% (w/v) suspension of formalin-fixed *Stapholococcus aureus* was added, and incubation continued for 1h at 4°. The bacteria were washed three times by centrifugation at 10,000 rpm for 20 sec followed each time by resuspension in 600µl of lithium chloride washing buffer. Samples were finally resuspended in 100µl of 200mM glycine (pH2.5 at 4°) to elute the antigen from the bacteria. After incubation on ice for 30min, the bacteria were pelleted by centrifugation at 10,000rpm for 5 min and the supernatant transfered to a vial containing 50µl 3X disruption buffer.

2.11.3 SDS PAGE

Proteins were separated on two types of slab

polyacrylamide gels either Single concentration gel, containing 10% polyacrylamide gels in which the acrylamide was cross linked with 1 part in 40 (w/w) N,N'-methylene bisacrylamide in resolving gel buffer, or gradient gels, composed of a 6-12.5% or 6-15% linear gradient of polyacrylamide in which the acrylamide was cross linked with 1 part in 20 (w/w) N,N'-methylenebisacrylamide in resolving gel buffer. Gradient gels were formed using a proportioning pump (Technicon Ltd.). APS and TEMED at a final concentration of 0.006% (w/v) and 0.004% (v/v) respectively were added to gel solution just before pouring to polymerise the gel. The gel was overlaid carefully with butan-2-ol to ensure a smooth interface on polymerisation. The butan-2-ol was removed once the gel had polymerised and the interface washed twice with ¼ strength resolving gel buffer. The stacking gel, which contained 5% polyacrylamide (in which the acrylamide was cross linked with 1 part in 40 (w/w) N,N'-methylenebisacrylamide in stacking gel buffer) was poured on top of the resolving gel. Wells were formed with teflon combs. Protein samples were separated by electrophoresis at either 0.45 mA/cm² for 3-4h or 0.09 mA/cm² for 18h in tank buffer.

2.11.4 Autoradiography

Gels were removed from glass plates after electrophoresis and treated with gel fixative and subsequently soaked in destain solution. Gels were either dried down under vacuum and exposed to X-Omat XS-1 film, or were soaked in En²hance (Dupont, Boston, USA) for 30 min at

RT, rehydrated in several changes of water for 30 min and then dried under vacuum. Dried gels treated with En²hance were placed in contact with flashed Kodak X-Omat XS-1 at -70° and exposed for at least 18h.

2.12 ANTIPEPTIDE ANTIBODIES

2.12.1 Production of polyclonal antibodies to synthetic oligopeptides

Since low mw peptides are poor immunogens, their immunogenicity was enhanced by coupling to a carrier protein. The coupling reaction was performed essentially as described by Bassiri *et al.* (1979) with a few modifications. Briefly, 40mg of BSA or β-galactosidase we re added to the peptide (5-10mg) in 2-4ml of 1X borate buffer pH9. While the sample was being stirred on ice 1ml of DAB was added dropwise until the solution turned dark brown. After the pH was adjusted to pH 8.7-9.0 with 0.5N NaOH, the sample was left on ice for 2h prior to dialysis. If, however, a precipitate began to form, dialysis was started earlier. The sample was dialysed for 2 days at 4° with five changes of PBS. The dialysed sample was diluted with PBS and 1ml aliquots, containing approximately 150ug of coupled peptide, were stored at -20°. 1ml of either co mplete or incomplete Freund's adjuvant was mixed with 1ml of coupled peptide and the sample emulsified. Six month old New Zealand white female rabbits (two rabbits for each peptide) were immunised intradermally at 5 sites in the scruff of the neck. Pre-immune sera were obtained from all

rabbits before immunisation was started. The first injection contained antigen in complete Freund's adjuvent, followed by a total of four booster injections of antigen in incomplete Freund's adjuvent, each at 10-20 day intervals. Prior to each injection, a 20ml blood sample was taken from each rabbit. After 50-60 days the rabbits were killed and the whole blood collected. The clotted blood was centrifuged at 2,000 rpm for 10 min, and the serum removed and stored in aliquots at -20°.

2.12.2 Removal of BSA antibodies from oligopeptides antiserum using K50 column

Serum, prepared from rabbits injected with an oligopeptide coupled to BSA, was passed through a K50 column (Pharmacia Fine Chemicals) containing BSA linked to CNBr activated Sepharose 4B to remove antibodies to BSA. Prior to the addition of serum, the K50 column was washed overnight with 1-2L of PBS. The serum was first pumped through a small column containing 10ml of BSA linked to CNBr activated Sepharose 4B to remove any large particles, and then into a large column. Once the serum had reached the middle section of the second column the pump was switched off, the column clamped off, and the serum left for at least 1h at RT. After that time the pump was switched on, the clamps removed, column washed with PBS while the serum was collected, and stored at -20°.

2.12.3 Fast protein liquid chromatography (FPLC)

This technique was used to purify IgG from rabbit

serum. A column was packed with DEAE-Trisacryl^{R} M beads (LKB instruments, Ltd.), equilibrated with a low salt solution (0.025M Tris-HCl, 0.035 NaCl pH 8.8), and inserted into an automated FPLC machine (Pharmacia). 10ml serum was dialysed in 2L of the low salt overnight prior to addition to the column. After the 1-2h of incubation, the column was first washed with the low salt solution, which removed the IgG bound to the beads in the column, and then with a high salt solution (100mM Tris-HCl, 1000mM NaCl pH 8.8), which removed other serum proteins bound to the beads. Fractions were collected and stored at -20°.

2.12.4 Radioimmunoassay (RIA)

The method used to detect the presence of antibodies to oligopeptides or BSA was based on a modified method of Green *et al.*(1982). Each well of a microtitre plate (96-well) was coated with 1µg/well of peptide dissolved in 25µl of PBS, or 25µl/well of 10% (w/v) BSA in PBS. The antigens were adsorbed on to the round bottom plates overnight at 37°. The solutions were shaken off the wells and peptides were fixed to the wells with 200µl methanol per well. After 5 min incubation at RT the methanol was removed. Non-specific protein binding sites were blocked with 200µl 0.5% polyoxyethylene sorbitan monolaurate (Tween 20)in PBS for 1h. Wells were then washed with PBS, and dried. BSA solution was shaken off the BSA treated wells, wells were washed once with PBS, and dried. 25µl of diluted serum in 0.05% Tween 20 in PBS was added to each well. Plates were sealed and incubated at

37° for 1h. This step was followed by three washes of 200µl of 0.05% Tween 20 in PBS. Protein A conjugated to [^{125}I] was diluted in PBS containing 0.05% Tween 20, and 25µl (20,000 cpm) added to each well. After the plates had been incubated for 30 min at 37°, the wells were washed three times with PBS by flooding the plates. Finally, 200µl of 5M NaOH was added to each well to elute bound protein A, and the plates left for 25 min at RT. Samples were transferred to plastic tubes and the amount of [^{125}I] monitered by a Gamma counter (NE 1600, Nuclear Enterprises Ltd., Edinburgh, Scotland).

2.12.5 Western blotting technique

The method used was based on the procedure described by Towbin *et al.*(1979). Proteins from infected-cell extracts, labelled with [^{35}S]-methionine were separated by SDS PAGE. After electrophoresis, proteins were transferred onto nitrocellulose paper using a Bio Rad Transblot apparatus. A sandwich was prepared in which the gel, equilibrated in blotting buffer, was placed on a sheet of 3mm Whatman's filter paper, soaked in the same buffer. Underneath the filter paper was a fiber pad. Strips of nitrocellulose, also presoaked in buffer, were carefully placed on the gel and covered with a sheet of moistened 3mm Whatman's filter paper. After air bubbles were removed, a fiber pad was placed on top of the filter paper and the sandwich held together in a plastic holder. The holder was placed in a tank filled with blotting buffer. The strips faced the anode while the gel faced the cathode. Proteins

were removed onto the nitrocellulose strips by electrophoresis at 250mA. After 3h, strips were carefully removed to plastic dishes, containing 200ml of preheated blocking buffer, which were placed in a shaking water bath for 1h at 37°. After one change of blocking buffer, the strips were removed, air dried, and stored at -70°.

2.12.6 Detection of Antibodies bound to protein on nitrocellulose strips using [^{125}I] protein A

Strips were placed protein side up in shallow slots of a perspex block, and covered with different dilutions of serum in 1X NT buffer containing 0.05% Tween 20 and 1% BSA. The blocks were shaken at 37° for 2-4h or overnight at RT. Strips were then transferred to plastic dishes, washed twice with wash buffer at RT for 10 min, and incubated in protein A solution containing [^{125}I] protein A at 37°. After 2h, strips were washed twice with KI solution for 1h, and rinsed several times with wash buffer. Finally, strips were air dried, aligned on a piece of cardboard and placed in contact with three X-Omat XS-1 films. A screen of black paper was inserted between the first and the second sheets of film sheet,and an image intensifying screen placed after the third sheet. This procedure, described by Haarr et al.(1985), was used to determine which of the [^{35}S]-methionine labelled proteins the antibody reacted with as [^{125}I] protein A bound to that antibody. The first film was used to detect [^{35}S]-methionine labelled proteins. The [^{35}S]-methionine does not penetrate the second film, whereas the high energy emission from the

[^{125}I] passes through the three films to interact with the intensifying screen and produce photons which affect mainly the third film, and to an extend the second film but are prevented from reacting to the first film by the black paper. The films were exposed for at least 18h before developing.

2.12.7 Immunoprecipitation with polyclonal antibodies

Antibody (50µl) was incubated with 20µl (1X10^6 cpm) of [^{35}S]-methionine labelled virus-infected cell extracts overnight at 4°. Protein A sepharose (60µl), which had been swollen and washed with RIPA buffer three times, was added to the antigen-antibody mixture and samples shaken gently for 1h at 4°. This step was followed by four washes with 600µl washing buffer. Disruption buffer (50µl) was added to the samples, and tubes boiled for 5 min. Samples were then centrifuged, and 40µl of each supernatant was loaded into a well of a protein gel.

2.13 ISOLATION AND PURIFICATION OF HSV VIRIONS AND NUCLEOCAPSIDS

The method of Gibson and Roizman (1972) was used for the isolation and purification of nucleocapsids. BHK cells grown in 850cm^2 culture bottles were infected with 0.03 pfu of virus per cell. After 7-8hpi incubation at 31° 1uCi of [^{35}S]-methionine was added to each bottle. When extensive CPE had developed cells were shaken off into medium, and pelleted at 2,000 rpm for 10 min at 4°. Virions were purified from the supernatant, and capsids

were isolated from the cell pellet.

The supernatant was centrifuged at 12,000 rpm for 2h at 4°. The pellet was resuspended in 2ml Eagle's medium without phenol red (E w/o PR)and loaded onto 2X12ml, 5-15% Ficoll gradients in E w/o PR. After 2½h centrifugation at 13,000 in 4°, the tubes were placed under a strong light source and the virion band was removed using a syringe needle pierced through the side wall of the tube. The band was suspended in E w/o PR and pelleted in a TST41 rotor at 21,000 for 2h at 4°. The pellet was resuspended carefully in 500µl PBS.

The cell pellet obtained from one burrler of virus-infected cells was resuspended in 50ml PBS containing 0.5% NP40, incubated for 10 min at 4°, and the nuclei concentrated by centrifugation at 2,000 rpm for 10 min at 4°. The nuclei were resuspended in 3.8ml PBS, and lysed with a final concentration of 1% (w/v) sodium deoxycholate. 50µl of DNase 1 (10mg/ml) was added, and the sample incubated for 30 min at RT to reduce the viscosity of the solution. Urea, at a final concentration of 500mM was added, and incubation continued for a further 15-20 min. The sample was loaded onto a 10-40% (w/v) sucrose gradient in PBS, and the tubes centrifuged at 40,000 rpm for 20 min at 4°. The tubes were placed under a strong light source and the nucleocapsid bands removed by puncturing the side with a syringe. The nucleocapsids were suspended in PBS, and centrifuged at 40,000 for 30 min at 4°. The supernatant was discarded, and the pellet resuspended in 250µl of PBS.

2.14 Bacterial expression assay

A (60µl) sample of bacteria carrying the expression vector was removed from an overnight culture, diluted in 3ml of L-broth containing ampicillin and shaken for 30 min at 37°. The culture was then divided between two vials and 100mM IPTG added to one of the samples to enhance the expression of the foreign gene. Incubation was continued until the bacteria reached an OD_{590nm} of 0.2. The bacteria were then pelleted by centrifugation, resuspended in 1X disruption buffer and boiled for 15min. A sample of the boiled solution was loaded on a 6-15% SDS polyacylamide gradient gel. After electrophoresis the gels were stained in gel fixative solution containing coomassie blue stain and then destained.

2.15 ELECTRON MICROSCOPY

2.15.1 Preparation of samples in Epon-resin

The growth medium was removed from mock- and virus-infected HFL cells in 30mm dishes (at 14 hpi for PT samples, 12 hpi for NPT samples and 16 hpi for samples shifted at 12 hpi from the NPT to the PT in the presence of cycloheximide) and the monolayers washed three times with PBS. Cells were scraped into 0.5ml PBS, transferred to beem capsules and the samples centrifuged at 5000 rpm for 10 min. The supernatant was removed, 500µl of 2.5% (v/v) gluteraldehyde in PBS added to each sample, and the capsules left at 4°. The next day, the pellets were washed

three times with PBS and post fixed with 1% (w/v) OsO_4. After 1h the OsO_4 was removed by three washes with PBS. The pellets were subsequently dehydrated through a series of increasing ethanol concentrations (30%, 50%, 70%, 90%, 100% (v/v) in PBS). The pellets were infiltrated with 50% (v/v) epon resin in ethanol, followed by two changes of epon resin alone. Epon resin, added to the samples at the final stage, was polymerised by incubation at 65° for three days.

2.15.2 Thin sectioning

Pelleted cells embedded in polymerised epon resin were cut either with a glass knife or with a diamond knife on a ultra-micotome (Ultracut E, Reichert-Jung), and thin sections were collected on parlodium-coated copper grids. Sections were stained with saturated uranyl acetate in 50% (v/v) ethanol for 1h, rinsed with deionised water and counter-stained with lead citrate for 1h. The stained thin sections were examined at 80KV in a Jeol 100S electron microscope.

2.16 COMPUTING AND ANALYSIS OF SEQUENCE DATA

Sequence handling was carried out in a DEC PDP11/44 computer under RSX11M operating system or a DEC VAX computer. The programmes used in the PDP11/44 computer were either designed or implemented by Dr. P. Taylor. In the 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).

3.1 CHARACTERISATION OF THE HSV-1 MUTANT, *TS*1233 WITH A DEFECT IN ENCAPSIDATION OF VIRAL DNA AT THE NPT

3.1.1 Introduction

The sequence of events leading to the assembly of HSV capsids is still unclear. At least seven polypeptides have been identified in capsids purified from the nuclei of virus-infected cells and two of these, $V_{MW}155$ and $V_{MW}50$, are required for the assembly of capsids (section 1.9.1). Several structural proteins have been directly or indirectly implicated in DNA encapsidation. The mechanism by which HSV packages the genome into a preformed capsid is not known. It is clear from the analysis of defective virus genomes and *ts* mutants that DNA encapsidation is a complex process which requires a variety of functions including both cis-acting sequences and trans-acting elements (see sections 1.7.4 and 1.7.5). Work on *ts* mutants of HSV-1 and PRV has established that cleavage of high mw concatemeric DNA to unit length genome is linked to DNA encapsidation (Ladin *et al.*, 1980; 1982; Preston *et al.*, 1983; Addison, 1986; Sherman and Bachenheimer, 1987; 1988), and that several gene products are involved in the process of viral DNA packaging. The following chapter deals with the characterisation of a DNA positive *ts* mutant of HSV-1, *ts*1233, which fails to package DNA at the NPT, and the identification of the gene in which the lesion lies.

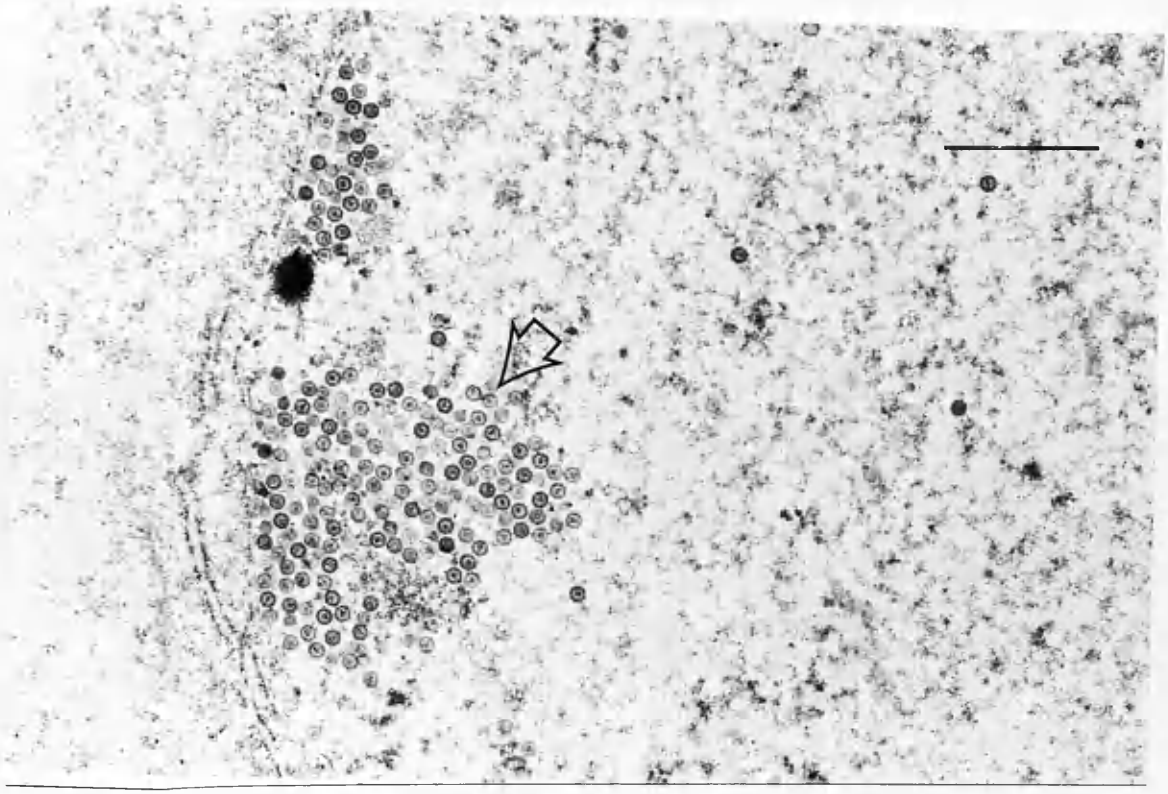
3.1.2 Electron microscopic analysis of *ts*1233-infected cells reveals that the mutant has a defect in DNA encapsidation

Thin section preparations of *ts*1233 and 17*syn*$^+$ HFL-infected cells were examined under the electron microscope. At the NPT the nuclei of *ts*1233-infected cells contained large numbers of intermediate capsids which had electron-translucent cores. These capsids were present as aggregates or as single capsids (figure 9A). No dense-cored capsids or empty capsids lacking an internal core were observed in the mutant-infected cells. By contrast all three capsid forms were present in the nuclei of *wt* virus-infected cells at both the PT and NPT and in the nuclei of *ts*1233-infected cells at the PT. Mature virions were also detected in these virus-infected cells (figures 9 b, c,d). These results suggest that *ts*1233 has a block in assembly of full nucleocapsids. When the mutant virus-infected cells were shifted from the NPT to the PT at 12 h.p.i. in the presence of cycloheximide (a protein synthesis inhibitor) and incubated for a further 4h before being harvested, no full nucleocapsids were detected in the nuclei and no enveloped virions were present in the cytoplasm or on the surface of infected cells, indicating that the effect of the mutation could not be reversed by transferring the sample to the PT (figures 9 F, G).

3.1.3 Quantitation of different capsid types in virus-infected cells

The number of empty, partially-cored, and full

A



B

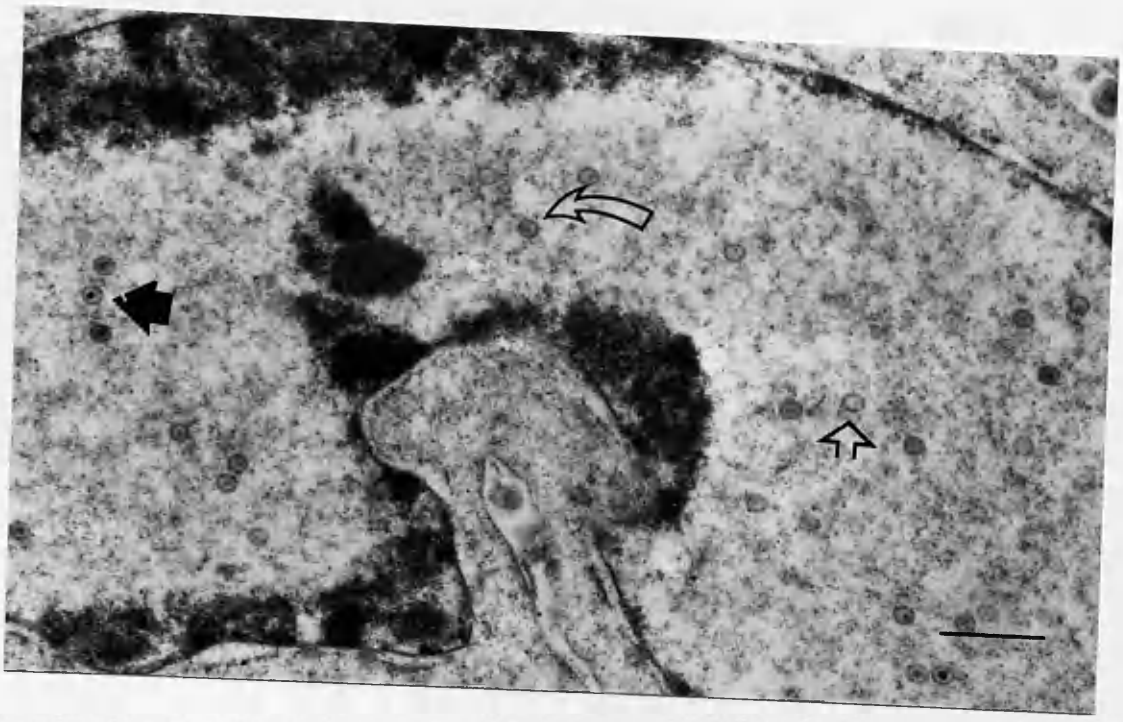


Figure 9

Electron microscopic analysis of *ts*1233- and 17*syn*$^+$ - infected cells

Electron micrograph of thin section preparations of HFL cells infected with a moi of 5pfu of *ts*1233 or *wt* HSV-1 17*syn*$^+$.

A) Nucleus of a cell infected with *ts*1233. Cells were harvested after 12h incubation at the NPT (39°).

B) Nucleus of a cell infected with *wt* 17*syn*$^+$. Cells were harvested after 14h incubation at the PT (31°).

C) Nucleus of a cell infected with *wt* 17*syn*$^+$. Cells were harvested after 12h incubation at the NPT (39°).

D) Nucleus of a cell infected with *ts*1233. Cells were harvested after 14h incubation at the PT (31°).

E) Nucleus of a mock infected HFL cell. Cells were harvested after 12h incubation at the NPT (39°)

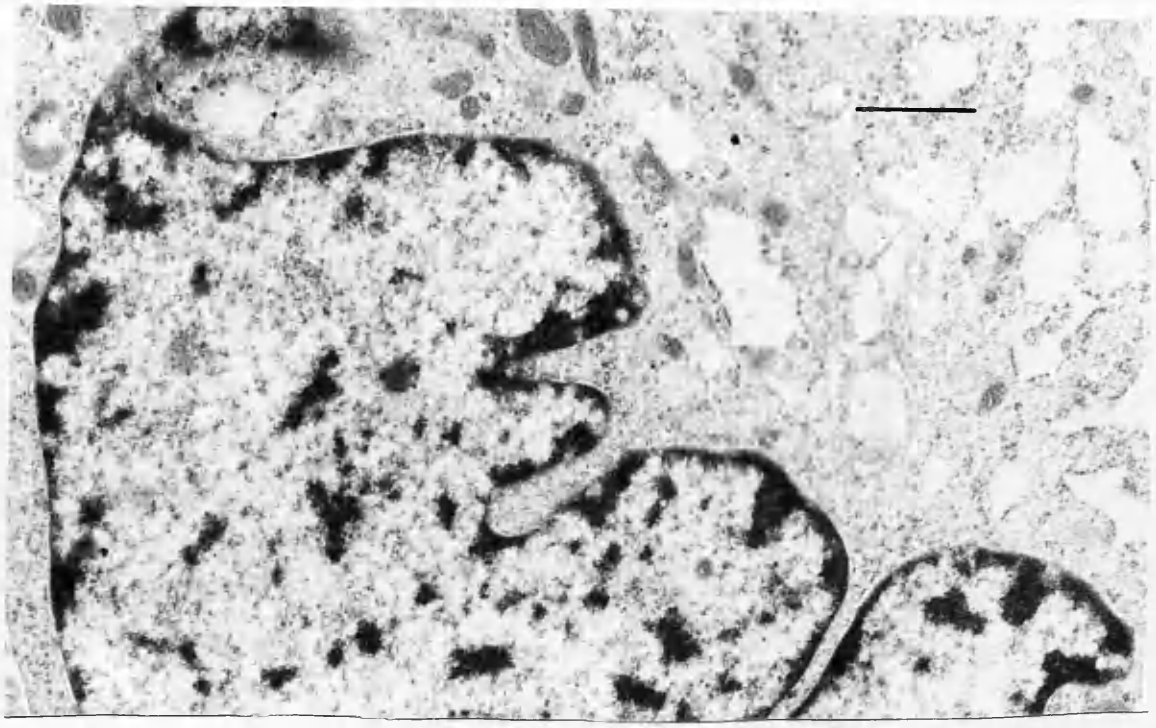
F) Nucleus of a cell infected with *ts*1233 at the NPT. Cells were shifted to the PT at 12hpi in the presence of cycloheximide and harvested after a further 4h incubation at the PT.

G) Nucleus of a cell infected with *wt* virus at the NPT. Cells were shifted to the PT at 12hpi in the presence of cycloheximide and harvested after a further 4h incubation at the PT.

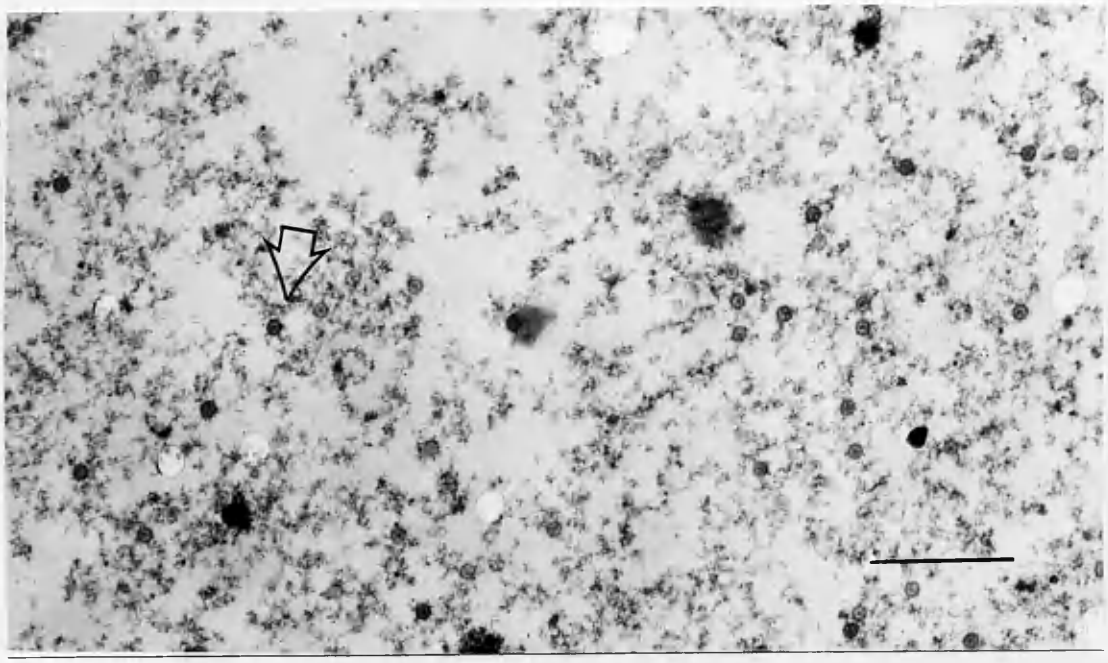
Full capsids are indicated by The solid arrows. Partially-cored capsids are indicated by closed arrows.Empty capsids are indicated by an open arrow.

The bar represents 0.5µm.

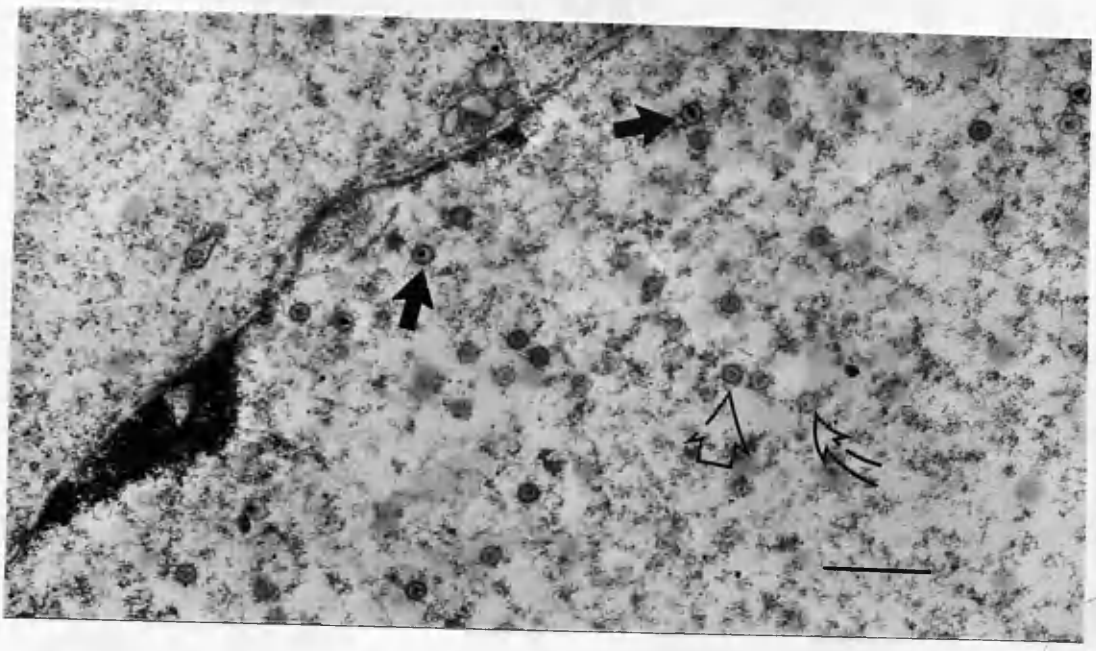
E



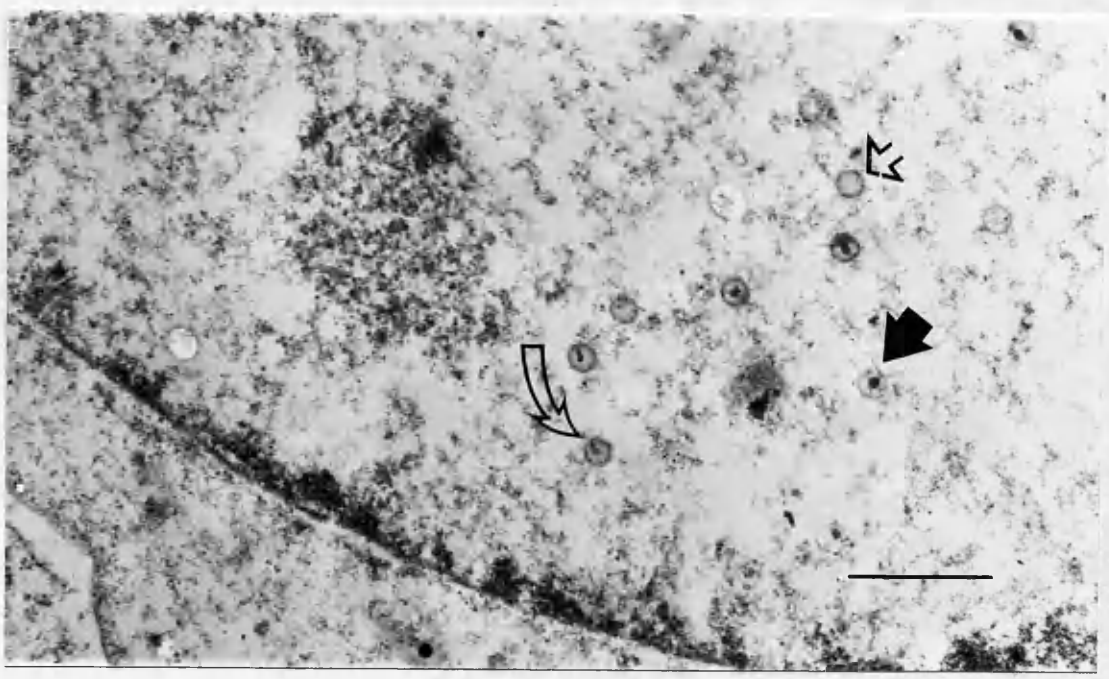
F



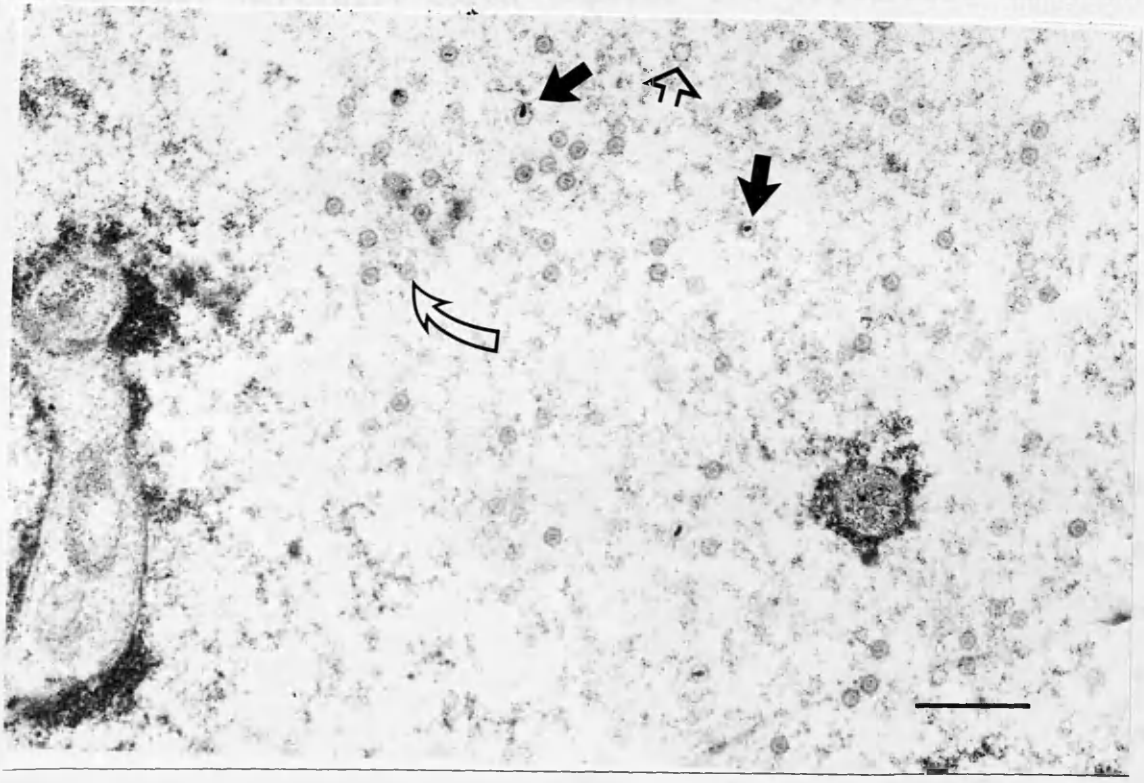
C



D



G



capsids present in the nuclei of virus-infected cells was determined by counting capsids seen in thin section preparations under the electron microscope. 50 nuclei from both 17*syn*$^{+}$-, and *ts*1233-infected cells were chosen at random. Results represented in table 4 revealed that mean total number of capsids varied from 33.42 to 100.56 per thin section of nucleus. The predominant capsid type was the partially-cored capsid. This is consistent with the idea that partially-cored capsids are precursors of full capsids. No empty capsids were detected in *ts*1233-infected cells at at the NPT and this finding supports the proposal that empty capsids are breakdown products of full capsids and are not precursors of DNA containing capsids. At the NPT the total number of capsids/nucleus of *ts*1233-infected cells was greater than that of *wt* virus-infected cells (53 cf 62). This could be due to capsids remaining in the nucleus in *ts*1233-infected cells, whereas in *wt* virus full capsids are rapidly enveloped. In *wt* virus-infected cells treated with cycloheximide at 31° or 39° a slight decrease in the proportion of partially-cored capsids was observed, and this was accompanied at 31° by a slight increase in proportion of empty capsids and full capsids. The major reason for the decrease in partially-cored capsids was a decrease in the absolute number of total capsids/nucleus of thin section. This result suggested that in *wt* virus-infected cells an unstable gene product might be important for the production of partially-cored capsids. In *ts*1233-infected cells treated with cycloheximide at 39°, on the other hand, an increase in the absolute number of capsids/nucleus was

TABLE 4

Quantitation of different capsid types in virus-infected cells

Temp.	Virus	E	P	F	Total no.
31° (1)	17*syn*+ (%)	3.52 (5.71)	53.64 (87.10)	4.42 (7.17)	61.58 (100.00)
	*ts*1233 (%)	5.80 (8.99)	49.38 (76.55)	9.32 (14.44)	64.50 (100.00)
39° (2)	17*syn*+ (%)	4.10 (7.72)	42.68 (80.40)	6.30 (11.86)	53.08 (100.00)
	*ts*1233 (%)	0 0	76.58 (100.0)	0 0	76.58 (100.00)
31° \CH 31° (3)	17*syn*+ (%)	6.32 (11.40)	42.34 (76.42)	6.74 (12.16)	55.40 (100.00)
	*ts*1233 (%)	8.76 (17.46)	33.70 (67.18)	7.70 (15.35)	50.16 (100.00)
39° \CH 31° (3)	17*syn*+ (%)	1.80 (5.38)	28.40 (84.97)	3.22 (9.63)	33.42 (100.00)
	*ts*1233 (%)	0 0	100.56 (100.00)	0 0	100.56 (100.00)
39° \CH 39° (3)	17*syn*+ (%)	3.78 (9.26)	30.76 (75.39)	6.26 (15.34)	40.80 (100.00)
	*ts*1233 (%)	0 0	81.44 (100.00)	0 0	81.44 (100.00)

Table 4

The table represent the proportion of dense-cored (F), partially-cored (P) and empty (E) capsids in the nuclei of infected cells determined from the examination of thin section preparation of cells under the electron microscope (50 cells were screened for each type of capsids at:

1) Cells infected with *wt* virus or *ts*1233 at the PT (31°) and harvested after 14hpi.

2) Cells infected with *wt* virus or *ts*1233 at the NPT (39°) and harvested after 12hpi.

3) Cells infected with *wt* virus or *ts*1233 at the NPT (39°) followed by a shift to the PT (31°) at 12hpi in the presence of cycloheximide, and harvested after further 4h.

Top figure represent the average number of each type of capsids per nucleus, brackets represent percentage of capsid type per nucleus.

observed which could possibly be due to the stability of a gene product (this is discussed in more details in section 3.4).

3.1.4 Analysis of viral DNA processing

Electron microscopic observation of thin section preparations of *ts*1233-infected cells suggested that *ts*1233 did not encapsidate DNA at the NPT. To confirm that the mutant DNA was not packaged into capsids at the NPT, and to determine whether the DNA was cleaved, processing of *ts*1233 DNA was investigated using the technique of Southern blotting (refer to Section 2.7).

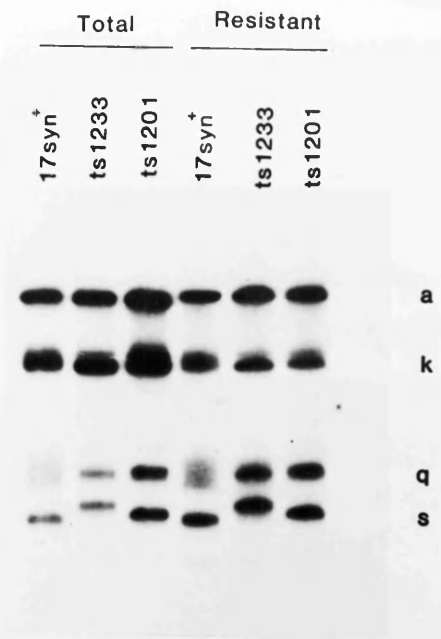
Total and encapsidated viral DNA from *ts*1233, *ts*1201, and *wt* virus-infected HFL cells were prepared as described in sections 2.3.2.a; 2.3.2.b. *Ts*1201 was included as a control in this study since this mutant fails to cleave and package DNA at the NPT. The viral DNAs were digested with BamHI which cleaves not only the unique regions but also the L and S repeats. The terminal fragments *q* and *s*, and the joint spanning fragment *k* were identified by hybridisation to [^{32}P]-labelled plasmid pGX22, which contains BamHI *k*. [^{32}P]-labelled pGX24, containing the HSV-1 U_L fragment BamHI *a*, was included in the hybridisation mix to determine the relative amount of total and encapsidated viral DNA.

The rationale for using BamHI *k* as a probe is that the presence of terminal fragments *q* and *s* gives an indication that cleavage of concatemeric DNA is occurring. Virion DNA, digested with BamHI, contains fragments *k*, *q*

and *s* in equimolar amounts since the genome is present in virus particles as a linear DNA molecule. Late in infection, however, in *wt* virus-infected cells most of the unpackaged DNA is present as high mw concatemers and, as a consequence, the proportion of BamHI *k* increases relative to terminal fragments *q* and *s*. In Southern blot analysis using the probe BamHI *k*, the intensity of BamHI *k* should be equal to the intensity of *q+s* bands in virion DNA.

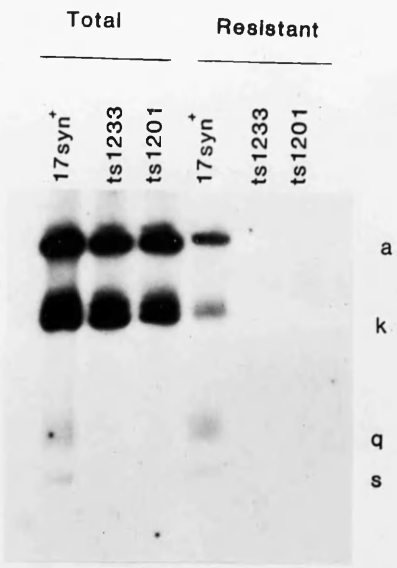
Figure 10a shows an autoradiograph of a Southern blot of BamHI digested total virus DNA and encapsidated DNA. The terminal *s* and *q* fragments were present in 17*syn*$^+$, *ts*1233, and *ts*1201 total virus-infected cell DNA prepared from cells grown at 31°, indicating that endless DNA was cleaved. Analysis of encapsidated DNA samples showed that cleaved DNA was packaged at 31° (figure 10a). Examination of total virus-infected cell samples prepared from cells grown at 39° revealed that the terminal fragments BamHI *s* and *q* were detectable in *wt* virus-infected cells only, suggesting that *ts*1233 and *ts*1201 DNA were endless (figure 10b), and were not cleaved to unit length DNAs. The failure to detect packaged DNA in mutant encapsidated DNA samples at 39° confirmed that *ts*1233 and *ts*1201 had an encapsidation defect at 39°. When virus-infected cells were transferred from 39° to 31° in the presence of cycloheximide at 12 hpi and incubated for a further 4h before being harvested, the terminal fragments were detected in both wt virus- and *ts*1201 total virus-infected cell DNA samples. The effect of *ts*1201 lesion was, however, only partially reversible upon downshift of

(A)



DNA analysis at 31°C

(B)



DNA analysis at 39°C

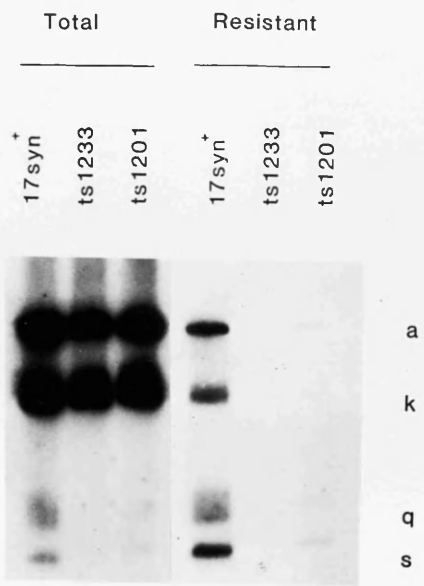
Figure 10

Autoradiograph of the processing of total and encapsidated (DNase-resistant) virus-infected cell DNA

Unlabelled DNA was prepared from HFL cells infected at a moi of 5 pfu/cell with *wt* virus, *ts*1233 or *ts*1201 as described in section 2.3.2.

Virus-infected cells were incubated at 31° (A), and 39° (B) and harvested at 18hpi. 1/10 of the samples from 50mm petri dish was cleaved with BamHI and the resulting fragments were separated by electrophoresis through 0.8% agarose gel. The fragments were transferred to nitocellulose sheets and hybridised to *in vitro* labelled [^{32}P]-pGX22 (containing BamHI *k*) and pGX24 (containing BamHI *a*).

(C)



DNA analysis upon shiftdown

39°C-31°C

Figure 10C

Autoradiograph of the processing of total and encapsidated (DNase-resistant) virus-infected cell DNA

Unlabelled DNA was prepared from HFL cells infected at a moi of 5 pfu/cell with *wt* virus, *ts*1233 or *ts*1201 as described in section 2.3.2.

Virus-infected cells were transferred from the NPT to the PT at 12hpi in the presence of cycloheximide and incubated for a further 4h prior to harvesting.

1/10 of the sample from 50mm petri dish was cleaved with BamHI and the resulting fragments were separated by electrophoresis through 0.8% agarose gel. The fragments were transfered to nitrocellulose sheets and hybridised to *in vitro* labled [^{32}P]-pGX22 (containing BamHI *k*) and pGX24 (containing BamHI *a*).

*ts*1201-infected cells from the NPT to PT (figure 10c) and only a low proportion of *q+s* fragments were detected in comparison with the *wt* virus *q+s* fragments. In *ts*1233 total infected cell DNA sample treated in the same manner no terminal fragments were detected suggesting that the DNA was not cleaved. This result was confirmed by the observation that no packaged *ts*1233 DNA could be detected in mutant-infected cells transferred from 39° to 31° (figure 10c) and supported the electron microscopic observation that the effect of *ts*1233 lesion could not be reversed by transfer of the sample from the NPT to PT. Hence, *ts*1233 has a block in the assembly of functional capsids.

3.1.5 Densitometric analysis of Southern blots of total virus-infected cell DNA and encapsidated DNA

The relative amount of viral DNA packaged was determined by analysis of autoradiographs of Southern blots of total virus DNA and encapsidated DNA. The amount of the U_L fragment BamHI *a* hybridised to total virus infected cell DNA was compared to the amount hybridised to encapsidated DNA. The intensity of BamHI *k* , *q* and *s* was not determined because quantitation of these bands probably gives a less reliable estimate of packaging. Plasmids which do not contain the *a* sequence but have ori$_S$ are able to form concatemers in the presence of helper virus. This result raises the possibility that the ends of the uncleaved viral DNA concatemers in mutant virus-infected cells are different from cleaved unit length DNA in *wt* virus-infected cells.

Results of the densitometric analysis revealed that at 31° *wt* virus packaged DNA very efficiently. More than 50% of the viral DNA synthesized had been encapsidated by 12 hpi. At this temperature *ts*1233 and *ts*1201 also packaged DNA efficiently (see table 5a). At the NPT of 39°, about 8% of *wt* virus DNA was encapsidated (table 5b). It is clear from this result that at 39° packaging of *wt* virus DNA is much less efficient than at 31°, indicating that there is a thermolabile step in this process. This conclusion is supported by the finding that the amount of *wt* virus DNA packaged increased when the sample was transferred from 39° to 31° in the presence of cycloheximide. Less than 1% of *ts*1233 and *ts*1201 was encapsidated at the NPT. This result suggests that these mutants have a packaging defect at this temperature. Results of the analysis of shiftdown experiment showed that the effect of *ts*1201 mutation was not fully reversible upon downshift from 39° to 31° in the presence of cycloheximide. Only about 4% of *ts*1201 DNA was encapsidated (table 5c). This figure is lower than the value determined elsewhere (Addison, 1986) possibly because a high NPT of 39° was used in this experiment whereas 38.5° had been used in the previous work on this mutant. Less than 1% of *ts*1233 DNA was packaged after transfer of mutant-infected cells from 39° to 31°.

3.1.6 Ts1233 defines a new complementation group

To determine whether *ts*1233 belonged to an existing complementation group or not, complementation analysis was

TABLE 5

Densitometric analysis of total and encapsidated virus-infected cell DNA

A

PT 31°	17*syn*+	*ts*1233	*ts*1201
Total DNA synthesised (%)	100.00 100.00	121.69 182.00	189.08 166.00
Encapsidated DNA (%)	57.50 50.14	61.00 22.60	32.80 43.96

B

NPT 39°	17*syn*+	*ts*1233	*ts*1201
Total DNA synthesised (%)	100.00 100.00	65.35 50.90	50.00 75.66
Encapsidated DNA (%)	7.65 8.59	<1.00 <1.00	<1.00 <1.00

C

NPT-PT 39°-31° *	17*syn*+	*ts*1233	*ts*1201
Total DNA (%)	100.00	61.69	80.05
Encapsidated (%)	17.95	<1.00	4.12

(*) cycloheximide treated

Table 5

Densitometric analysis of Southern blot of total and encapsidated virus DNA. The amount of DNA synthesised or encapsidated by each virus represented by the amount of [^{32}P] labelled probe hybridising to the BamHI *a* fragment.

Total virus DNA is represented as a percentage of the DNA synthesised by *wt* 17*syn*$^+$ virus at the PT (A), or NPT (B), or following a shift from the NPT to the PT in the presence of cycloheximide (*). In each case synthesis of 17*syn*$^+$ is taken as 100%.

Encapsidated DNA is presented as a percentage of the total virus-infected cell DNA synthesised at the PT (A), NPT (B) or following a shift from the NPT to the PT in the presence of cycloheximide (*). The figures in A and B represent two sets of experiments.

carried out. Since *ts* mutants belonging to complementation group 1-13 had defects which mapped close to the *ts*1233 mutation, a member, *ts*N20, from this group was used in the complementation yield test. The TsN20 lesion maps within a 500bp SalI-EcoRI fragment at the right hand end of EcoRI *m* (Coen *et al.*, 1984). Another mutant in this cistron *ts*W44 has a mutation within EcoRI *o* (Pancake *et al.*, 1983). Originally these mutants were placed in two different complementation groups (Pancake *et al.*, 1983), but recently they have been assigned to a single cistron c(1-13) (Schaffer *et al.*, 1987). *Ts*N20 made large numbers of aberrant, partially-cored and empty capsids as well as some dense-cored capsids and enveloped virus particles at 39° (Schaffer *et al.*, 1974). From this electron microscopic analysis, its phenotype appeared to be very similar to that of glycoprotein B mutant *ts*J12. *Ts*N20 was chosen for complementation analysis with *ts*1233 because this mutant was readily available at the Institute. *Ts*1201, which has a mutation in UL26 and like *ts*1233 failed to package DNA at the NPT (Preston *et al.*, 1983), was used as a control. The results of two independent experiments (Table 6) revealed that *ts*1233 complemented both *ts*N20 and *ts*1201, suggesting that *ts*1233 belongs to a different complementation group from that of *ts*N20 and *ts*1201.

3.1.7 Analysis of *ts*1233-infected cell polypeptide

Polypeptides synthesized by *ts*1233 were analysed by SDS PAGE to determine whether the mutant polypeptide profile contained any polypeptide bands which had an

TABLE 6

Complementation test between *ts*1233 and two mutants with packaging defects at the NPT

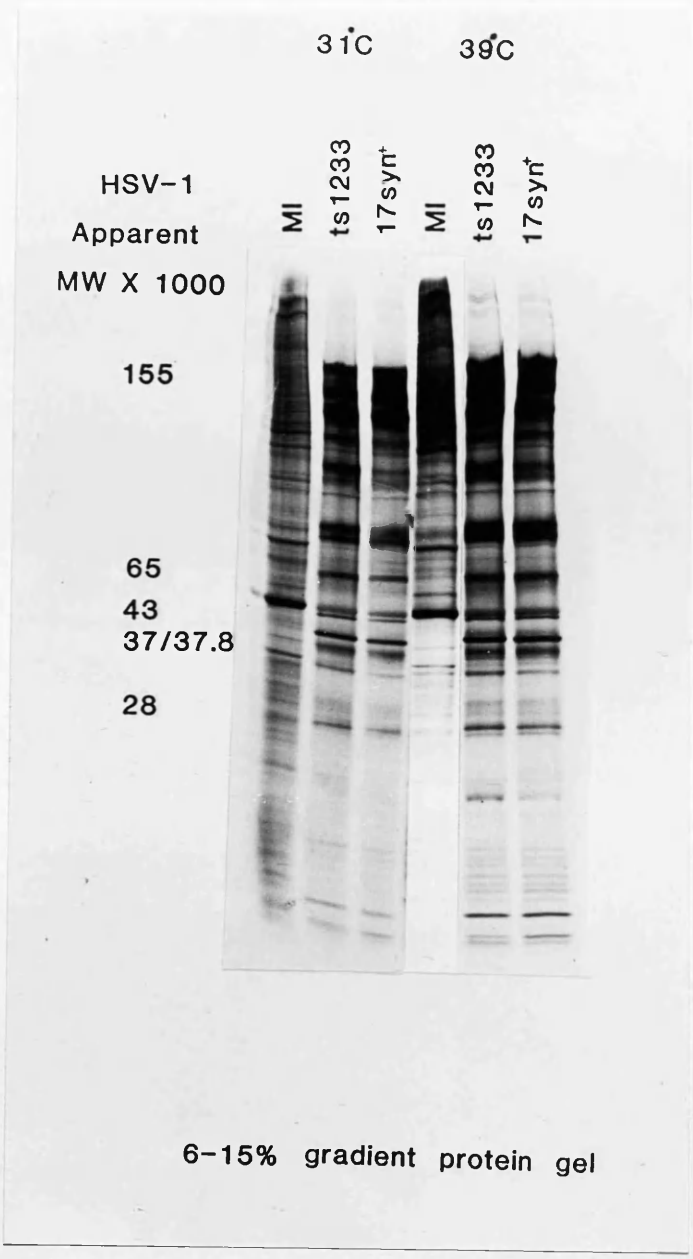
Mutant	*ts*N20	*ts*1201	*ts*1233
***ts*N20**	1 1	12.06 35.90	12.95 43.20
***ts*1201**		1 1	71.85 211.00
***ts*1233**			1 1

Complementation yield test was performed between *ts*1233, *ts*1201, *ts*N20. The values in the table represent complementation indices of two separate experiments calculated from the formula used by Brown *et al.* (1973). Values greater than four were considered positive.

altered mobility, or intensity in comparison with *wt* virus polypeptide bands. Virus and mock-infected cells were grown at the NPT or PT, labelled with [^{35}S]-methionine for 30 min at 5 hpi and the proteins separated by SDS-PAGE. Autoradiographs of polypeptides revealed that *ts*1233 had a similar polypeptide pattern to that of wt virus both at the PT and the NPT when the polypeptides were separated on single concentration (data not shown) or gradient polyacrylamide SDS gels (figure 11).

3.1.8 Processing of p40 in *ts*1233 infected cells

The inability of *ts*1201 to package DNA into capsids at the NPT correlates with the failure of the mutant to process p40 (gene product of UL26) to its lower mw forms. Since it is not known if other proteins are required for modification of p40, processing of p40 in infected cells was examined to determine whether the lesion in *ts*1233 affected any of the processing steps of p40. *Wt* virus-, *ts*1233- and *ts*1201-infected cells, together with mock-infected cells were pulse-labelled with [^{35}S]-methionine for 20 min at 5 hpi and samples either harvested immediately or incubated for a further 5h in the absence of [^{35}S]-methionine prior to harvesting. P40 in virus-infected cell extracts was immunoprecipitated with a p40 specific monoclonal antibody, 5010B, and the immunoprecipitates analysed by SDS-PAGE. At the PT at least four virus-infected polypeptide bands were detected in *wt* virus-, *ts*1201- and *ts*1233-infected cell samples pulse labelled with [^{35}S]-methionine. After a 5h chase,



6-15% gradient protein gel

Figure 11

Autoradiograph of polypeptides induced at 31° and 39° in HFL cells infected with *wt* HSV-1 or *ts*1233 or in mock infected cells. Virus-infected cells were labelled with [^{35}S]-methionine for 30min at 6hpi and harvested. Protein samples were analysed on a 6-15% gradient SDS PAGE. Numbers represent apparant mw.

two lower mw p40 specific bands appeared, indicating that p40 was processed normally at the PT in all three virus-infected cell samples (figure 12). At the NPT only *ts*1201 failed to process p40 to the lower mw forms after 5h chase. Both *wt* and *ts*1233 processed p40 normally. The results show that the mutation in *ts*1233 did not affect the processing of p40 at the NPT (figure 13).

3.1.9 Isolation of the *ts*$^+$ revertants for growth of *ts*1233

Two independent revertants for growth of *ts*1233, *ts*1233 rev1 and rev2, were isolated and high titre virus stocks were prepared. The revertants were titrated both at the PT and the NPT, together with the wt virus and a *ts*$^+$ virus isolated by marker rescue of *ts*1233 with cloned wt EcoRI *o* (table 7). The eop NPT/PT of rev1, rev2, the *ts*$^+$ marker rescued virus were similar to that of *wt* 17*syn*$^+$ virus. This information strongly suggests that *ts*1233 has a single *ts* mutation.

3.1.10 Map location of the *ts*1233 lesion

Preliminary marker rescue studies localised the *ts*1233 mutation to the region shared by EcoRI *o* and BamHI *b'* (I.McDougall, unpublished results). Separated XbaI restriction endonuclease fragments from cloned *wt* HSV-1 17*syn*$^+$ EcoRI *o* fragment derived from the plasmid pGX74 were used in marker rescue experiments to map the *ts*1233 lesion to a smaller region of the genome (table 8). The results showed that the lesion mapped within a 941 bp fragment at the end of XbaI *f*. To further refine the map

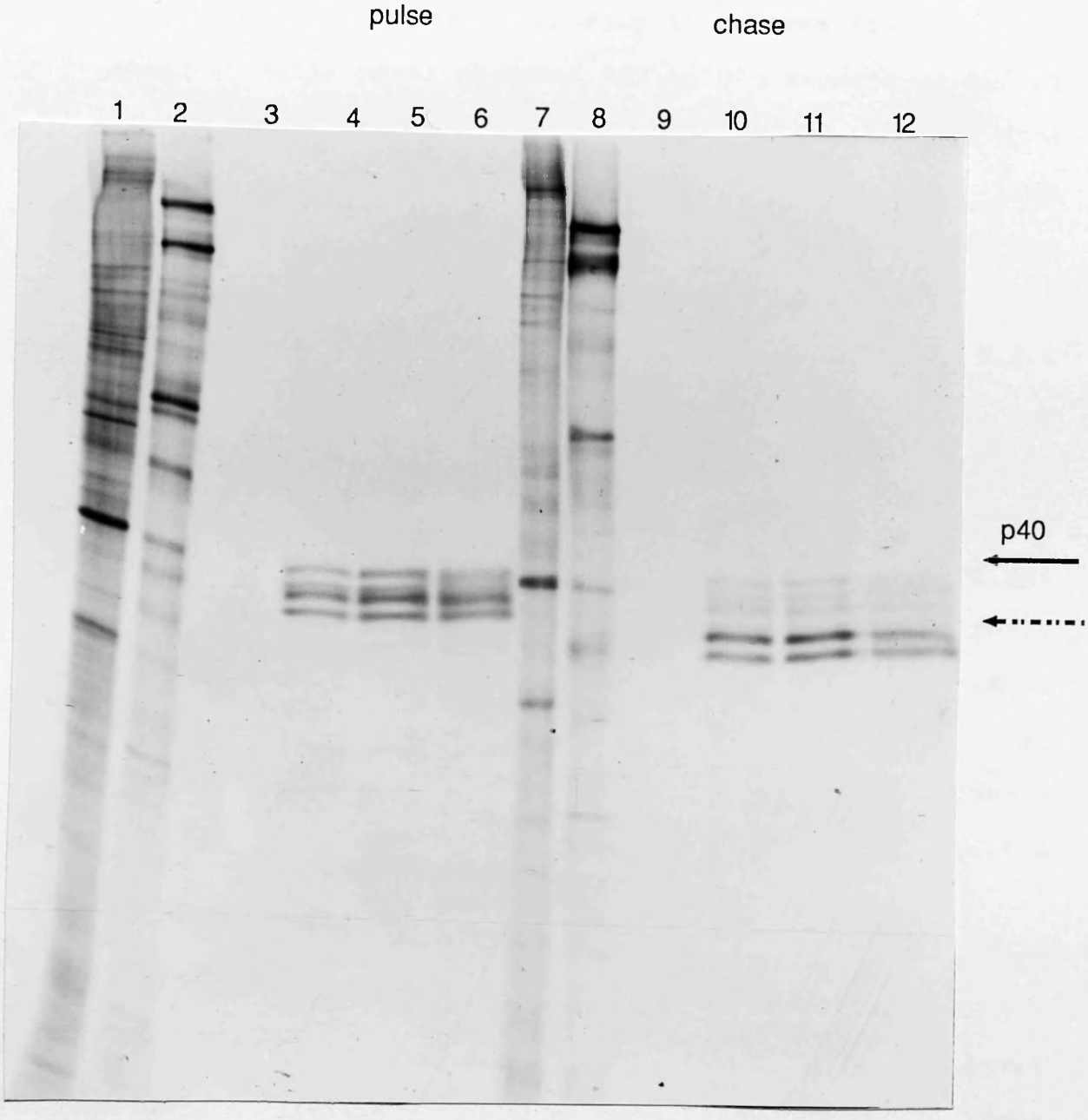

pulse
chase
1
2
3
4
5
6
7
8
9
10
11
12
p40

Figure 12

Autoradigraph of immuneprecipitates of p40 separated by SDS PAGE. HFL cells were either mock-infected or infected with *wt* virus, *ts*1201 or *ts*1233 at 31°. At 6 hpi, cells were pulsed-labelled with [^{35}S]-methionine for 30 min and harvested either immeadiately (pulse) or after incubation for a further 5h at 31° in EFC_{10} (chase). Immunoprecipitation was performed as described in section 2.11.2 using a ma5010B, which is specific for p40. As a control, *wt* virus-infected cell extract was incubated with non-immune rabbit serum instead of ma5010B (track 3 and 9). Tracks 1 and 7 represent moc infected cell extracts, tracks 2 and 8 represent *wt* virus-infected cell extracts, tracks 3, 6, 9, 12, represent immune precipitates from *wt* virus-infected cells, tracks 4 and 10, immune precipitates from *ts*1201-infected cells, tracks 5 and 11, immune precipitates from *ts*1233 infected cells. The immune precipitated polypeptides were analysed on an 8% SDS polyacrylamide gel.

Solid arrows represent high mw forms of p40.

Dotted arrows " low " " " " .

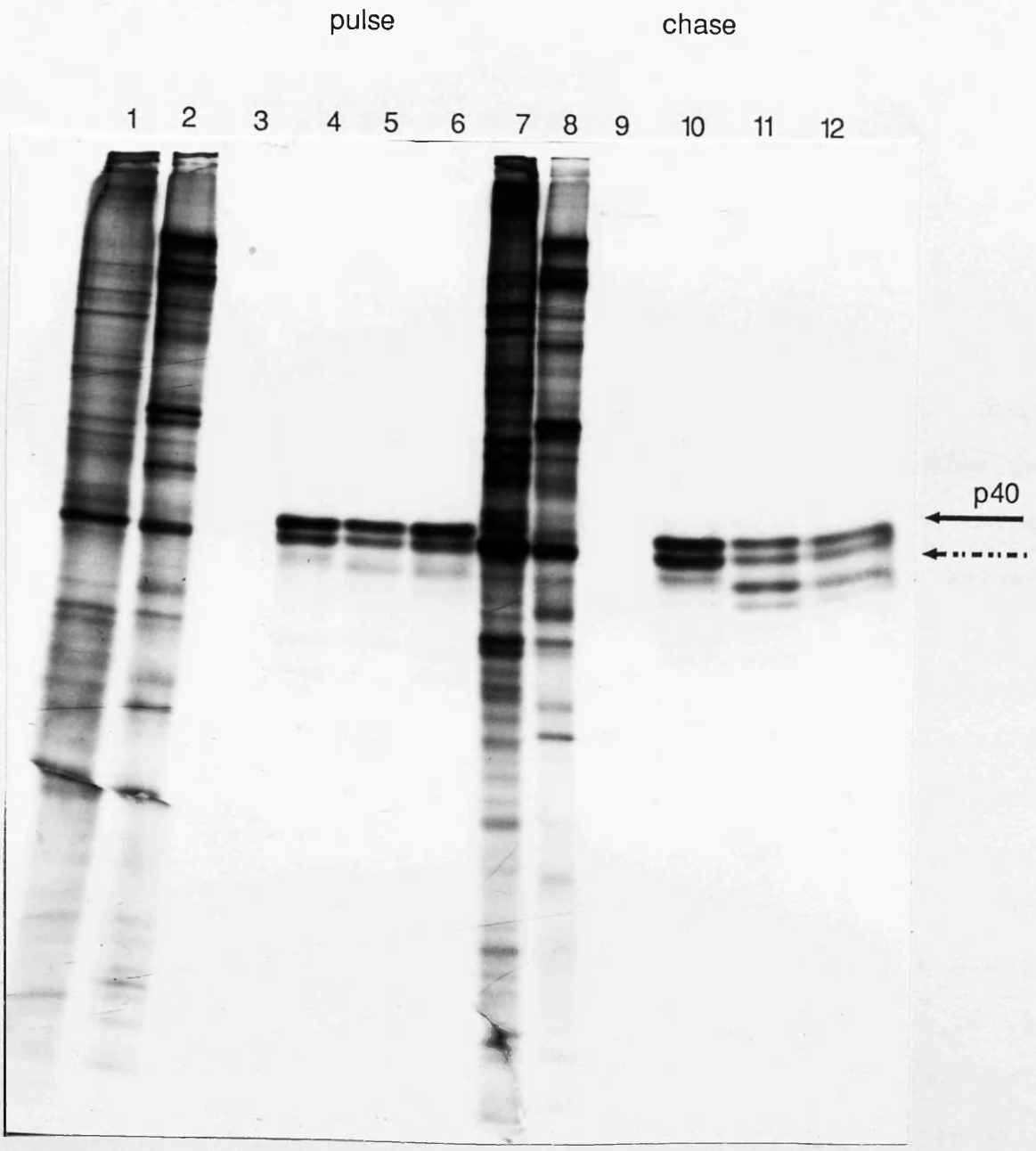

pulse
chase
1 2 3 4 5 6 7 8 9 10 11 12
p40

Figure 13

Autoradigraph of immuneprecipitates of p40 separated by SDS PAGE. HFL cells were either mock-infected or infected with *wt* virus, *ts*1201 or *ts*1233 at 39°. At 6 hpi, cells were pulsed-labelled with [^{35}S]-methionine for 30 min and harvested either immeadiately (pulse) or after incubation for a further 5h at 39° in EFC_{10} (chase). Immunoprecipitation was performed as described in section 2.11.2 using a ma5010B, which is specific for p40. As a control, *wt* virus-infected cell extract was incubated with non-immune rabbit serum instead of ma5010B (track 3 and 9). Tracks 1 and 7 represent mock-infected cell extracts, tracks 2 and 8 represent *wt* virus-infected cell extracts, tracks 3, 6, 9, 12, represent immune precipitates from *wt* virus-infected cells, tracks 4 and 10, immune precipitates from *ts*1201-infected cells, tracks 5 and 11, immune precipitates from *ts*1233 infected cells. The immune precipitated polypeptides were analysed on an 8% SDS polyacrylamide gel.

Solid arrows represent high mw forms of p40.

Dotted arrows " low " " " " .

Table 7

Eop(NPT/PT) of *ts*1233 rev1 and rev2

Virus	EOP(NPT/PT)
17*syn*$^+$	0.98
*Ts*1233 rev1	1.00
*Ts*1233 rev2	0.84
Ts$^+$ virus isolated by marker rescue	1.00

NPT is 39°
PT is 31°

TABLE 8

Summary of marker rescue of *ts*1233

Fragments obtained from pGX74 (HSV-1 EcoRI *o*)	Efficiency of plating NPT/PT X10³	Rescue
pGX74 digested with EcoRI	2.66	+
pGX74 digested with XbaI		
Unseparated XbaI frags.	1.73	+
Separated XbaI frags.*/ contains part of XbaI *f*	1.64	+
" " " XbaI *c*	<0.02	-
pGX74 digested with PvuII		
Unseparated PvuII frags.	0.22	+
Separated PvuII frags.*/ contains part of PvuII *k'*	0.16	+
" " " PvuII *o*	<0.02	-
" " " PvuII *o'*	<0.02	-
None	<0.01	-

(*) XbaI digest gives two fragments each containing part of the vector sequences. Similarly with a PvuII digest.

location of the mutation, separated PvuII fragments were used in marker rescue experiments. A low level of *ts*$^+$ virus progeny was obtained with a 602 bp PvuII fragment (containing part of the PvuII *k'* fragment). The lesion of *ts*1233, therefore, lies within a 152 bp region common to XbaI *f* and PvuII *k'* (figure 14) between map coordinate 0.45-0.46 mu.

3.1.11 Cloning EcoRI *o* of *ts*1233, *ts*1233 rev1 and rev2

Marker rescue experiments showed that the *ts*1233 mutation mapped within a 152bp region common to PvuII *k'* and XbaI *f*. The 5' ends of two genes, UL32 and UL33, oriented in opposite directions, lie in this part of the genome (McGeoch *et al.*, 1988). To determine which gene the *ts*1233 lesion mapped in, the sequence alteration responsible for the *ts*1233 mutation was determined. The first step in this analysis was to clone the fragment containing the *ts* mutation and the corresponding fragments from *ts*1233 rev1 and rev2. EcoRI *o* fragments from an EcoRI digest of *ts*1233, *ts*1233 rev1 and rev2 DNA were cloned into the EcoRI site in pUC8 (figure 15). Two independent chimeric plasmids, pGX200 and pGX201, containing EcoRI *o* fragment of *ts*1233, and two chimeric plasmids pGX203 and pGX204 containing EcoRI *o* of *ts*1233 rev1 and rev2 respectively, were constructed. All the plasmid inserts appeared to have identical EcoRI, BamHI, XbaI and PvuII sites to those present in *wt* HSV DNA sequence (data not shown). Plasmids were digested with BamHI and XbaI and tested for their ability to rescue the mutation of *ts*1233

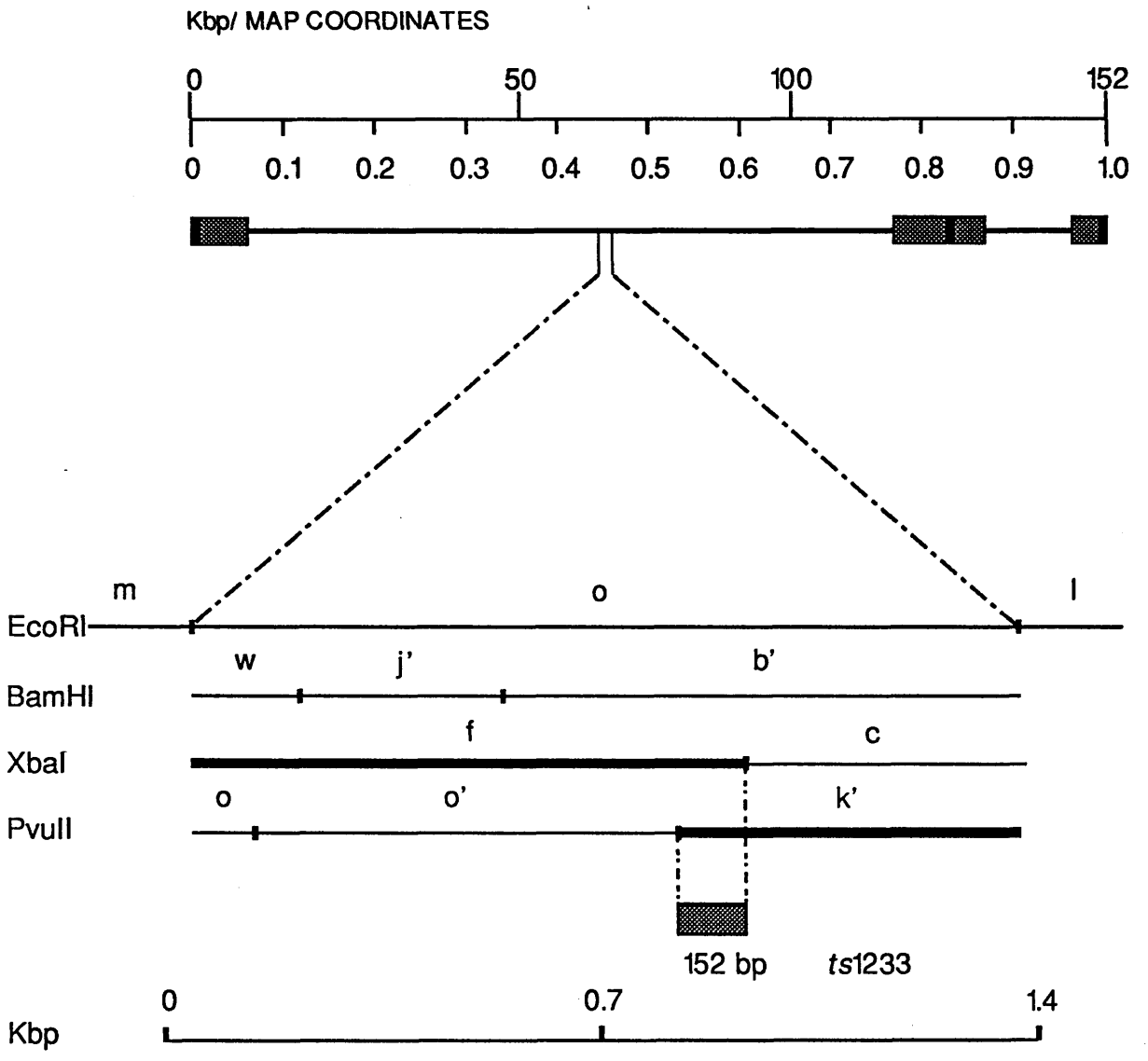
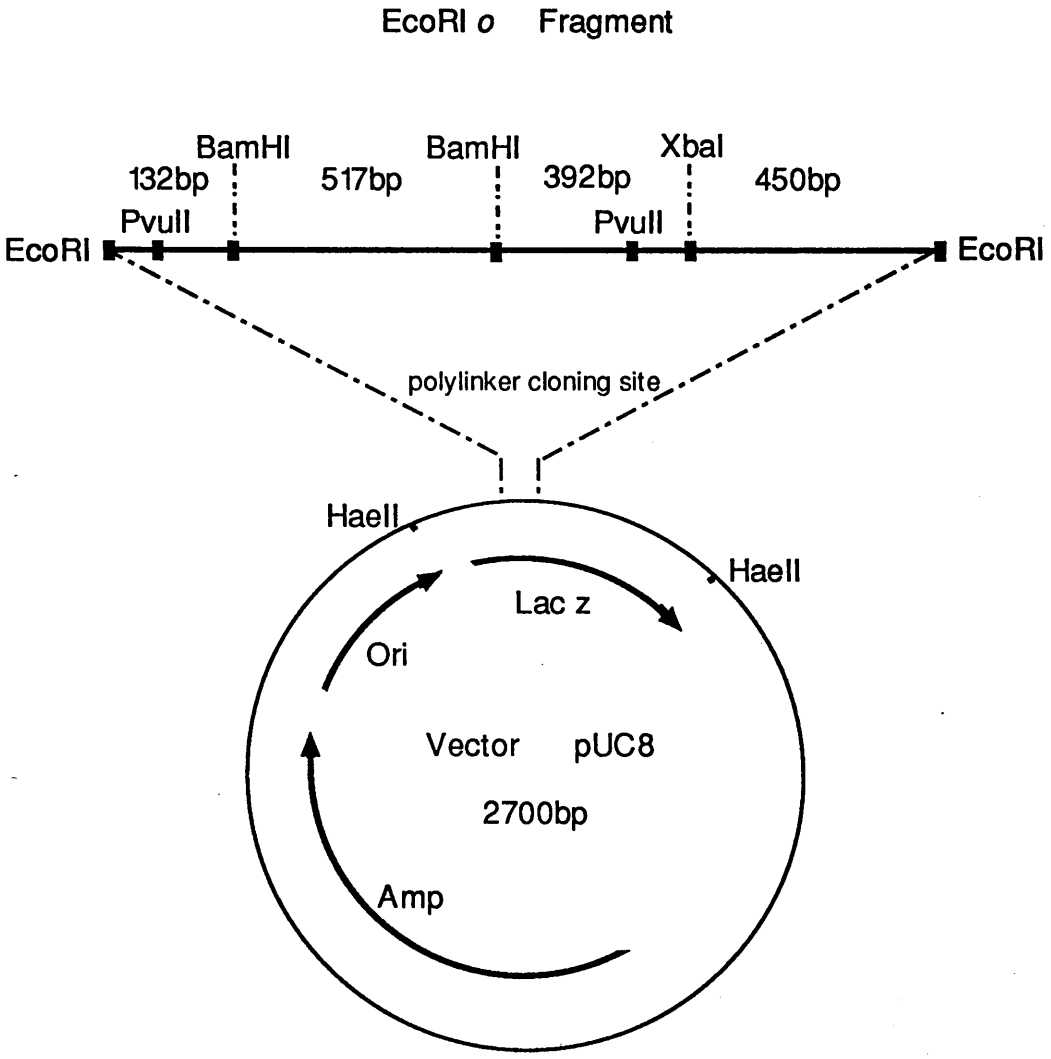

Kbp/ MAP COORDINATES
0
50
100
152
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
m
o
l
EcoRI
w
j'
b'
BamHI
f
c
XbaI
o
o'
k'
PvuII
152 bp
ts1233
0
0.7
1.4
Kbp

Figure 14

Map location of the *ts*1233 mutation.

The EcoRI, BamHI, XbaI and PvuII cleavage sites in the region where *ts*1233 lesion lies are given. The solid XbaI and PvuII lines represent the fragments which rescued the *ts*1233 mutation. The 152bp cross hatched box represents the region common to XbaI *f* and PvuII *k'*.



pGX200 *ts*1233

pGX201 *ts*1233

pGX203 Rev 1

pGX204 Rev 2

Figure 15

A diagram representing the EcoRI *o* fragment taken from *ts*1233, *ts*1233 rev1,or *ts*1233 rev2 and cloned into the EcoRI site of pUC8 polylinker. Numbers on top of the fragment represent the size of the four fragments obtained from a BamHI-XbaI double digest. The 392bp fragment was used in sequencing analysis.

DNA in a marker rescue experiment (table 9). The two mutant clones, pGX200 and pGX201 failed to rescue *ts*1233 lesion, whereas pGX203 and pGX204 both gave positive values in the marker rescue test. These results confirmed that the correct fragment had been cloned in each case.

3.2 GENE UL33 IS REQUIRED FOR DNA ENCAPSIDATION

The first part of this section deals with the identification of the gene responsible for the defect in *ts*1233, while the subsequent parts of the section deal with attempts to identify the gene product of this gene.

3.2.1 *Ts*1233 lesion maps within UL33

The 392bp fragment common to XbaI *f* and BamHI *b'* in each of the following plasmids pGX200, pGX203 and pGX204 was ligated into M13mp18 and M13mp19 DNA cleaved with XbaI-BamHI. These two bacteriophage DNAs have the polylinker cloning sequence in opposite orientations to one another. The ligated DNA was transfected into competent JM101 bacteria and bacteriophage forming white plaques were isolated. Bacteriophage ssDNA was prepared and sequenced using the chain termination method of Sanger. The nucleotide sequence of the *ts*1233 BamHI-XbaI cloned fragment was identical to the sequence in the corresponding cloned fragment from *wt* virus except for a single bp change at residue 69210 where a transversion from an A to a T had occurred (figure 16 refer to *ts*1233 A and T tracks). Sequence analysis of the BamHI-XbaI from the two *ts*$^+$ revertant clones revealed that these DNAs each had an

TABLE 9

Marker rescue of *ts*1233 using pGX74, pGX200, pGX201, pGX203, pGX204

Clones /Fragment used	Efficiency of plating NPT/PT X 10^3	Rescue
pGX74 digested with BamHI-XbaI		
Unseparated fragments	0.20	+
Separated frag obtained from a BamHI-XbaI dig.		
392bp frag.	0.30	+
517bp "	<0.02	-
1874bp "	<0.02	-
2505bp "	<0.02	-
pGX200 digested with BamHI-XbaI Unseparated fragments	<0.02	-
pGX201 digested with BamHI-XbaI Unseparated fragments	<0.02	-
pGX203 digested with BamHI-XbaI		
Unseparted fragments	0.35	+
Separated frag obtained from a BamHI-XbaI dig.		
392bp frag.	0.20	+
460bp "	<0.02	-
517bp "	<0.02	-
2822bp "	<0.02	-
pGX204 digested with BamHI-XbaI		
Unseparetd fragments	0.20	+
Separated frag obtained from a BamHI-XbaI dig.		
142bp frag.	<0.02	-
392bp "	0.12	+
517bp "	<0.02	-
3140bp "	<0.02	-
None	<0.02	-

Table 9

Marker rescue of *ts*1233 using pGX74, pGX200, pGX201, pGX203, pGX204

					Vector
pGX74	contain	HSV-1 17*syn*+	EcoRI	*o* fragment.	pACYC184
pGX200	"	*ts*1233	"	"	. pUC8
pGX201	"	*ts*1233	"	"	. "
pGX203	"	*ts*1233 rev1	"	"	. "
pGX204	"	*ts*1233 rev2	"	"	. "

The insert in pGX204 is in opposite orientation. When the clones are digested with BamHI-XbaI, four fragments are obtained which contain the vector sequences attached to the 450bp fragment and 132bp fragment. For the sizes of the fragment refer to figure 15.

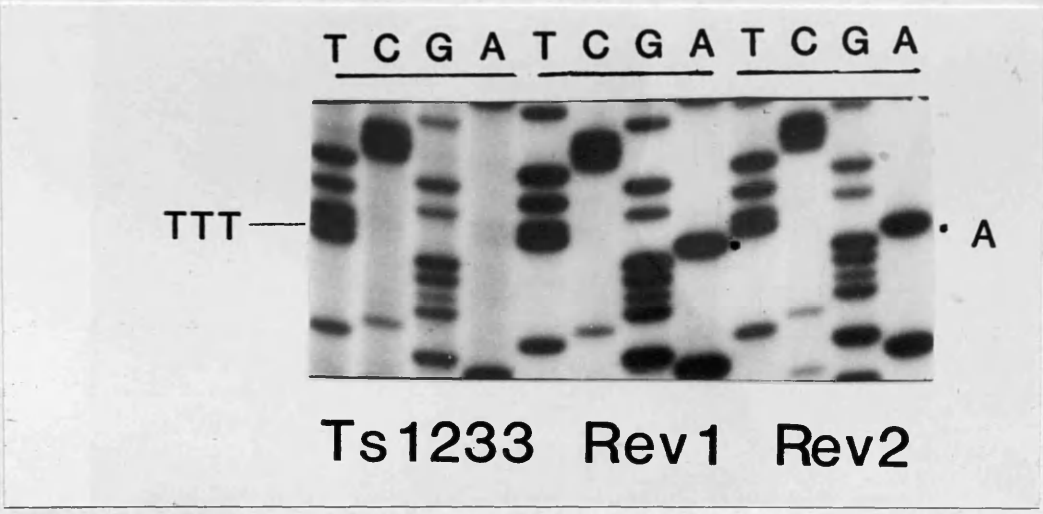

T C G A T C G A T C G A
TTT
A
Ts1233 Rev1 Rev2

Figure 16

A portion of the autoradiograph of a DNA sequencing gel. Sequence products were separated on a 6% denaturing polyacrylamide gel containing 9M urea. The base pair change in *ts*1233 is indicated by TTT (thymine), whereas both the revertants shows the presence of adenine (A track).

identical sequence to that of wt virus DNA (Figure 16 refer to *ts*1233 rev1 and rev2 tracks A and T).

As mentioned previously, within the 152bp region lie the 5' ends of two genes transcribed in opposite directions. One of the genes, UL32, encodes a 65,000 mw polypeptide, and the other, UL33, encodes a 14,000 mw polypeptide. DNA sequence analysis of 17*syn*$^+$ virus (figure 17) has shown that residue no. 69210 lies 50 bp downstream from the ATG initiation codon of gene UL33 (McGeoch *et al.*, 1988), and therefore *ts*1233 lesion must lie in gene UL33 (figure 18).

3.2.2 Production of polyclonal antibodies against UL33 gene product

DNA sequence and electron microscopy analyses showed that *ts*1233 contained a *ts* mutation in UL33 which affected virus assembly at the NPT. The next step was to identify the gene product of UL33 to obtain further information about the function and role of this protein. Antisera raised against synthetic oligopeptides, representing small portions of the amino acid sequence of a protein, have been extremely useful in identifying the product of a gene whose DNA sequence is available (Palfreyman *et al.*, 1984; Frame *et al.*, 1986; McLean *et al.*, 1987; Parris *et al.*, 1988). It was therefore decided to make antisera using two synthetic oligopeptides, one of which was directed towards the amino terminus and the other of which was specific to the carboxy terminus. The two oligopeptides were chosen because of the high success rate

Translation of: UL33

```
M  A  G  R  E  G  R  T  R  Q  R  T  L  R  D  T  I  P  D      19
                                  *
ATG GCT GGG CGG GAG GGG CGC ACG CGC CAG CGA ACT TTA CGG GAC ACA ATC CCC GAC  69217

 C  A  L  R  S  Q  T  L  E  S  L  D  A  R  Y  V  S  R  D      38
TGC GCG CTG CGG TCC CAG ACC CTG GAG AGT CTA GAC GCG CGC TAC GTC TCG CGA GAC  69274

 G  A  H  D  A  A  V  W  F  E  D  M  T  P  A  E  L  E  V      57
GGC GCG CAT GAC GCG GCC GTC TGG TTC GAG GAT ATG ACC CCC GCC GAG CTG GAG GTT  69331

 V  F  P  T  T  D  A  K  L  N  Y  L  S  R  T  Q  R  L  A      76
GTC TTC CCG ACT ACG GAC GCC AAG CTG AAC TAC CTG TCG CGG ACG CAG CGG CTG GCC  69388

 S  L  L  T  Y  A  G  P  I  K  A  P  D  D  A  A  A  P  Q      95
TCC CTC CTG ACG TAC GCC GGG CCT ATA AAA GCG CCC GAC GAC GCC GCC GCC CCG CAG  69445

 T  P  D  T  A  C  V  H  G  E  L  L  A  A  K  R  E  R  F     114
ACC CCG GAC ACC GCG TGT GTG CAC GGC GAG CTG CTC GCC GCC AAG CGG GAA AGA TTC  69502

 A  A  V  I  N  R  F  L  D  L  H  Q  I  L  R  G  -           130
GCG GCG GTC ATT AAC CGG TTC CTG GAC CTG CAC CAG ATT CTG CGG GGC TGA          69553
```

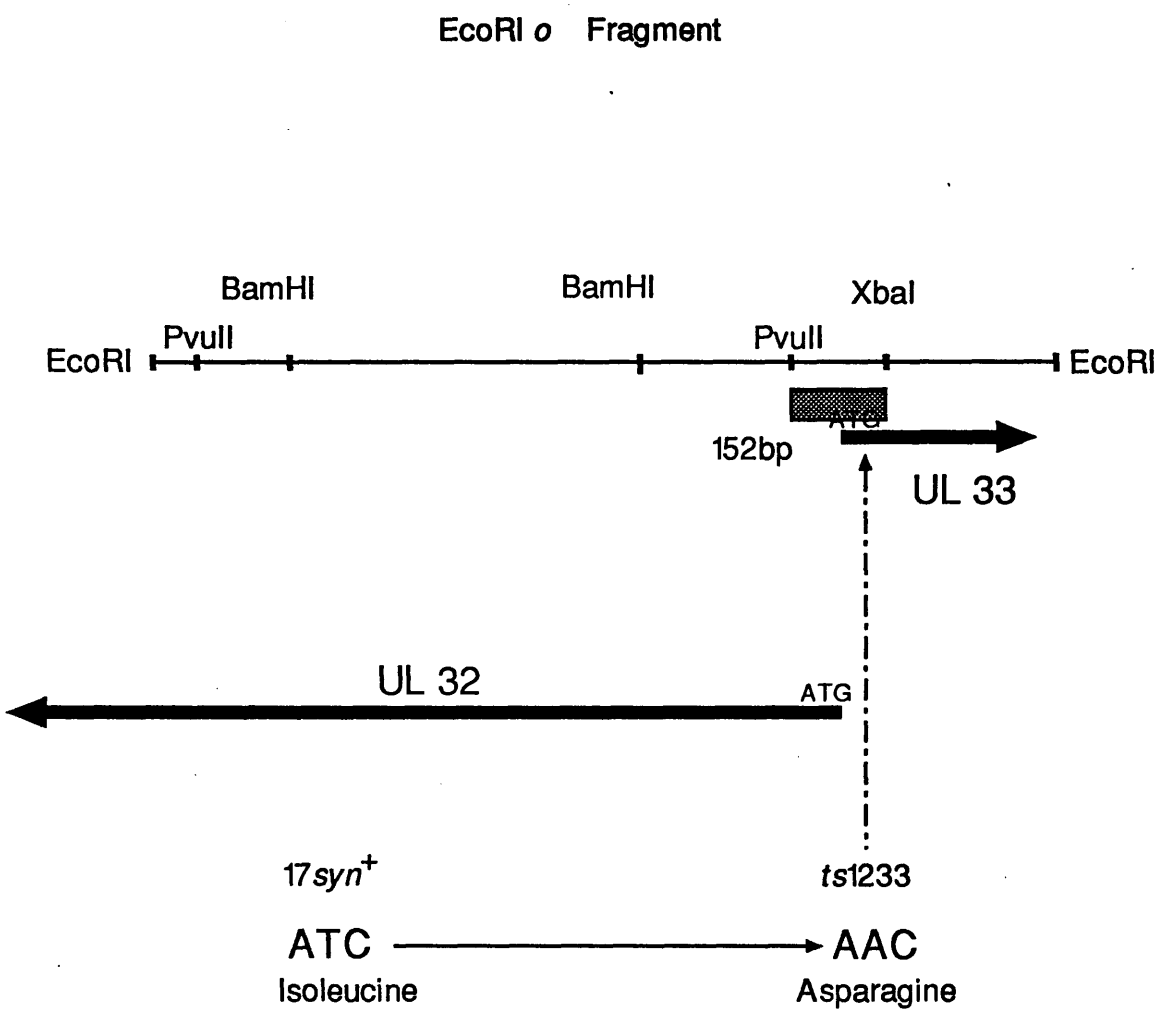
----- AMINO ACID COMPOSITION -----

Ala	19	14.6%	Val	6	4.6%	Leu	15	11.5%	Ile	4	3.1%
Gly	6	4.6%	Pro	7	5.4%	Cys	2	1.5%	Met	2	1.5%
His	3	2.3%	Phe	4	3.1%	Tyr	3	2.3%	Trp	1	0.8%
Asn	2	1.5%	Gln	5	3.8%	Ser	5	3.8%	Thr	11	8.5%
Lys	3	2.3%	Arg	14	10.8%	Asp	11	8.5%	Glu	7	5.4%

Approximate Molecular Weight = 14436.49

Figure 17

The EcoRI *o* fragment showing 152bp region were the 5' end of two genes map. HSV-1 17*syn*$^+$ sequence is compared to *ts*1233 sequence. Bold letters represent the thymidine nucleotides observed in the sequencing results. The underlined region represents the change in the amino acid sequence from an isoleucine to asparagine. The crossed-hatched region is where the *ts*1233 mutation was located by marker rescue.



HSV-1 17*syn*$^+$ seq. 5' TCGGGACACAATCCCCGACTGC 3'
AGCCCTGTGTTAGGGGCTGACG

*TS*1233 seq. 5' TCGGGACACAAACCCCGACTGC 3'
AGCCCTGTGTTTGGGGCTGACG

Figure 18

Translation of gene UL33 (McGeoch *et al.*, 1988). The diagram shows the nucleotide and amino acid sequence of this gene and its predicted mw. The (*) at residue 69210 represents the position of the mutation in *ts*1233. The boxes indicate the amino acid sequence of the two peptides synthesised.

Tyrosin was added to the 3'end and 5'end of the first and second oligopeptide respectively to facilitate coupling to carrier protein.

with antisera raised against oligopeptides specific to the ends of the protein. Antisera often contained antibodies which cross reacted with the intact protein and the regions contained amino acid sequence that were able to be synthesised easily on the oligopeptide synthesiser (H. Marsden, personal communication). The first peptide represented the first 11 amino-acids from the amino-terminus of gene UL33, and the other represented the last 11 amino-acids from the carboxy-terminus of gene UL33 (Figure 18). The oligopeptides were coupled to either BSA or β-galactosidase, and injected into rabbits (table 10) (also refer to section 2.12.1).

Antibodies made against BSA were removed by passing the sera through a column containing BSA linked to CNBr activated Sepharose 4B (Section 2.12.2, 2.12.4). A radio-immunoassay was performed prior to and after BSA antibody isolation to check that BSA antibodies had been removed (data not shown).

Figures 19a, and 19b represents results of the radio-immunoassays of serum from animals injected with the carboxy-terminal peptide coupled to β-galactosidase. Antisera from post-immune bleeds reacted to the antigen (peptide) coating the microtitre wells and gave high [^{125}I] counts in comparison to preimmune serum, suggesting that the antisera raised against the peptides were recognising the antigen. Similar results were obtained with both peptides coupled to BSA from which BSA antibodies had been removed (data not shown).

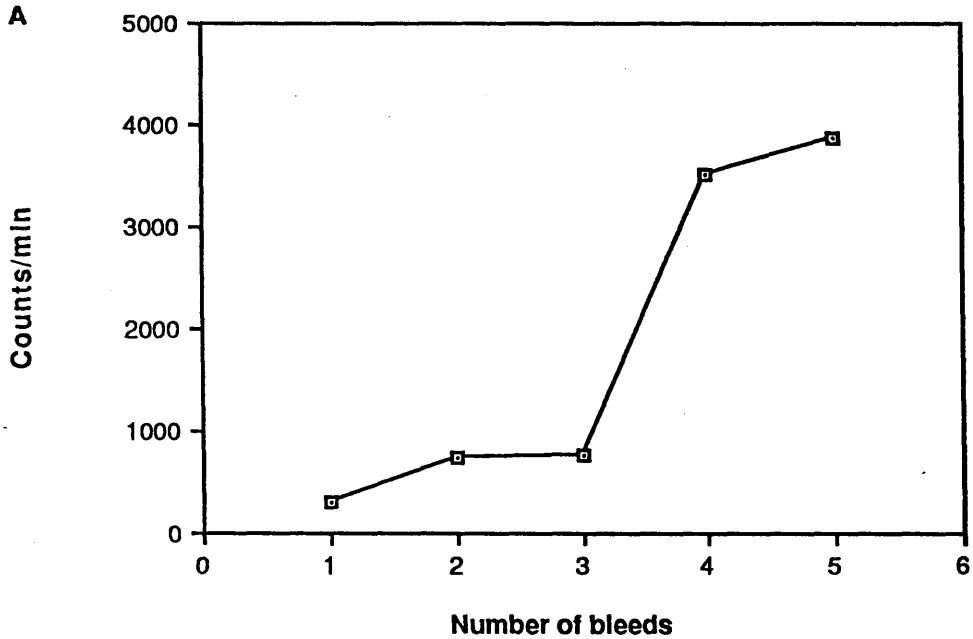
TABLE 10

The injection protocol of UL33 peptides coupled to β-galactosidase

Peptide code	Peptide representing	injections					peptide/ml
		1	2	3	4	5	
20988	The first 11 A.A from the NH_2-terminus	*	/				150µg
		*	*	*	/		"
20994	The last 11 A.A from the COOH-terminus	*	*	*	*	*	"
		*	*	*	*	*	"

/ : no injections were given due to the death of animals
* : injection

A


5000
4000
3000
2000
1000
0
Counts/min
0
1
2
3
4
5
6
Number of bleeds

B

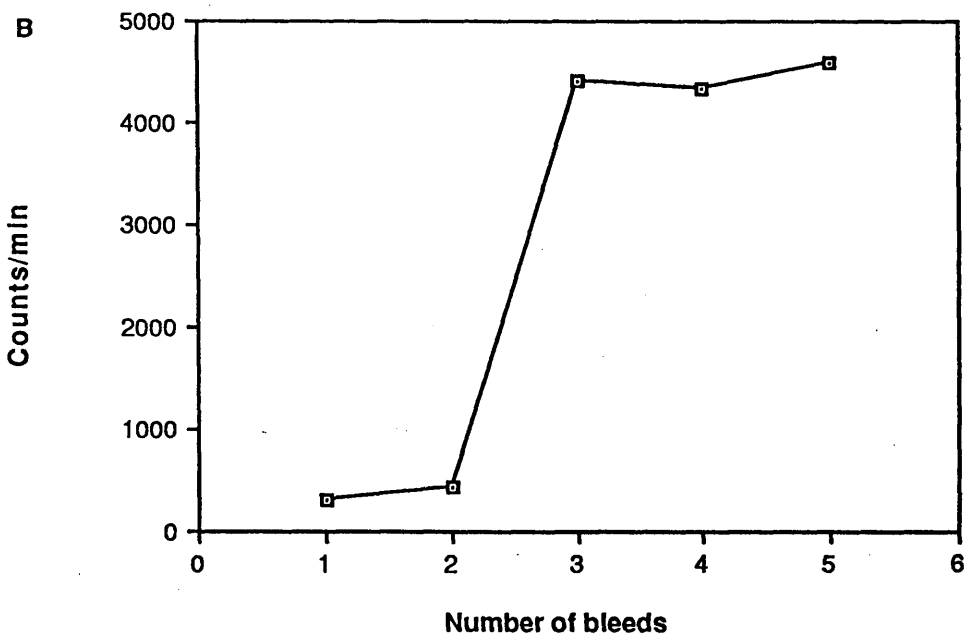

5000
4000
3000
2000
1000
0
Counts/min
0
1
2
3
4
5
6
Number of bleeds

Figure 19

The graphs represent the results obtained from radio-immunoassay experiment using sera from two animals injected with the carboxy terminal peptide coupled to β-galactosidase. The pre-immune serum is the first bleed. Post immune bleeds were obtained before booster injection with the peptide.

3.2.3 Detection of antibodies using western blots

All antisera prepared by coupling peptide to either BSA or β-galactosidase were tested on western blots (as described in section 2.12.5, 2.12.6). None of the antisera reacted specifically with a virus-infected cell protein band (figure 20), suggesting that the oligopeptide antibodies did not recognise UL33 gene product immobilised on nitrocellulose.

3.2.4 Immunoprecipitation of UL33 gene product

Since the oligopeptide antisera did not react with a virus-specific polypeptide on a protein blot, immunoprecipitation experiments were performed to determine whether antibodies specific to UL33 gene product could be detected by this method. Oligopeptide antisera were incubated with extracts of [^{35}S]-methionine labelled virus-infected cell polypeptides and the antibody-antigen complexes precipitated by binding to protein A sepharose. The bound protein was eluted and analysed on SDS 6-15% polyacrylamide gradient gels. The antisera raised against oligopeptides coupled to BSA did not appear to contain antibodies which immunoprecipitated a virus-specific polypeptide suggesting that the oligopeptide antibodies did not recognise UL33 gene product. Further experiments were performed using IgG purified from one of the antisera by fast protein liquid chromotography. Although purified IgG reacted with the oligopeptide in a radioimmunoassay, no virus-specific polypeptides were selectively precipitated from virus-infected cell extracts. However, one of the

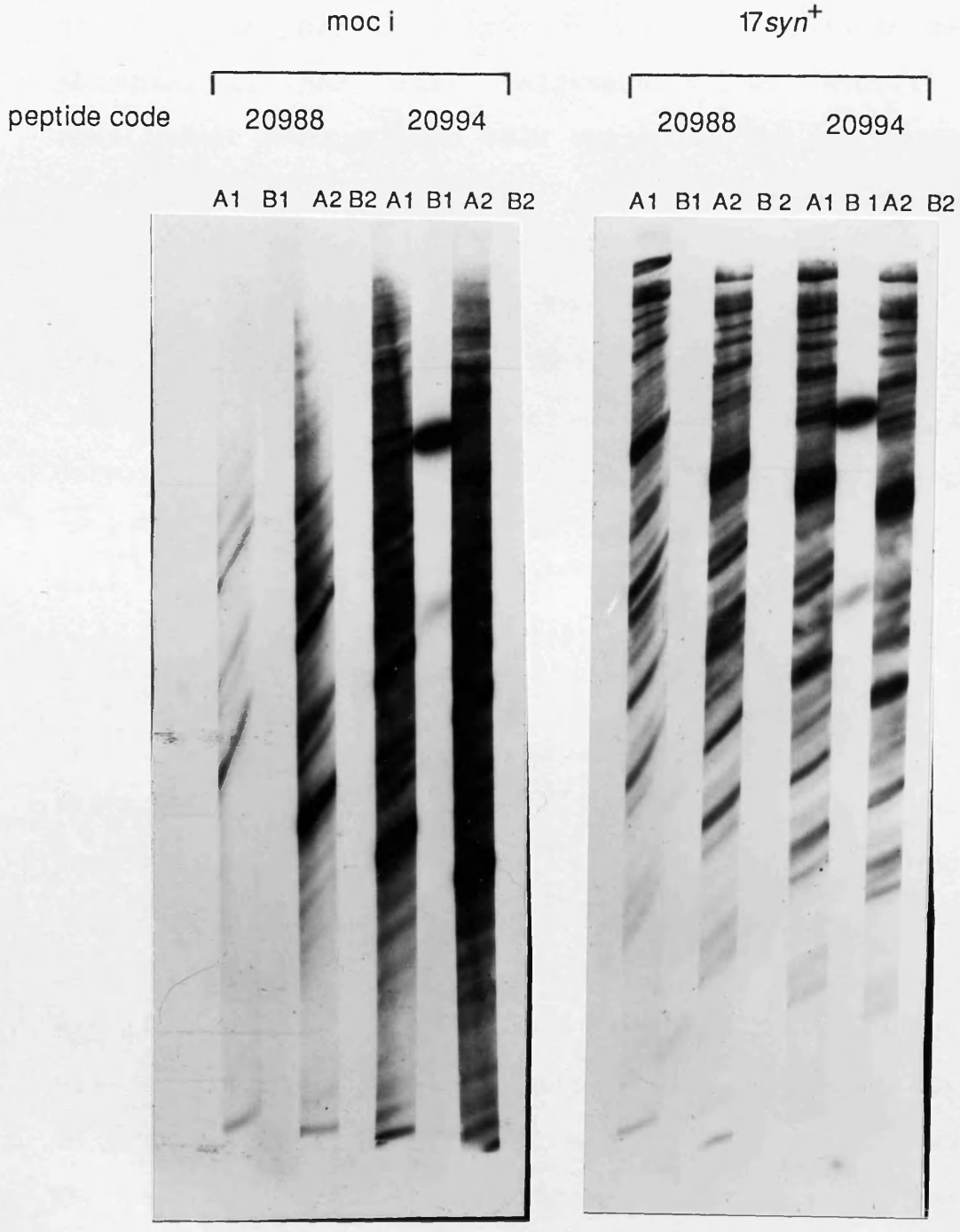

moc i
17syn+
peptide code
20988
20994
20988
20994
A1 B1 A2 B2 A1 B1 A2 B2
A1 B1 A2 B 2 A1 B 1 A2 B2

Figure 20

Immunoblot experiment using anti-carboxy terminal peptide serum, carried out as described in section 2.12.5 and 2.12.6. The A track represent the [^{35}S]-methionine of labelled mock-infected (moc i) or *wt* virus-infected cell polypeptides (17*syn*$^{+}$) transferred to nitrocellulose. The B track represent the reaction of antiserum bound to [^{125}I] protein A with mock-infected or *wt* virus infected cell polypeptides. Sera obtained from two rabbits were diluted either 1/10 (A1 and B1) or 1/100 (A2 and B2). Bands seen on the [^{125}I] track represents non-specific reaction between the serum and mock- or virus-infected cells.

antisera raised against the carboxy-terminus peptide coupled to β-galactosidase showed a weak reaction with a protein of around 14,000 MW in immunoprecipitation experiments (Figure 21).

3.2.5 Nucleocapsid and virion polypeptide profiles of HSV-1 , HSV-2, and the HSV-1/HSV-2 recombinant BX1(31-1)

A monoclonal antibody to $V_{MW}155$ reacted not only with free $V_{MW}155$ but also with $V_{MW}155$ present in capsids, providing cell extracts were prepared using a mild solubilising buffer which did not disrupt capsids. Thus in immunoprecipitates five capsid proteins, as well as $V_{MW}155$ were detected. The genomic region specifying the low mw capsid protein $V_{MW}12$ was determined by analysis of HSV-1/HSV-2 recombinants, taking advantage of the observation that $V_{MW}155$ antibody immunoprecipitated capsids. $V_{MW}12$ was shown to map between 0.44-0.52 mu (J.W. Palfreyman, personal communication). This region of the genome contained coding sequences of genes UL32 to UL36. Only two of these genes, UL33 and UL35, however, specify low mw polypeptides. The HSV-1/HSV-2 recombinant BX1(31-1) has HSV-2 sequence spanning UL33 gene (figure 22) (Marsden *et al.*, 1978). Since the recombinant induces $V_{MW}28$ with a mw intermediate between HSV-1 and HSV-2 counterparts, it is likely that the crossover site on the right hand side lies within gene UL34 which encodes $V_{MW}28$. The crossover site on the left hand side is probably located within UL32. The recombinant BX1(31-1) should therefore specify an HSV-2

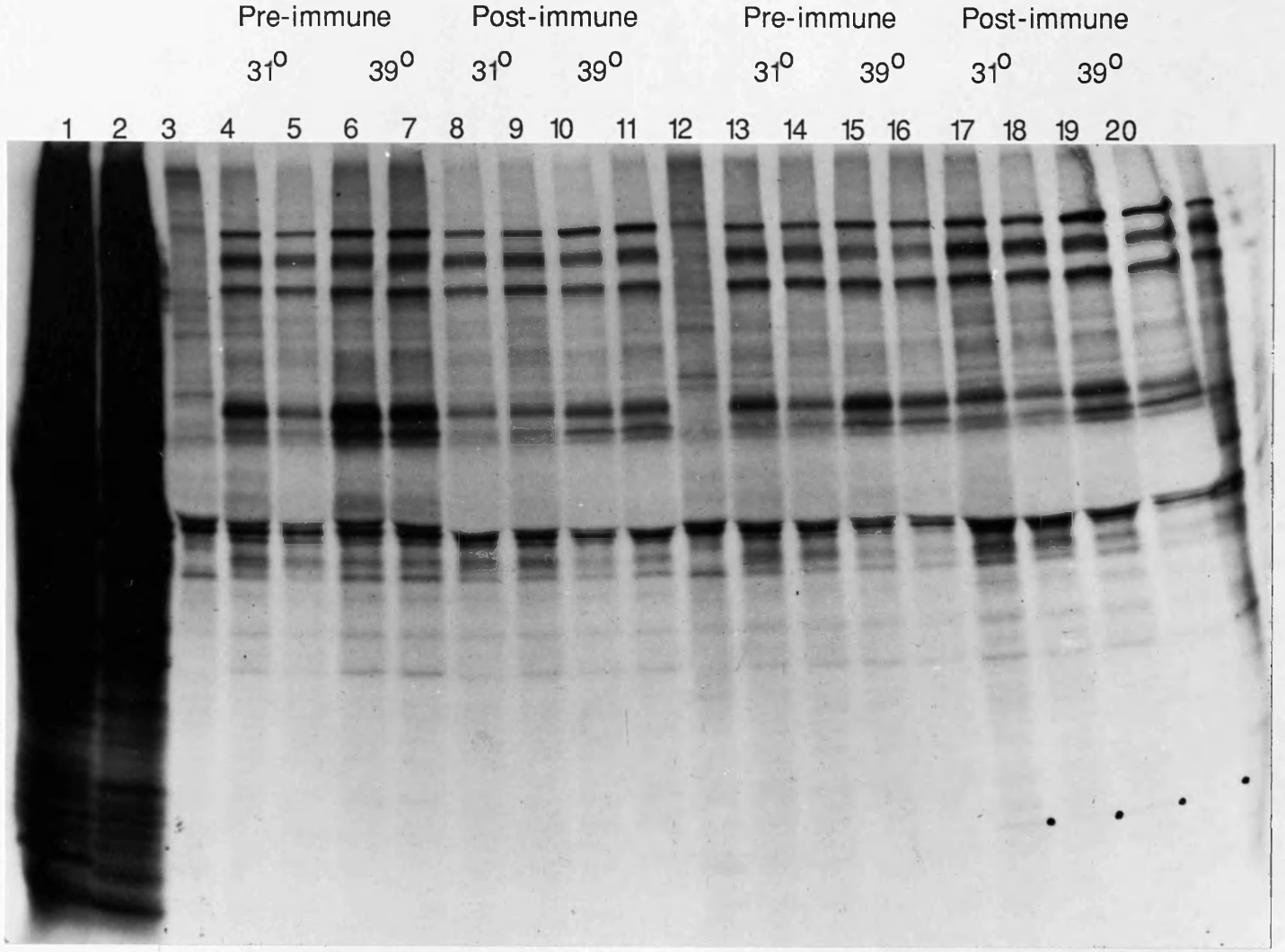

Pre-immune
Post-immune
Pre-immune
Post-immune
31°
39°
31°
39°
31°
39°
31°
39°
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Rabbit code
20988
20994

Figure 21

Autoradiograph of immunoprecipitates, using UL33 anti-carboxy terminus peptide antibody. Mock-infected cells or cells infected with *wt* virus-, or *ts*1233 were incubated at 31° or 39° for 9h, labelled with [^{35}S]-methionine for 1h and harvested. Immunoprecipitation was performed as described in section 2.12.7 and the immunoprecipitates were analysed on a 6-15% polyacrylamide gradient SDS gel. The polypeptide recognised by the oligopeptide antisera raised against the carboxy terminus (rabbit 20994) is marked. Track 1 represent mock-infected cell extracts, track 2 represent *wt* virus-infected extracts, tracks 3 and 12 mock-infected extracts with preimmune sera taken from either rabbit no. 20988 or 20994, track 4, 8, 13, and 17 represent extracts of *wt* virus-infected cells at 31°, tracks 5, 9, 14 and 18 represent extracts of *ts*1233-infected cell at 31°, tracks 6, 10, 15, 19 of extracts of *wt* virus-infected cells at 39°, tracks 7, 11, 16, 20 of extracts of *ts*1233 virus-infected cells at 39° with either sera from rabbit 20988 (tracks 3-11), or sera taken from rabbit no.20994 (tracks 12-20).

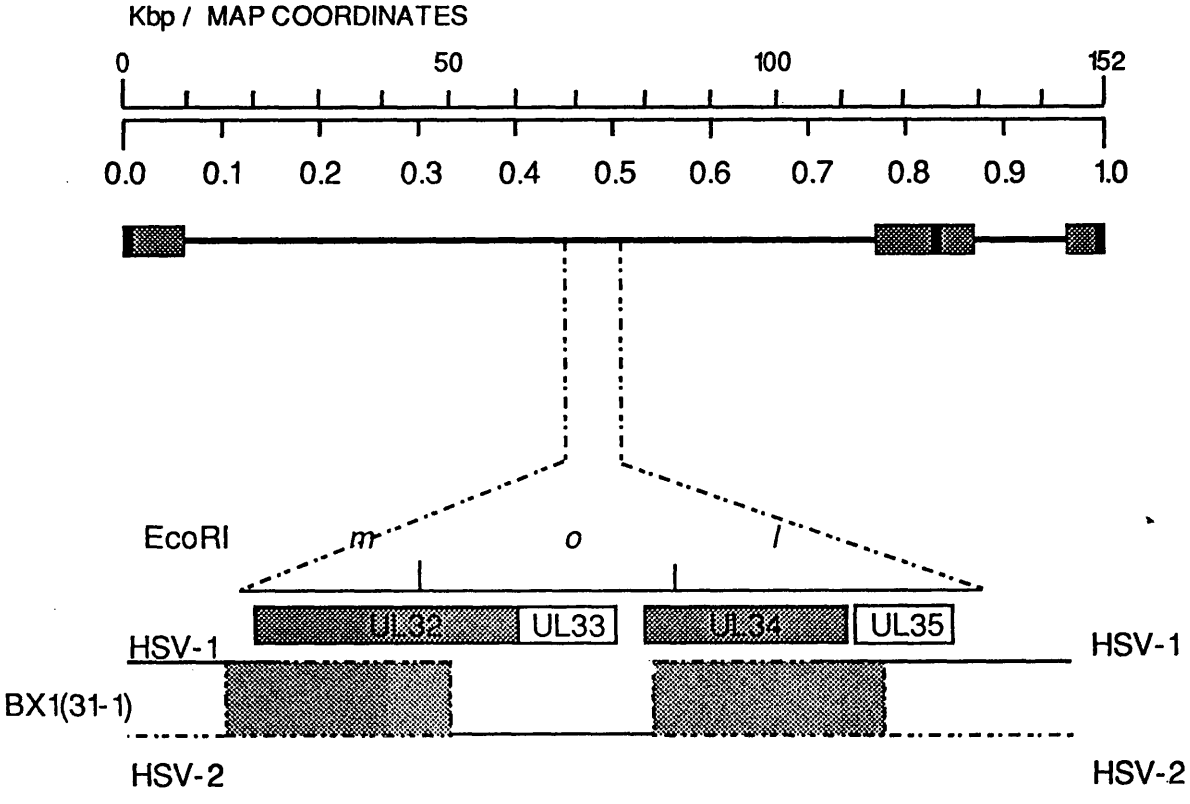

Kbp / MAP COORDINATES
0
50
100
152
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
EcoRI
m
o
l
UL32
UL33
UL34
UL35
HSV-1
HSV-1
BX1(31-1)
HSV-2
HSV-2

Figure 22

The structure of BX1(31-1).

The regions where a crossover has occurred in BX1(31-1) are denoted by a cross hatched area. The upper line represents HSV-1 sequences and the lower line represents HSV-2 sequences. The HSV-1 EcoRI sites and the position of genes UL32, 33, 34 and 35 are also included.

UL33 gene and an HSV-1 UL35 gene. An experiment was carried out to determine whether UL33 gene product was a capsid protein. Nucleocapsids and virions were purified from cells infected with either *wt* HSV-1 17*syn*$^+$ virus, wt HSV-2 HG52 virus or the recombinant virus BX1 (1-31). The method of purification was essentially the same as that described by Gibson and Roizman (1972) (refer to section 2.13). Capsids and virions, labelled with [^{35}S]-methionine, were analysed on SDS 6-15% polyacrylamide gradient gels. Figure 23 shows an autoradiograph of virion and capsid profiles of HSV-1, HSV-2 and the recombinant. The recombinant BX1(31-1) appeared to specify a 12,000 mw capsid protein of the same mobility on SDS polyacrylamide gels as HSV-1 *wt* virus. These results suggest that UL33 does not encode a capsid protein.

3.3 UL33 GENE EXPRESSION

Antisera raised against synthetic oligopeptides representing the carboxy-terminus and amino-terminus of UL33 either failed to give a specific reaction with UL33 gene product or only weakly recognised the protein. Therefore, the main objective for expressing the protein was to use the expressed protein to make antisera specific for UL33 gene product. Two different techniques were used during the course of this study in an attempt to identify the gene product of gene UL33.

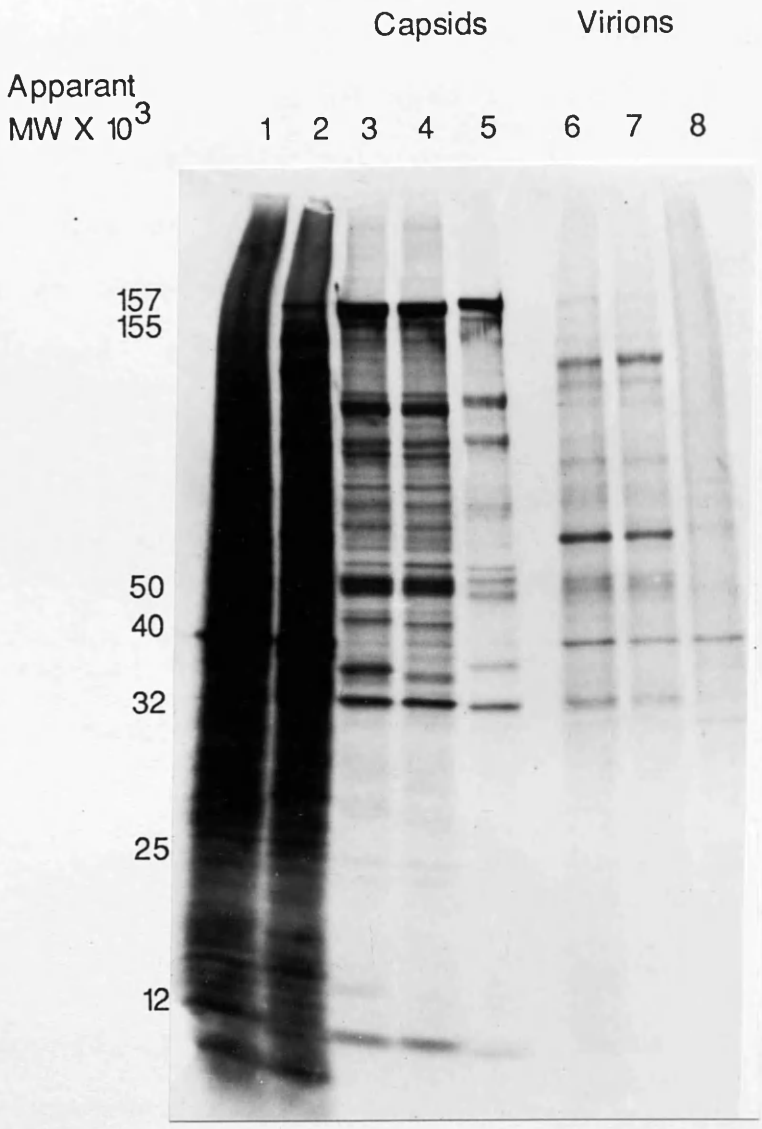

Capsids
Virions
Apparant
MW X 10^3
1 2 3 4 5 6 7 8
157
155
50
40
32
25
12

Figure 23

Autoradiograph of polypeptide profile obtained from [^{35}S]-methionine labelled capsids and virions prepared according to Gibson and Roizman (1972). Polypeptides were separated on a 6-15% polyacrylamide SDS gel.

Lanes 1 and 2 represent mock-infected and *wt* virus-infected cell extracts respectively.

Lanes 3 and 6 represent 17*syn*$^+$ capsids and virions respectively.

Lane 4 and 7 represents BX1(31-1) recombinant capsids and virions respectively.

Lane 5 and 8 represents HG52 capsids and virions respectively.

3.3.1 Cloning and expression of intact UL33 in bacterial expression vector

Initially, intact UL33 gene was inserted into bacterial expression vector. Two vectors, pKK240-11 which contains the *tac* promoter, and pKK233-2 which contains the *trc* promoter (*trp-lac* fusion promoter with a 17bp consensus spacing between the *trp* -35 region and the *lac*UV 5-10 region) followed by *lacZ* ribosome binding site -10 region, an ATG initiation codon and a transcription terminator were used. PKK233-2 has been shown to express high levels of HSV-1 gD (Amann and Brosius, 1985). The ATG codon in the vectors is located within a unique NcoI site which, when digested with NcoI, exposes a 4bp overhang containing the ATG codon. Since sequencing data of HSV-1 genome has shown that the ATG initiation codon of UL33 (McGeoch *et al.*, 1988) coincides with an NcoI site, the 472bp NcoI fragment containing the coding sequence of gene UL33, was cloned into the NcoI site of pKK233-2 and pKK240-11 and transfected into *E. coli* strain W3110 $lacI^q$. Recombinant clones were screened with various restriction endonucleases to check that the correct sequence had been cloned and to determine the orientation of the insert. Cultures of bacteria containing the clones with the insert in the correct orientation were prepared and expression in duced from *tac* or *trc* promoter by the addition of IPTG. The plasmid p*cI*15, containing the N-terminal deletion mutant of the bacteriophage λ cI repressor inserted into pKK233-2, was used as a control. This truncated repressor protein is stable in *E. coli* and expressed at high levels (Amann and

Brosius, 1985). Polypeptides from the bacteria were analysed by SDS PAGE. The protein profiles of the bacteria containing the recombinant plasmids were similar to these of bacteria containing the expression vector. No novel protein of the size around the predicted mw for UL33 polypeptide was detected when the recombinant bacteria were treated with IPTG. Bacteria carrying pcI15, however, expressed high levels of a 26,000 mw, mutated *cI* repressor when culture were treated with IPTG, confirming that the expression assay was working (Figure 24).

3.3.2 Expression of gene UL33 under IE-Vmw175 control

Since the attempt to express gene UL33 in a prokaryotic expression system did not reveal any novel protein it was decided to express UL33 in a eucaryotic expression system. Enhanced expression of a gene can be obtained by placing the gene of interest under IE gene regulation in the HSV-1 virus *ts*K. This mutant has a defect in Vmw175 (Davison *et al.*, 1984), and as a consequence overproduces IE proteins, and fails to induce other classes of viral proteins at the NPT (Preston, 1979a; 1979b).

3.3.2.a Cloning and insertion of UL33 into the IE expression vector p23

The IE expression vector p23 consists of the Vmw175 promoter and regulatory region inserted into the cloned HSV-1 TK gene. A brief description of the construction of this plasmid is given here. The plasmid pGX153 contains the

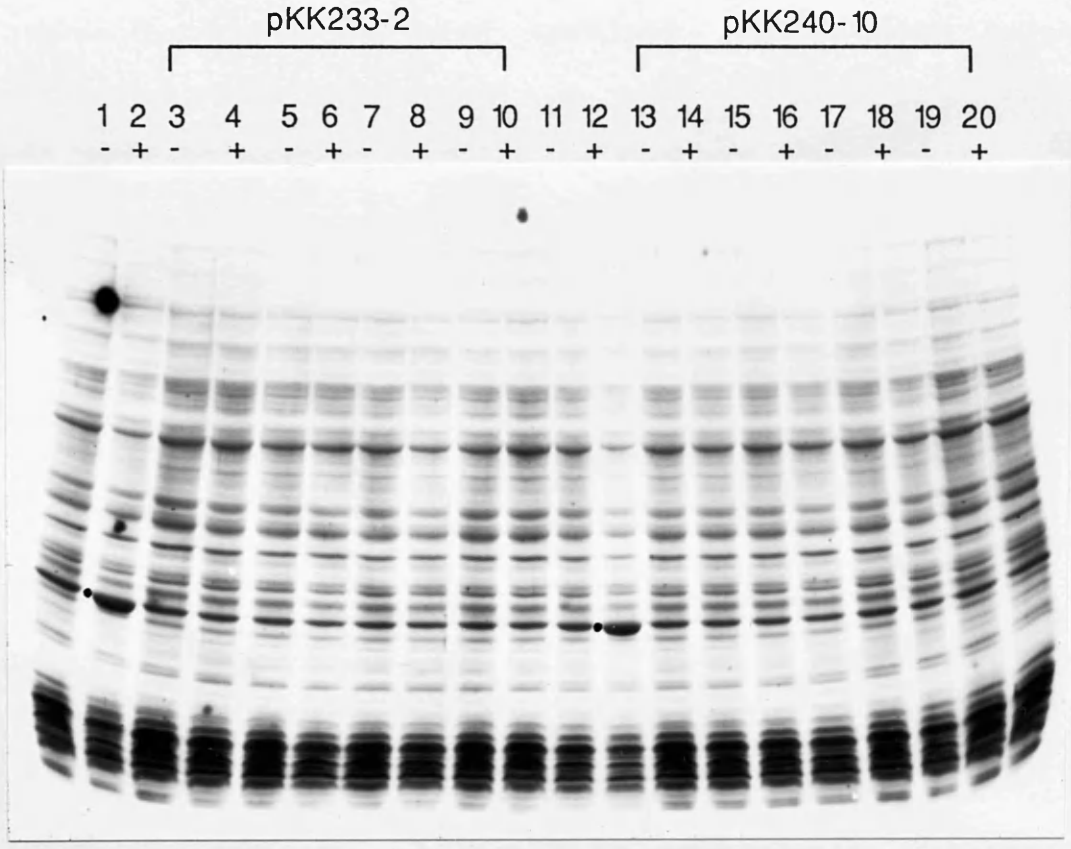

pKK233-2
pKK240-10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
- + - + - + - + - + - + - + - + - + - + - +

Figure 24

Comassie brilliant blue stain of a protein gel. Gene UL33 was cloned into pKK233-2 and pKK240-10. Bacterial cultures containing the clones were assayed as described in section 2.14. Polypeptides were separated on a 12.5% polyacrylamide SDS gel.

(-) without IPTG added

(+) with IPTG added

Tracks 1, 2, 11 and 12 represent bacteria carrying the plasmid p*cI*.

tracks 3, 4 , 13 and 14 represent bacteria carrying pKK233-2 and pKK240-10 without any insert.

Tracks 5, 6, 7, 8 represent bacteria carrying pKK233-2 containing UL33 inserted in the correct orientation required for expression.

Track 9 and 10 represent bacteria containing UL33 inserted in the opposite orientation.

Tracks 15, 16 , 17, 18 represent bacteria carrying pKK240-10 containing UL33 in the correct orientation.

Tracks 19, 20 represents bacteria carrying pKK240-10 containing UL33 in the opposite orientation. Expressed p*cI* is marked.

HSV-1 BamHI *p* fragment inserted into the BamHI site of pAT153. An XhoI site was created within the coding sequences of TK in BamHI *p* by insertion of an XhoI oligonucleotide linker at the SstI site (Fisher and Preston, 1986). The mutated plasmid was referred to as pGX166. A 360bp BamHI-HindIII fragment, containing the promoter and upstream regulatory sequences of the Vmw175 gene, was derived from the plasmid A494#23 constructed by Dr. C. M. Preston (see figure 25). This fragment was treated with Klenow in the presence of all four dNTPs to create a blunt ended fragment and inserted into pGX166, cleaved with XhoI and treated with Klenow in the presence of dNTPs. The XhoI site was recreated by the ligation of the filled in BamHI site from the 360bp fragment with the filled in XhoI site from pGX166. Similarly, the HindIII site was created at the other end of the inserted fragment. The plasmid containing the 360bp BamHI-HindIII fragment was referred to as p23 and generously supplied by Dr. C. M. Preston.

Since the EcoRI *o* fragment did not contain any suitable restriction endonuclease sites for cloning the UL33 gene into the XhoI site in p23, the 555bp EcoRI-SmaI fragment, containing UL33, was subcloned from pGX74 (this plasmid contains the EcoRI *o* of HSV-1 17*syn*$^+$) into EcoRI-SmaI cleaved pUC8 (figure 26). Two clones, referred to as pGX215 and pGX216, were isolated and digested with various restriction endonucleases to check that the correct fragments had been inserted. PGX215 was linearised with EcoRI, and the EcoRI site was filled in with T4 DNA polymerase in the presence of 200µM dGTP, dCTP (1 unit per

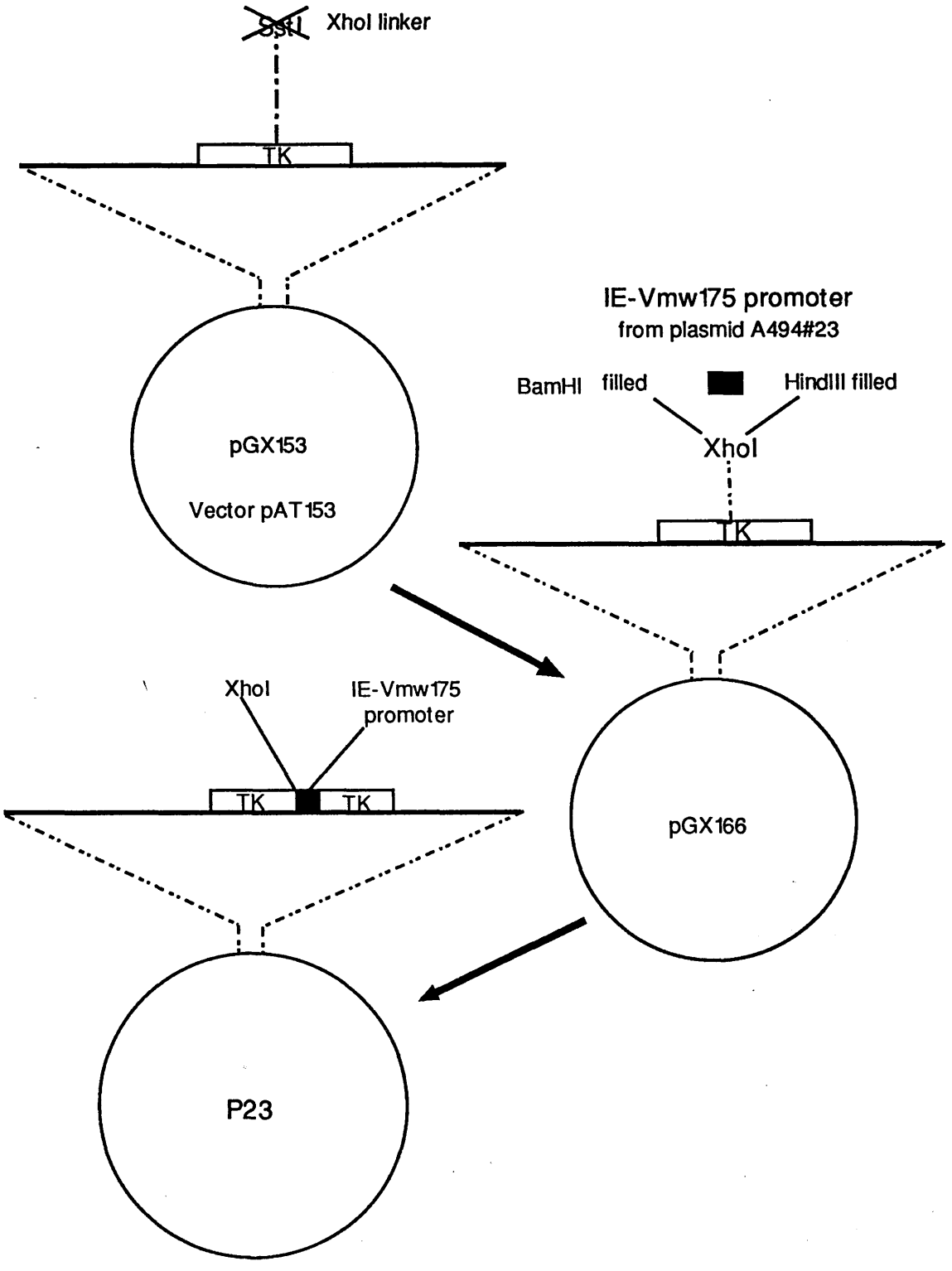

SstI
XhoI linker
TK
pGX153
Vector pAT153
IE-Vmw175 promoter
from plasmid A494#23
BamHI
filled
HindIII filled
XhoI
TK
XhoI
IE-Vmw175
promoter
TK
TK
pGX166
P23

Figure 25

Diagram showing the construction of p23 vector into which the UL33 gene was inserted. An XhoI linker was inserted into an SstI site and the IE $V_{MW}175$ promoter and regulatory sequences cloned into the XhoI site to give p23 (for details of the construction method refer to the text).

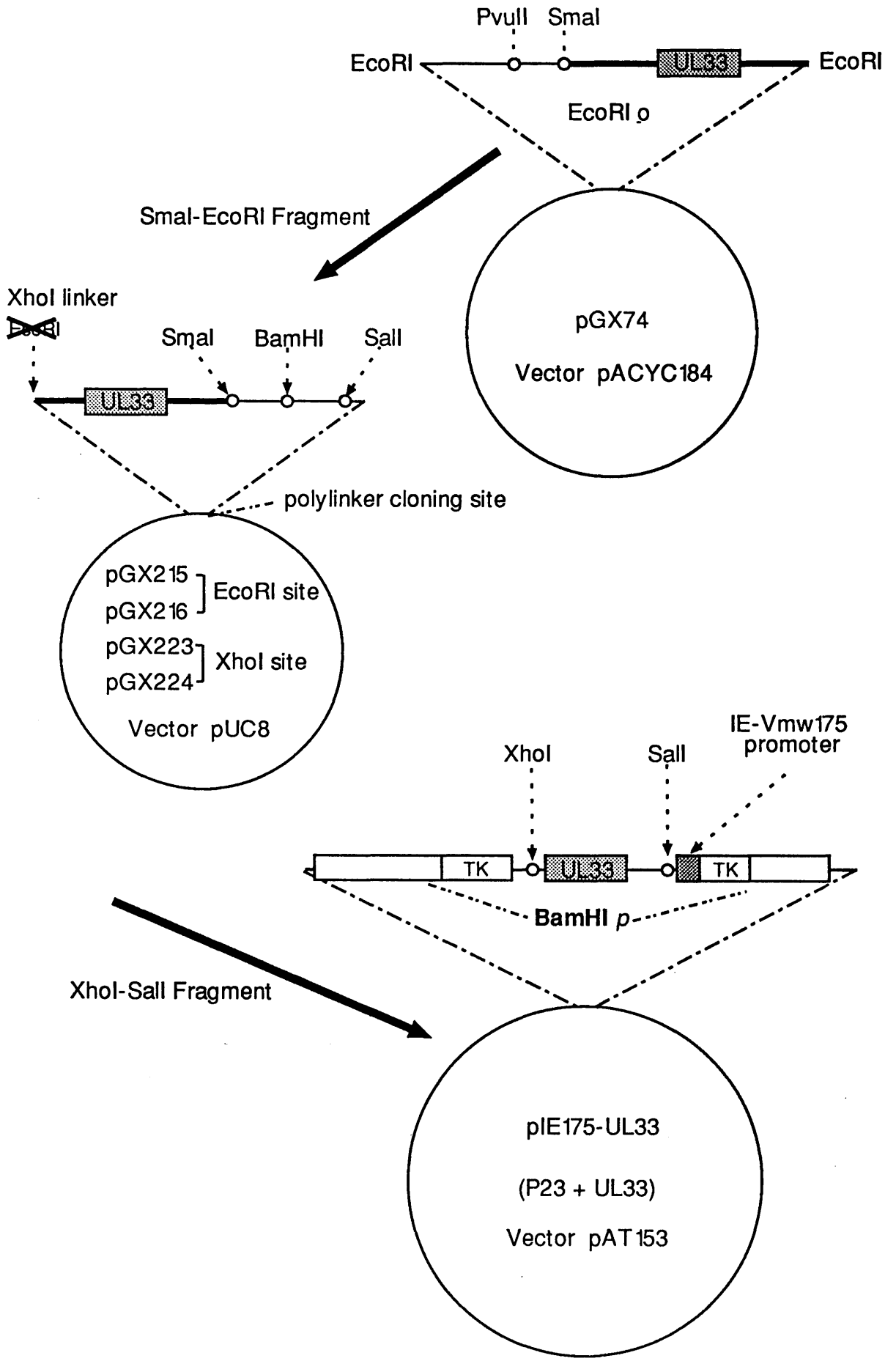

PvuII
SmaI
EcoRI
UL33
EcoRI
EcoRI p
SmaI-EcoRI Fragment
pGX74
Vector pACYC184
XhoI linker
SmaI
BamHI
SalI
UL33
polylinker cloning site
pGX215
pGX216
EcoRI site
pGX223
pGX224
XhoI site
Vector pUC8
IE-Vmw175
promoter
XhoI
SalI
TK
UL33
TK
BamHI p
XhoI-SalI Fragment
pIE175-UL33
(P23 + UL33)
Vector pAT153

Figure 26

A diagram showing the construction of pIE-UL33 used for inserting UL33 into TK gene of *ts*K virus. The EcoRI-SmaI fragment containing UL33 gene was sub-cloned from pGX74 (contains the *wt* virus EcoRI *o*) into pUC8. The SalI-XhoI fragment was subcloned from pGX224 into XhoI cleaved phosphatased p23. The resulting clone was pIE-UL33 (for further details on the construct refer to section 3.3.2a.

µg plasmid DNA) in a final concentration of 1X T_4 polymerase buffer. The linearised plasmid was then treated with 10 units of calf intestinal phosphatase. The phosphophatased treated plasmid DNA was ligated to XhoI phosphorylate oligonucleotide linker d(pCCGCTCGACGG) and transfected into competent DH1 bacteria. Two clones containing the XhoI linker, pGX223 and pGX224, were isolated and the presence of restriction endonuclease sites checked. The XhoI-SalI fragment (SalI site is present in the polylinker cloning site of pUC8 vector) from pGX224 was ligated into XhoI cleaved, phosphatased p23 plasmid. Isolated clones (referred to as pIE-UL33) were digested with various restriction endonucleases to confirm the correct fragment had been cloned and to determine the orientation of the insert.

3.3.2.b Selection and isolation of a TK$^-$ recombinant virus containing gene UL33 under IE-V_{MW}175 gene regulation

HSV, lacking a functional TK gene, will grow in the presence of 5-bromo-2'-deoxycytidine (referred to as BCdR). In cells infected with *wt* virus, however, the TK phosphorylates this compound converting it into a toxic drug which interferes with viral DNA replication. BCdR was therefore used to select TK$^-$ recombinants containing UL33 gene under IE gene regulation. BHK cells were cotransfected with *ts*K and pIE-UL33 DNA as in marker rescue experiments. When extensive CPE had developed, cells were harvested, and progeny virus titrated on duplicate sets of BHK cell

monolayers in 30mm dishes. After incubation for 45min at 31°, the first set was overlaid with Eagle's medium containing new born calf serum, and BCdR. The second set was overlaid with Eagle's medium containing both new born calf serum, and human serum. Plates were incubated for 3 days at 31°. Thereafter cells of the first set were harvested into medium while the second set was stained with Giemsa stain and plaques counted. Virus progeny from the first set of plates were titrated on BHK cells and well isolated virus plaques picked and virus titrated. Virus was plaque purified three times, and large scale virus stocks prepared. Virus DNAs labelled with [^{32}P] were prepared using the Lonsdale technique as described in section (2.10) and the viral DNAs analysed with BamHI, XhoI and KpnI. Figure 27 represents an autoradiograph of separated restriction endonuclease fragments of *ts*K and two isolated recombinant viruses VIE-(UL33-1) and VIE-(UL33-2) following digestion with either BamHI or XhoI enzymes. The recombinant virus DNAs lacked BamHI *p* fragment and contained instead two smaller BamHI fragments. This was expected since the XhoI-SalI fragment inserted into the XhoI site of p23 contained a BamHI site from the polylinker of pUC8 (compare track 2,3 to track 1). The XhoI *f* fragment in *ts*K (track 1) was not present in the two recombinants. Instead two smaller fragments appeared, consistent with the presence of an XhoI site within XhoI *f* (track 2 and 3). Thus, these results suggested that the gene UL33 linked to IE-3 promoter had been recombined successfully into the TK gene of *ts*K.

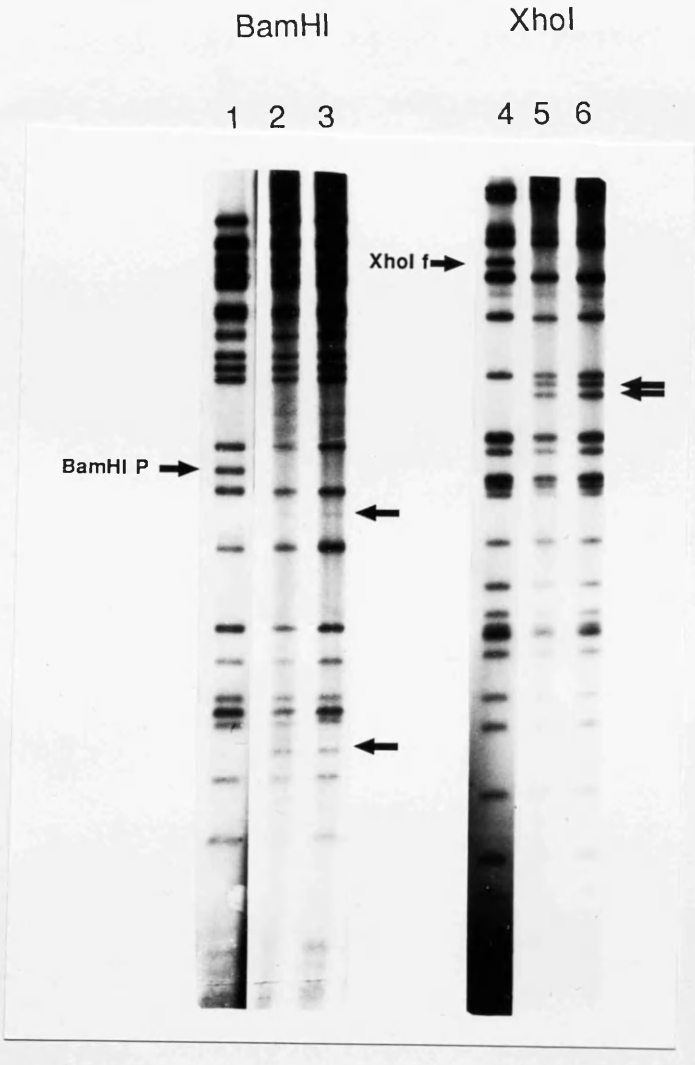

BamHI
XhoI
1 2 3
4 5 6
XhoI f
BamHI P

Figure 27

Autoradiograph showing restriction endonuclease digests of viral DNA labelled *in vivo* with [^{32}P]. BamHI (lane 1-3) and XhoI (lanes 4-6) digestion of *ts*K (lane 1, 4) and two separate recombinant viruses (lane 2, 3, 5, 6). The location of the BamHI *p* and XhoI *f* in *ts*K is indicated by the left hand arrows (lane and 4). The novel fragments in the two recombinants are indicated by right hand arrows.

3.3.2.c UL33 expression by the recombinant virus

BHK cells were infected with recombinant virus or *ts*K at the PT and the NPT. Virus-infected cells were labelled with [^{35}S]-methionine, and the proteins separated on SDS 6-15% polyacrylamide gradient gels. Results obtained from the autoradiographs revealed that the two recombinant viruses each fail to induce TK (about 39,000 mw protein) at the PT. In addition, more than one band was detected under IE conditions at the NPT, one with a mw slightly higher than the predicted MW of UL33 and the other band was about 35,000-30,000 (figure 28). However, subsequent experiments using a recombinant virus containing the VMW175 promoter and upstream regulatory sequences inserted into the TK gene of *ts*K is showed that this virus induced a similar sized band of about 16,000 mw. Therefore, it was concluded that UL33 was probably not expressed under these conditions.

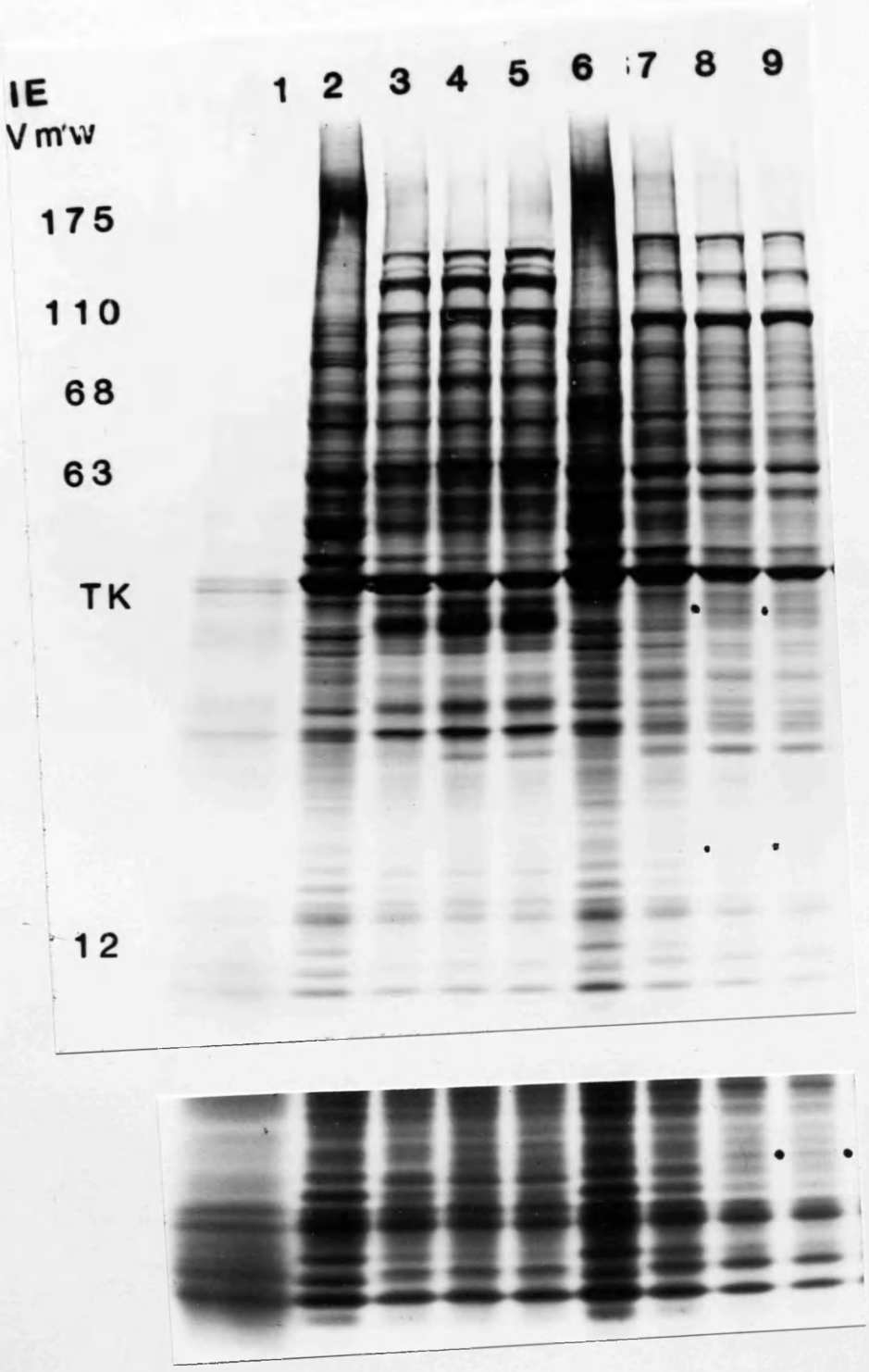

IE
1 2 3 4 5 6 7 8 9
Vm'w
175
110
68
63
TK
12

Figure 28

Autoradiograph of polypeptides induced at 31° and 38.5° in BHK cells infected with *ts*K or VIE-(UL33-1) or VIE-(UL33-2) or in mock infected cells. Virus-infected cells were labelled with [^{35}S]-methionine for 1h at 4 hpi and harvested. Protein samples were analysed on a 6-15% gradient SDS PAGE. Numbers represents apparant mw of IE polypeptides. Track 1 represents *wt* virus-infected cell extracts, tracks 2 and 6 represent mock-infected cell extracts, tracks 3 and 7 *ts*K infected cell extracts at 31° and 39° respectively, tracks 4 and 8 of VIE(UL33-1) virus-infected cells at 31° and 38.5° respectively and tracks 5 and 9 represent VIE(UL33-2) virus-infected cells at 31° and 38.5° respectively. TK band in *ts*K (track3) is marked. The two bands mentioned in the text are marked. Below is a longer exposure of the same area where the induced 16,000mw protein is marked.

3.4 DISCUSSION

In the previous sections a DNA positive mutant of HSV-1 *ts*1233 has been characterised. The lesion of *ts*1233 was shown to lie in gene UL33. When work on *ts*1233 was initiated no mutations had been assigned to UL33 and no information was available about the function of the gene. The analysis of this mutant suggests that gene UL33 is required for the assembly of nucleocapsids and may be important for cleavage and encapsidation of HSV DNA since *ts*1233 failed to package DNA into capsids at the NPT.

3.4.1 DNA processing in *wt* virus, *ts*1233 and *ts*1201 infected cells

Electron microscopic analysis of virus-infected cells and Southern blot analysis of total viral and encapsidated DNA present in *ts*1233-infected cell extracts at the NPT and PT revealed that *ts*1233 failed to package DNA at the NPT. More than 99% of the DNA synthesised by *ts*1233 was endless at the NPT. This finding is consistent with the information obtained from the analysis of *ts* mutants of PRV and HSV which have encapsidation defects and suggests that viral DNA is cleaved as it is inserted into the capsid (Ben-Porat and Veach, 1980; Ladin *et al.*,1980; Preston *et al.*, 1983; Addison, 1986; Sherman and Bachenheimer, 1987).

*Ts*1233 did not cleave DNA when mutant-infected cells were downshifted from the NPT to the PT in the presence of cycloheximide and therefore, the effect of *ts*1233 mutation could not be reversed upon shift down of virus-infected cells to 31°. Some cleavage of *ts*1201

concatemeric DNA, however, occurred following transfer of the mutant infected cells from 39° to 31° in the presence of cycloheximide, although not as much as reported previously (Addison, 1986). One explanation for this result is that some of the *ts*1201 mutant protein remains non-functional after transfer to 31° or the conformation of the mutant protein has been altered in such a way at 39° that it does not work as efficiently as the *wt* virus protein at 31°. Another explanation is that at the NPT all of the *ts*1201 DNA was present in an endless state and therefore, unless DNA cleavage was very efficient, there should not be as much viral DNA cleaved in *ts*1201-infected cell sample as in the *wt* virus-infected cell samples.

Although mutant and *wt* viruses packaged DNA efficiently at 31°, only about 8% of the DNA synthesised by the *wt* virus was cleaved and packaged at 39°. This observation is consistent with other work showing that *wt* 17*syn*$^+$ virus packaged DNA less efficiently at a NPT of 38.5° than at the 31° (Addison, 1986). This result suggests that there may be more thermolabile proteins involved in encapsidation.

DNA +ve mutants from five different complementation groups (*ts*F18, *ts*1208, *ts*1201, *ts*1203, *ts*N20) fail to encapsidate viral DNA at the NPT (Schaffer *et al.*, 1987). One of the mutants, belonging to complementation group 1-13, has not been characterised in detail. The mutations in viruses belonging to the other complementation groups, however, have been assigned to specific genes. Mutants with defects in UL6, UL26, UL28, UL32, and UL33, all

produced large numbers of partially-cored capsids at the NPT, whereas the mutant *ts*1208, with a defect in UL25, produced only low numbers of capsids at 39°. It is therefore, likely that UL25 is not directly involved in DNA encapsidation. With the exception of *ts*1201 defect, which could be reversed when mutant virus-infected cells were shifted to the PT in the presence of cycloheximide, the effect of mutation in all the other packaging deficient mutants was irreversible. The internal structure of the partially-cored *ts*1201 capsids seemd to differ from those of other mutants, although no information on the internal sructure of *ts*N20 and *ts*F18 is available. In contrast to *ts*1201 partially-cored capsids, *ts*1233 produced capsids at the NPT which had a small core structure similar to those seen in *ts*1203-infected cells at the NPT.

Effect of UV light-induced mutation in *ts*1233

As indicated previously, *ts*1233 was isolated from a UV-mutagenised *wt* virus stock. This section deals with UV-induction of the mutation in *ts*1233 DNA and the effect the lesion exerts on the protein. UV-induced mutagenesis has been intensively studied in other organisms such as *lambda* phage, *Escherichia coli* and, therefore, these systems have been used as general models for explaining the nature of mutation in this study, although it should be emphasised that the analysis of larger numbers of UV-induced mutations in HSV is vital for increasing our knowledge regarding the concepts of UV mutagenesis and understanding the nature of the repair mechanism

employed by HSV.

The mechanism of UV-induced lesion

UV light is known to induce a number of different classes of mutations ranging from base substitutions (including single, tandem double-base substitution), to frameshifts, deletions, and insertions (Wood and Hutchinson, 1984; Wood *et al.*, 1984; Miller, 1985; Schaaper *et al.*, 1987). However, the identity of the major cause of the premutational DNA lesions after UV irradiation is uncertain, and is thought to be one of two photoproducts. Cyclobutane dipyrimidine dimer is the product which has been widely implicated in UV mutagensis, and around two-thirds or more of the mutations in lambda phage are thought to be caused by this product (Hutchinson *et al.*, 1988). The second less frequent candidate is pyrimidine-pyrimidone [6-4] photoproducts (referred to as [6-4]) and these are defined as a series of products derived from unstable four-membered rings formed after the photo-induced addition of a carbonyl or imino group from one pyrimidine in a DNA strand to the 6 position in the 5,6 double bond of an adjacent pyrimidine. This product has been implicated as a cause of premutational DNA in bacteriophage lambda (Wood *et al.*, 1984) and in the *E.Coli lacI* gene (Gilkman *et al.*, 1986; Schaaper *et al.*, 1987) .

The damaged DNA is excised and mutations result from errors in repair synthesis. Among the base substitutions, transitions predominate over transversions and form the largest class of mutations. 85% of base substitutions are

found at sequences of two or more adjacent pyrimidines, leading to the conclusion that UV-mutagenesis is a targeted event at sites of dipyrimidine damage, either of cyclobutane or the [6-4] type in *E.Coli lacI* gene (Schaaper *et al.* 1987). Results obtained from previous studies showed that transversion normally occurred at pyr-pyr*, rather than at pur-pyr*-pyr (* represent the mutated pyrimidine) sites in the lambda phage *cI* gene (Wood *et al.*, 1984), and the *lacI* gene of *E. coli* (Miller, 1985). In comparison with the data of site specificity of UV-induced base substitution in the *E. Coli lacI* gene and lambda phage, it is concluded that the mutation in *ts*1233 has occured as a result of a targeted event at a pur-pyr*-pyr and is consistent with the nature of induction of the mutation by UV-light in other systems. However, since transversions are much less frequent than other base substitutions a wider statistical analysis on the occurance of such mutations in HSV is required to support this conclusion.

UL33 protein conformation

As mentioned earlier the lesion in *ts*1233 is caused by a base pair transversion of A.T to T.A which results in the substitution of isoleucine with asparagine at amino acid position 17 in the proposed UL33 gene product. Since this change affects the ability of the virus to produce DNA containing capsids at the NPT, it is reasonable to assume that the lesion affects an important functional domain in UL33 protein. To assess the impact of the mutation on the UL33 protein structure, the predicted secondary structure of the

UL33 gene product was determined using the Chou-Fasman computer programme Chou and Fasman (1978). This programme calculates 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 (also known as coil regions which cause the polypeptide to fold on itself by approximately 180°). The most striking difference observed between the predicted *wt* virus UL33 protein structure and that of *ts*1233 is the formation of a new β-turn at the position of the lesion near an α-helix pole (figure 29 and 30). Since *ts*1233 grows well at 31°, it is possible that this change does not affect the conformation of the protein at the PT, but higher temperatures (39°) cause an alteration in the secondary structure of the protein and as a consequence the protein becomes non-functional. It is possible that the altered protein is more stable than it was in its possible original configuration This idea provides an explanation for the observation that the effect of the *ts*1233 lesion was unable to be reversed when mutant- infected cells were transferred from the NPT to PT in the presence of cycloheximide.

The position of the lesion in *ts*1233 is located at the α-helix pole, and it is interesting to note that the thermostability of the *T4* lysozyme has been increased by amino-acid substitutions at the dipoles (Nicholson *et al.*, 1988). Since the the accuracy of prediction of the Chou

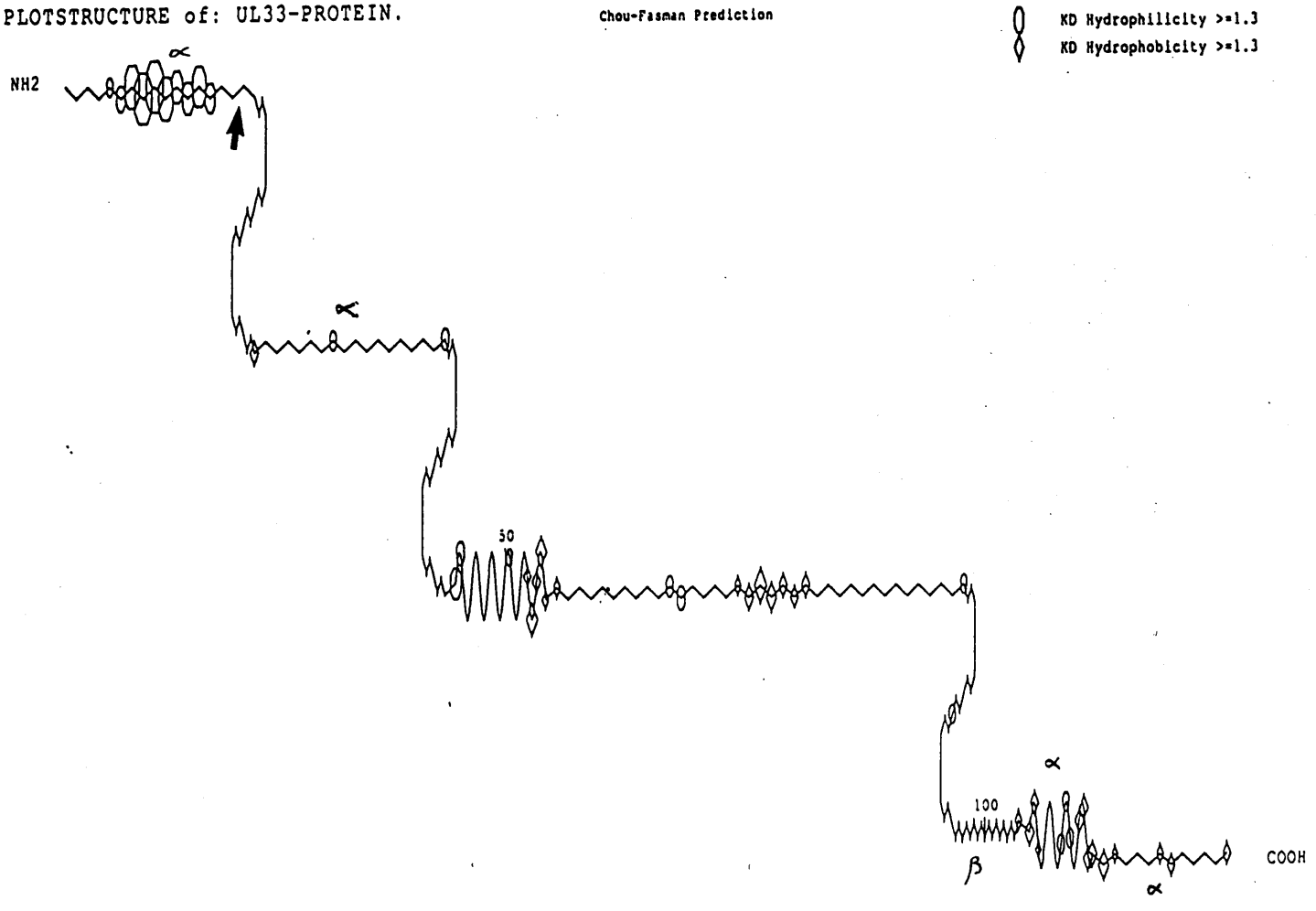

PLOTSTRUCTURE of: UL33-PROTEIN.
Chou-Fasman Prediction
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
α
α
50
α
100
β
α
COOH

Figure 29

Predicted secondary structure of UL33 protein.
The structure is based on Chou and Fasman computer programme which predicts helices, sheets and turns according to Chou and Fasman (1978).

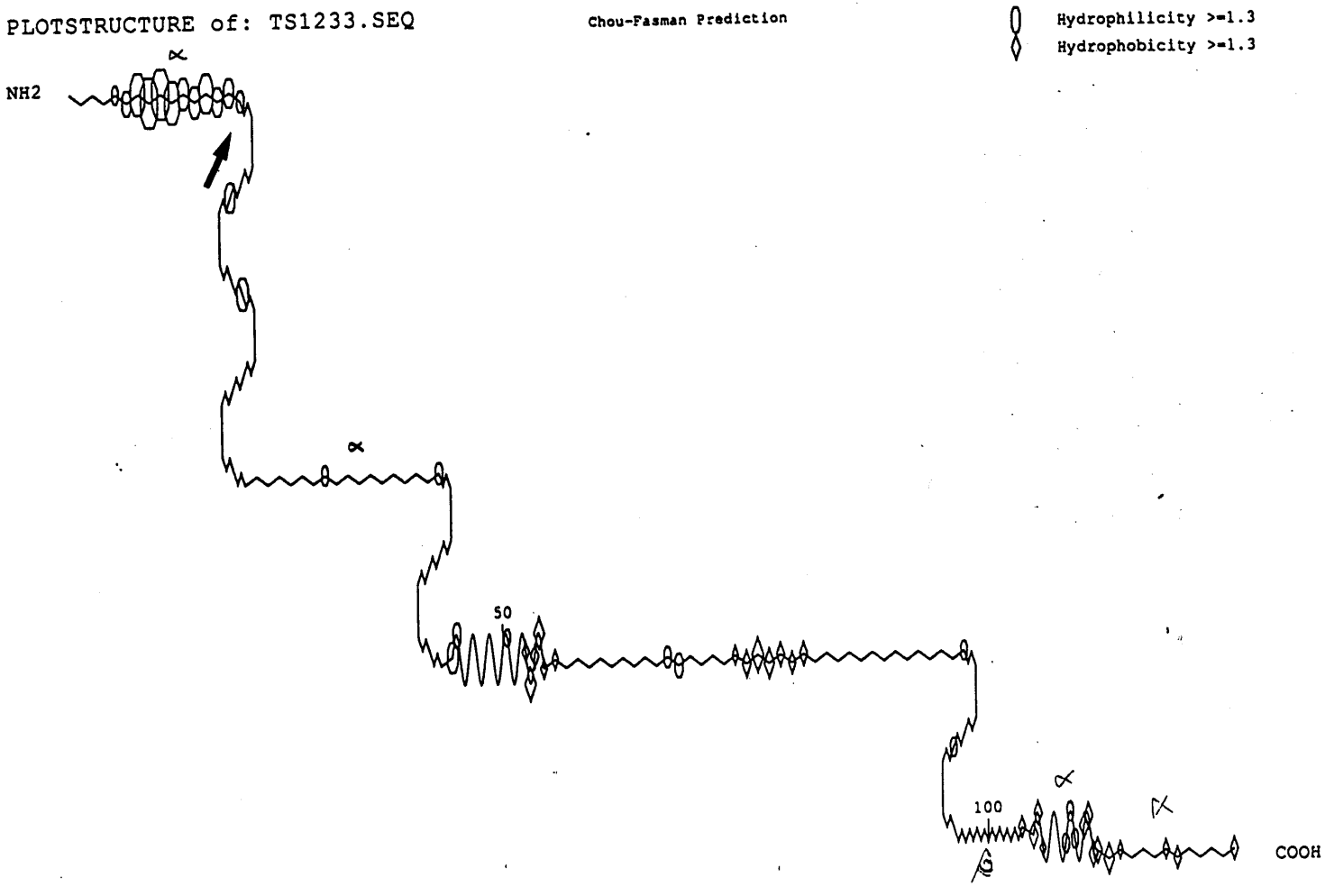

PLOTSTRUCTURE of: TS1233.SEQ
Chou-Fasman Prediction
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
50
100
COOH

Figure 30

Predicted secondary structure of *ts*1233 UL33 protein.

The structure is based on Chou and Fasman computer programme which predicts helices, sheets and turns according to Chou and Fasman (1978). (see figure 29)

The position of the mutation within the secondary structure is indicated by an arrow.

Helices are shown with a sine wave (∿∿∿), beta sheets with a sharp saw-tooth wave (⩘⩘⩘). turns with 180 degree turns, and coils with a dull saw-tooth wave. hydrophilicity (◊) and hydrophobicity (◊)are superimposed over the wave.

and Fasman programme is about 50-60% other explanations for the observation that the effect of the *ts*1233 lesion at the NPT is irreversible may exist, for example the protein could be unstable and degraded at the NPT, or alternatively the mutant protein at NPT might form a different structure to the *wt* protein upon transfer to the PT. Crystallographic analyses of UL33 protein would be of great help in determining the effect of the lesion on the protein conformation.

Polyclonal antibodies

Anti-peptide antibodies have been extremely useful for isolating and characterising gene products. Factors such as the length of the peptide, hydrophilicity, amino acids present in the oligopeptide, their location in the native molecule, and even the secondary and tertiary configuration of the peptide are thought to be important factors in generating protein-reactive antipeptide antibodies.

Two peptides were used in this study, one representing the carboxy-terminus (11 amino acids) and the other the amino-terminus (11 amino acids) of UL33. These peptides were chosen for several reasons. First, the carboxy-terminal and amino terminal regions of many proteins are relatively free to rotate and might be more exposed and free to react with antibodies than other regions of the molecule. Second, these peptides are reasonably hydrophilic. It was thought that antigenic regions are primarily hydrophilic regions on the surface of

the protein molecule, and often contain charged and polar residues. Not all hydrophilic regions, however, are antigenic and not all antigenic regions are hydrophilic. Nevertheless, many investigators have been able to raise antisera against short peptides that recognise native proteins and the majority of such short epitopes were shown to correspond to regions in the protein that possess a high segmental mobility (Tainer *et al.*, 1984). Another reason for choosing a resonable hydrophilic peptide is that the peptide is more likely to be soluble and therefore, more easily coupled to a carrier protein. One possibility for not obtaining a specific antibody could be that the length of the peptides chosen was not long enough to give an anti-protein immune response since this depends entirely on the probability that the free peptide would adopt the conformation that the identical sequence would assume in the native protein. Several studies have reported that not all antisera raised against peptides generated antibodies specific to the native protein (Tanaka *et al.*, 1985). The optimum length for an immunogenic oligopeptide likely to produce a positive antiserum has been estimated to be between 10-15 amino acids (Palfreyman *et al.*, 1984). Alternatively, it is possible that the weak response of the carboxy-terminus antipeptide antisera to putative UL33 gene product was due to other factors, for example, very small quantities of the gene product may be present in virus-infected cells and since the protein is low in methionine, UL33 gene product may not be easily detected even with a potent antiserum if the virus infected cell polypeptides

were labelled with [^{35}S]-methionine.

UL33 Gene expression

The bacterial plasmid vector pKK233-2 has been useful for the expression of high levels of foreign proteins from both prokaryotes and eukaryotes (Amann and Brosius, 1985). The reason for the failure to detect the putative UL33 gene product with this expression system is not known. The protein could be unstable or toxic. Alternatively, a mutation which affected gene expression could have been induced during the cloning of this gene into pKK233-2. The possibility that gene UL33 contains more than one intron can not be dismissed, especially since no transcripts have been characterised in the region where UL33 gene is located. It is, however, unlikely because RNA splicing is rare in HSV and, with one exception restricted to IE transcripts. Furthermore, a homologue of UL33 has been identified in VZV.

The UL33 gene was also placed under IE gene regulation and inserted into the TK gene of *ts*K. The results of this experiment were difficult to interprete because more than one novel protein band could be detected by SDS-PAGE when the recombinant virus was grown under IE conditions. One explanation for the presence of the high mw protein band is that the protein was modified or the promoter was altering the expression working on an adjacent open reading frame. It should be mentioned that in certain cases inversion can occur between sequences inserted in the TK gene and the endogenous gene provided the sequences

inserted into the TK gene are in opposite orientation to the original sequence. In view of difficulty in interpreting the results of expression of UL33 in *ts*K alternative methods of expressing UL33 gene product are required.

The functional role of UL33 gene product

Although the *ts*1233 mutant failed to package DNA at the NPT, the effect of the mutation of was irreversible, and therefore, it is not possible to conclude that the UL33 gene product is directly involved in DNA packaging. *Ts*1233 may produce abberrant capsids at the NPT which are unable to encapsidate DNA or the DNA may have been modified.

Several polypeptides have been implicated in the assembly of capsids. VMW155 is clearly an important component of capsids since the hexameric capsomeres and probably the pentamer capsomeres are composed of this protein (refer to section 1.8.1, on capsid proteins), and *ts* mutants with lesions in VMW155 have a capsid-negative phenotype at the NPT (Bone and Courtney, 1974; Schaffer *et al.*, 1974; Weller *et al.*, 1987). Like VMW155 *ts* mutants, the UL38 *ts* mutant HSV-1 A44*ts*2 fails to produce capsids at the NPT (Pertuiset *et al.*, 1989). This finding suggests that UL38 gene product is also an essential component of capsids, and recent results have shown that the capsid protein VMW50 (VP19C) is encoded by UL38 (F.J. Rixon, personal communication). Clearly, VMW155 and VMW50 are integral capsid proteins.

The correct processing of UL26 gene product is

probably important for assembly of partially-cored capsids since the mutant *ts*1201, which has a defect in UL26, produces capsids at the NPT that have a larger internal structure than the capsids produced at the NPT by packaging mutants with defects in other genes (Preston *et al.*, 1983; Sherman and Bachenheimer, 1988).

Nucleocapsid purification experiments suggested that UL33 protein is probably not the 12,000 mw capsid protein. The possibility that UL33 gene product is a virion non-capsid protein, possibly a tegument protein, which interacts with other structural proteins during assembly of capsids could not be ruled out, and in *ts*1233-infected cells at NPT such a protein was unable to interact with capsid proteins. Evidence obtained from electron microscopy favours the pathway of capsid assembly in which DNA is packaged into preformed nucleocapsids. No empty capsids were found in *ts*1233 infected cells at the NPT, therefore, demonstrating that empty capsids are probably derived from full or partially-cored capsids. This finding is consistent with the results obtained from the analysis of DNA +ve *ts* mutants of PRV and HSV, together with pulse-chase experiments showing that empty, coreless capsids are breakdown products of full, DNA-containing capsids (Ladin *et al.*, 1980; 1982; Addison, 1986; Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988).

The use of oligopeptides in preparing antisera directed against UL33 gene has not been fruitful to the extent of identifying the UL33 gene product, and since it was shown that the mutation in *ts*1233 was irreversible, the

functional role of the gene product could only be based on speculations in this situation. Work by Ladin *et al.* (1982) on PRV *ts* capsid- mutants suggested that the accumulation of the 35,000 mw polypeptide (equivalent to p40) was probably controlled by more than one viral gene product. Since the mutant *ts*1233 processed UL26 gene product normally at the NPT, the processing of UL26 gene product is not determined by UL33. Subsequent work has shown that the processing of this gene product does not depend on the presence of capsids since *ts* mutants that fail to make capsids at the NPT process p40 normally (V. Preston. personal communication). It is possible that the product of gene UL33 is required at a step leading to DNA encapsidation such as the anchoring of DNA into capsids (Poffenberger and Roizman, 1985), or it is a scaffolding protein required for the maintainance of the capsid. Alternatively, since it is thought that cleavage and packaging are linked together, UL33 protein could possibly be part of the postulated DNA complex mentioned by Diess *et al.* (1986) which binds and moves randomly along the DNA (See section 1.7.5). Recently it was shown that nuclear extracts of HSV-1 infected cells contain factors which formed two virus specific protein complexes with the *a* sequence (Chou and Roizman, 1989). The first complex, was composed of two proteins, one of which was thought to be the product of gene UL36 (MW 336,000), which is known to be important for the release of DNA from the capsids into the cytoplasm (Batterson *et al.*,1983), and the other, an unidentified protein with an approximate MW of 140,000.

The second complex contained a polypeptide which, on the basis of apparent mw of the protein, its enzymatic activity and reaction to a monoclonal antibody appeared to be the viral-encoded DNase. Since the binding of this DNase to viral DNA does not appear to be sequence specific, it was suggested that the DNase interacted with another protein that bound in a sequence specific manner to the *a* sequence and enabled the DNase to selectively cleave at the *a* sequence (Chou and Roizman, 1989). Although there is no evidence that the complexes are involved in DNA encapsidation, it is possible that other proteins such as UL33 gene product might be a component of this protein complex and interact with the DNase and the lesion in *ts*1233 could possibly affect a binding domain between UL33 gene product and the other protein.

3.5 FUTURE PROSPECTS

The main objective of characterising HSV-1 *ts* mutants is to identify the gene product in which the *ts* lesions are localised and determine the functions of the gene of interest. In this study the HSV-1 *ts*1233 mutant was shown to have a lesion in gene UL33. However, attempts to identify the gene product have been hampered by the production of weak antibodies to UL33 gene product and failure to express the gene in procaryotic expression systems. Oligopeptide antisera raised against synthetic oligopeptides specific for a small portion of the amino acid sequence of a protein have been used extensively to identify gene products. This technology, however,is still

at an early stage, and it would be interesting to use new developments in this field for preparing antibodies, such as longer peptides chosen from different regions or the use of branched peptides since preliminary studies have shown that a stronger immune response is obtained with branched peptides than with ordinary peptides (H.S.Marsden, personal communication). An alternative approach to the use of synthetic oligopeptides for raising antisera is a modified gene-fusion system developed by Löwenadler *et al.* (1987). Synthesis of oligodeoxynucleotides, representing short stretches of coding sequences of the protein of interest, are inserted into a prokaryotic expression vector to yield a fusion protein containing the peptide linked to a modified protein A carrier. Using a coupled excretion/expression vector system, the fusion protein is execreted in large amounts into the medium and is easily purified. This technique has several advantages over the traditional immunisation with a synthetic peptide chemically conjugated to a carrier protein. In particular, oligonucleotides encoding upto 50 amino acids can be synthesised. A synthetic peptide of this size would be difficult to produce. In addition peptide sequences which are difficult to synthesise should be able to be expressed in this system. Other gene fusion systems have been developed for the purpose of raising antisera. The advantage of these systems is that larger portions of the protein of interest can be expressed and therefore there is a great chance that the antiserum specific to the fusion protein will react with the intact protein. An alternative

approach would be to use a eucaryotic expression system, for example, the insect baculovirus vector or vaccinia virus for expression of larger amounts of the protein. Once an antiserum has been obtained to this protein further experiments could be designed to study the role of this protein, for example to determine whether it is a DNA-binding protein, whether it is a virion protein and if so, its location in the virus particle using immunoelectron microscopy on purified virions and capsids. It would also be interesting to see whether this protein interacts with other proteins to form complexes.

4.1 FURTHER CHARACTERISATION OF THE UL26 GENE PRODUCT AND THE HSV-1 MUTANT, *TS*1201, WHICH FAILS TO PROCESS UL26 GENE PRODUCT AT THE NPT

4.1.1 Introduction

Genetic analysis of HSV-1 *ts* mutants have led to the identification of several genes involved in DNA encapsidation and capsid assembly (See section 1.9.3). However, the precise role of each of these gene products is still obscure. *Ts*1201, a DNA positive mutant of HSV-1 17*syn*$^+$, has been widely characterised, and its mutation localised in UL26. This mutant fails to package DNA and process p40 the product of UL26, to its lower mw forms at the NPT. The correct processing of the protein could be achieved, however, when mutant-infected cells were shifted from the NPT to the PT (Preston *et al.*, 1983; Addison, 1986). Recent studies have shown that p40 is transiently associated with partially-cored capsids and is not a major component of full capsids or mature virions (Rixon *et al.*, 1988). The aim of the work presented in this section was to extend the characterisation of the mutant *ts*1201 and to investigate the processing of p40 to the lower mw species.

4.1.2 Marker rescue of the lesion in *ts*1201 with a 673 bp fragment obtained from BamHI *u*

Previous marker rescue experiments showed that the *ts*1201 lesion lies within a 673bp region BamHI-SalI fragment at the right hand end of BamHI *u* (Preston *et al.*,

1983). The 3' end of gene UL25 and the 5' end of gene UL26 are located within this part of the genome (McGeoch *et al.*, 1988). The failure of *ts*1201 to process p40 to its lower mw forms at the NPT together with the finding that two mutants, *ts*1204 and *ts*1208, which contain lesions within UL25 complement *ts*1201 suggested that the *ts*1201 lesion mapped within UL26. The 673 bp BamHI-SalI fragment from pGX56 (this plasmid contains 17*syn*$^+$ BamHI *u*), pGX198 (BamHI *u* of *ts*1201), and pGX208 (BamHI *u* of *ts*1201 rev2) (figure 31) were tested for their ability to rescue the mutation in *ts*1201 before being sub-cloned into M13mp18 and M13mp19 DNA. The results (Table 11) revealed that only the *wt* virus fragment rescued the lesion in *ts*1201. These data confirmed that the *ts*1201 mutation mapped in BamHI *u*. In addition, the results suggested that the mutation responsible for the *ts*$^+$ phenotype of *ts*1201 rev2 did not lie in BamHI *u*.

4.1.3 The lesion in *ts*1201 lies 89 bp downstream from the amino-terminus of gene UL26

The 673bp BamHI-SalI fragments from pGX198 and pGX208 were ligated to M13mp18 and M13mp19 DNA, cleaved with BamHI-XbaI and transfected into JM101 competent bacteria. White plaques, containing the insert, were isolated and ssDNA prepared and sequenced using the chain termination method (see section 2.9). The nucleotide sequence of the BamHI-SalI fragment from the mutant and the revertant was compared to that of 17*syn*$^+$ wt virus. The sequence in both the mutant and revertant was identical to

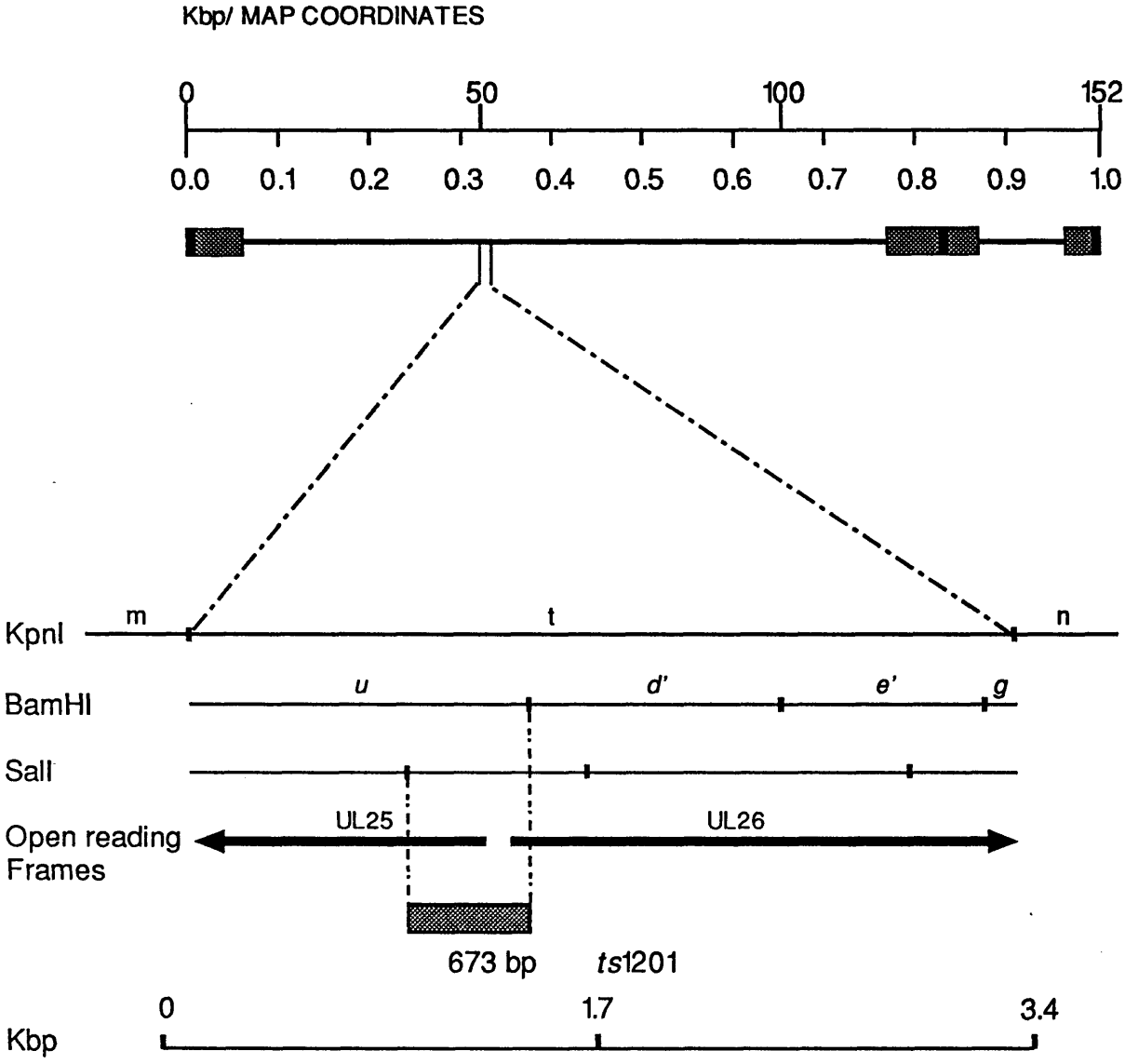

Kbp/ MAP COORDINATES
0
50
100
152
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
KpnI
m
t
n
BamHI
u
d'
e'
g
SalI
Open reading Frames
UL25
UL26
673 bp
ts1201
0
1.7
3.4
Kbp

Figure 31

Diagram showing the position of the *ts*1201 mutation within HSV genome as defined by marker rescue. The cross hatched box (673bp) BamHI-SalI (small) fragment containing the 5' end of two genes UL25 and UL26. This fragment was taken from *ts*1201 and *ts*1201 rev2, sub-cloned into M13mp18 and M13mp19 and used for sequencing analysis.

TABLE 11

Summary of marker rescue of *ts*1201

Marker rescue using clones digested with BamHI-SalI(*)	Efficiency of plating NPT/PT X10³	Rescue
pGX56 (17*syn*⁺ BamHI *u*)		
Unseparated fragments	0.818	+
Separated BamHI-SalI frag		
Small fragment	0.615	+
Large "	<0.02	-
pGX198 (*ts*1201 BamHI *u*)		
Unseparated fragments	<0.02	-
Separated BamHI-SalI frag		
Small fragment	<0.02	-
Large fragment	<0.02	-
pGX208 (*ts*1201 rev2 BamHI *u*)		
Unseparated fragments	<0.02	-
Separated BamHI-SalI frag		
Small fragment	<0.02	-
Large fragment	<0.02	-
None	<0.02	-

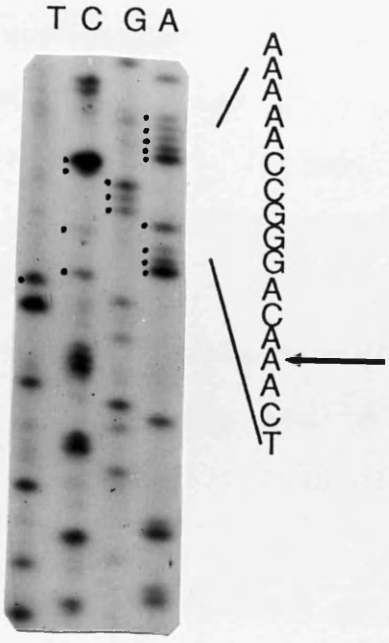
(*) The small fragment is the 673bp fragment obtained from a BamHI-SalI digest of BamHI *u*.

that of *wt* virus except for a single bp change observed in the nucleotide sequence of the mutant and the revertant corresponding to residue 50897 of the HSV-1 17*syn*$^+$ nucleotide sequence (figure 32, refer to the A track of *ts*1201 and rev2). Sequencing results obtained were consistent with the results of the marker rescue experiment showing that *ts*1201 rev2 BamHI-SalI fragment failed to rescue the lesion in the mutant and confirmed that the reversion event was located outside the 673bp BamhI-SalI fragment. Since residue 50897 was shown to be located 89 bp from the 5' end of UL26, it was concluded that *ts*1201 lesion lies in UL26 (figure 33).

4.1.4 Cloning gene UL26 (kpnI *t*)of *ts*1201 rev2 and rev3

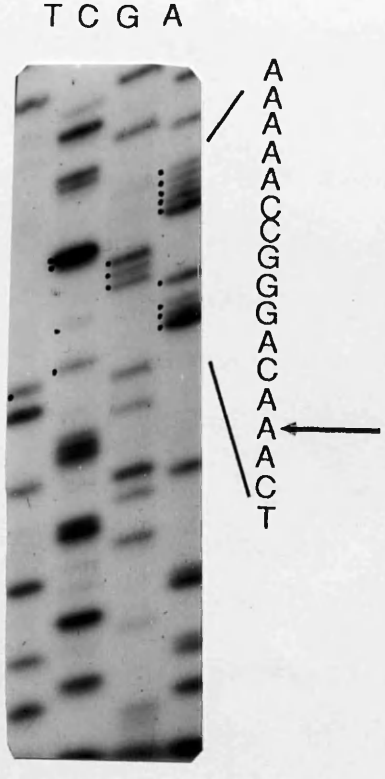
The results of marker rescue experiments and sequence analysis of *ts*1201 DNA and *ts*1201 rev2 DNA revealed that the *ts*$^+$ revertant for growth retained the bp change of the mutant. However, *ts*1201 rev2 and a number of other revertants were originally isolated at 38.5°. The *wt* virus, *ts*1201 rev2, and another *ts*$^+$ revertant *ts*1201 rev3 were therefore titrated at different temperatures to check whether the revertant viruses were *ts* at temperatures higher than 38.5°. Results of the titration revealed that *ts*1201 rev2 was slightly *ts* at 39° but at 39.5° had an $eop^{NPT/PT}$ of <0.0002. *Ts*1201 rev3, however, was only slightly *ts* at 39° and 39.5° (table 12). The KpnI *t* fragments, containing the whole nucleotide sequence of gene UL26 and part of the sequences of gene UL25, were isolated from KpnI digested *ts*1201 rev2 and rev3 virus DNAs and

(A)



*Ts*1201

(B)



*Ts*1201 rev2

Figure 32

Portion of autoradiographs of DNA sequencing gels. Sequence products were separated on a 6% denaturing polyacrylamide gels containing 9M urea.

A) Shows the base pair alteration in *ts*1201 (arrow) in the adenine track.

B) Shows the same base pair retained in *ts*1201 rev2 (arrow).

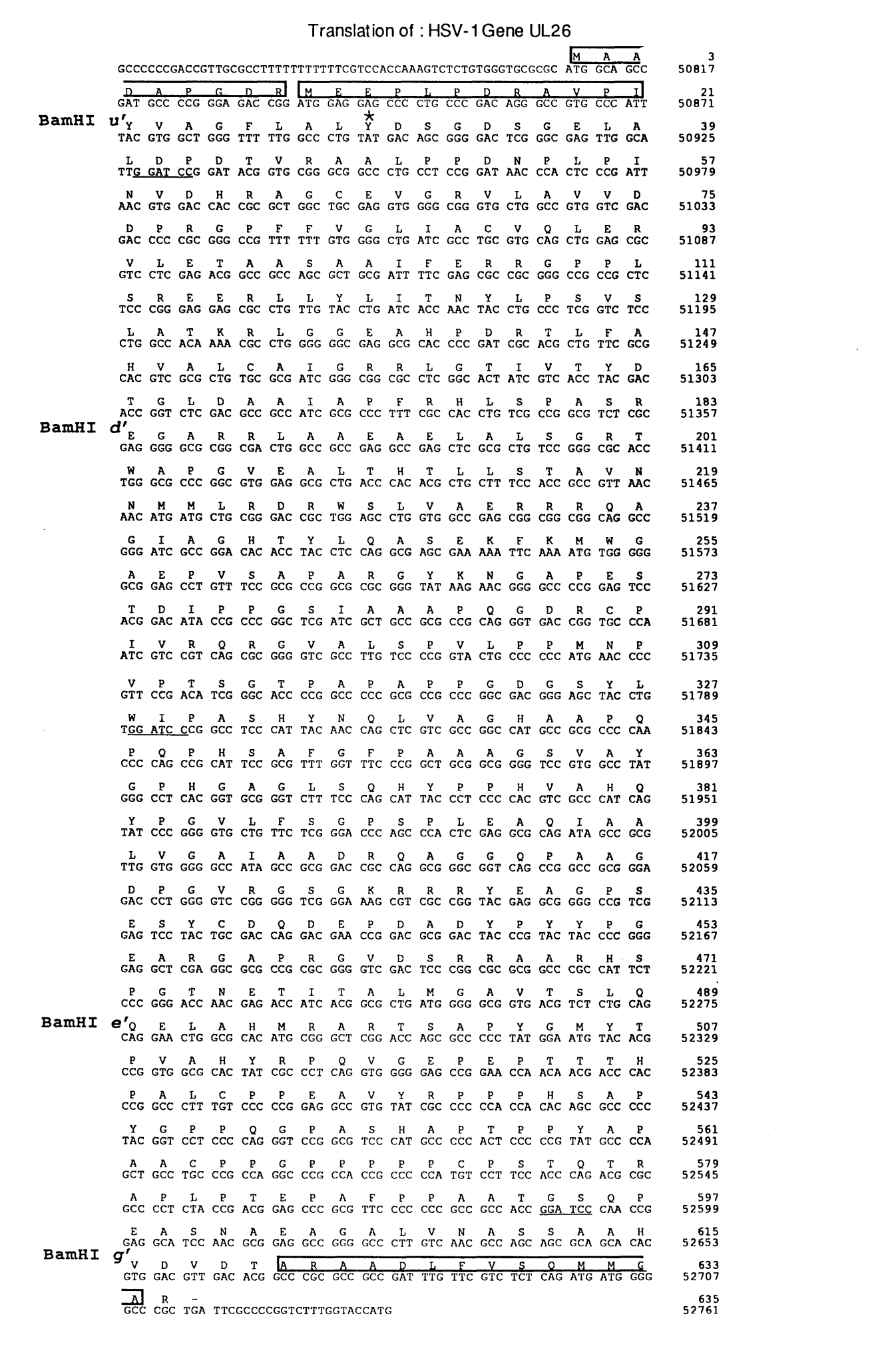

Translation of : HSV-1 Gene UL26
BamHI u'
BamHI d'
BamHI e'
BamHI g'

Figure 33

Translation of gene UL26 (McGeoch etal., 1988). The (*) at residue 50897 denotes the position of the base pair alteration in *ts*1201. The (boxes) represent the amino acid sequence of the peptide synthesised and used for preparing antipeptides antibody, Below is a comparison between *wt* and *ts*1201 sequence and the change in the amino acid sequence (bold). The amino acid composition of UL26 is also given.

HSV-1 17*syn*$^{+}$ seq. 5′ GCCCTG**TAT**GACAGC 3′
CGGGAGATACTGTCG

*Ts*1201 seq. 5′ GCCCTG**TTT**GACAGC 3′
CGGGACAAAGTGTCG

Change in amino acid sequence:

Tyrosine ⟶ Phenylalanine

------ UL26 AMINO ACID COMPOSITION ------

Ala	102	16.1%	Val	37	5.8%	Leu	47	7.4%	Ile	16	2.5%
Gly	57	9.0%	Pro	86	13.5%	Cys	8	1.3%	Met	11	1.7%
His	20	3.1%	Phe	12	1.9%	Tyr	23	3.6%	Trp	4	0.6%
Asn	11	1.7%	Gln	21	3.3%	Ser	39	6.1%	Thr	31	4.9%
Lys	5	0.8%	Arg	46	7.2%	Asp	28	4.4%	Glu	31	4.9%

Approximate Molecular Weight = 66466.92

TABLE 12

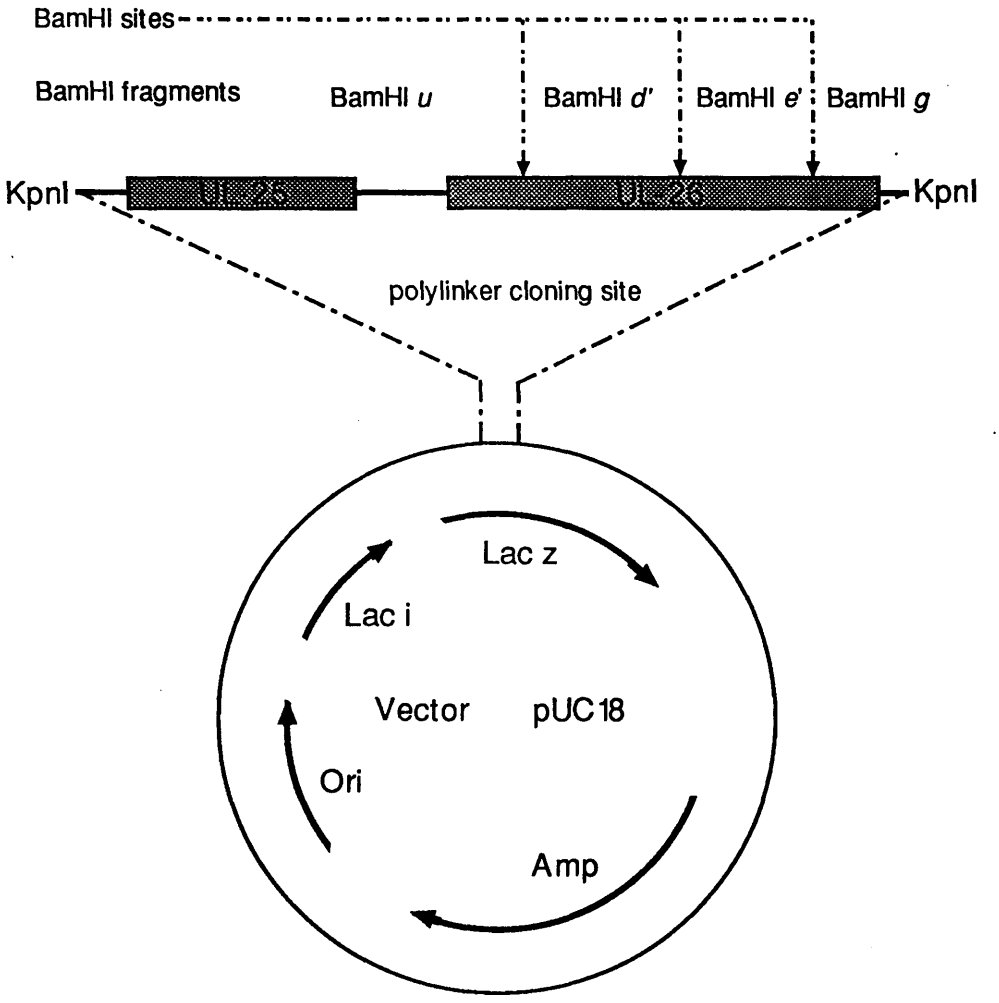
Efficiency of plating ($EOP^{NPT/PT}$) of HSV-1 17*syn*+, *ts*1201 rev2 and *ts*1201 rev3 at various NPT temperatures

$eop^{NPT/PT}$ Virus	NPT= 38.5°	NPT= 39°	NPT= 39.5°
HSV-1 17*syn*+	0.64	0.33	0.31
*ts*1201 rev.2	0.61	0.01	<0.0002
*ts*1201 rev.3	0.73	0.09	0.06

cloned into the KpnI site of pUC18 (figure 34). Two chimeric plasmids, one containing KpnI *t* of *ts*1201 rev2 (referred to as pGX209) and the other containing *ts*1201 rev3 (referred to as pGX210), were isolated. These clones were checked for the presence of various restriction endonuclease sites to confirm that the correct fragment had been cloned.

4.1.5 Marker rescue of the lesion of *ts*1201 with cloned KpnI *t*

Separated restriction endonuclease fragments were used in marker rescue experiments to determine the location of the reversion to *wt* virus temperature-sensitivity in *ts*1201 rev2 and rev3. Initially, plasmids pGX142 (contains *wt* virus KpnI *t*), pGX209 (contains *ts*1201 rev2 KpnI *t*) and pGX210 (contains *ts*1201 rev3 KpnI *t*) were digested with KpnI or BamHI and tested for their ability to marker rescue the lesion of *ts*1201. A high level of rescue was obtained with all three plasmids digested with KpnI. No significant level of rescue, however, was obtained with the KpnI *t* of *ts*1201 rev2 clone, digested with BamHI (table 13). Separated fragments obtained from BamHI digests of cloned KpnI *t* from pGX142, pGX209, and pGX210 were used in marker rescue experiments. The results revealed that the *wt* virus BamHI *u* fragment from pGX142 was able to rescue the lesion in *ts*1201 as expected but, interestingly, *wt* virus BamHI *d'* also rescued the *ts* mutation albeit at a lower efficiency. *Ts*1201 rev2 BamHI *u* from pGX209 failed to rescue the lesion, which is consistent with previous findings showing



pGX209 ts1201 Rev.2

pGX210 ts1201 Rev.3

Figure 34

A diagram representing the Kpn *t* fragment containing the whole UL26 gene and part of UL25 of *ts*1201 rev2 and *ts*1201 rev3 cloned into KpnI site of pUC18. Four BamHI fragments are obtained with a BamHI digest. The BamHI fragments were used in marker rescue experiments.

TABLE 13

Summary of marker rescue of *ts*1201 with different KpnI and BamHI fragments from cloned KpnI *t* of *wt*, *ts*1201 rev2, and rev3 viruses

fragments obtained from HSV-1 cloned KpnI *t*(*)	Efficiency of plating NPT/PT X10³	Rescue
pGX142 (contains 17*syn*⁺ KpnI *t*) digested with KpnI	30.00	+
BamHI *u* fragment	8.66	+
" *d'* "	0.49	+
" *e* "	<0.02	-
" *g* "	<0.02	-
pGX209 (contains *ts*1201 revertant 2 KpnI *t*) digested with KpnI	40.00	+
digested with BamHI	0.63	+
BamHI *u* fragment	<0.02	-
" *d'* "	<0.02	-
" *e'* "	<0.02	-
" *g* "	<0.02	-
pGX210 (contains *ts*1201 revertant 3 KpnI *t*) digested with KpnI	70.00	+
BamHI *u* fragment	<0.02	-
" *d'* "	3.76	+
" *e'* "	<0.02	-
" *g* "	<0.02	-
None	<0.02	-

(*) When clones are digested with BamHI, four fragments are obtained. The sequences of the vector are attached to BamHI *u* and BamHI *g*.

that the *ts*1201 lesion within BamHI *u* was retained in *ts*1201 rev2 DNA (Sections 4.1.2; 4.2.3). It was also clear from marker rescue data that *ts*1201 rev3 DNA also retained *ts*1201 mutation in BamHI *u*. The BamHI *d'* fragment from pGX210 rescued *ts*1201, suggesting that the reversion event in *ts*1201 rev3 mapped within BamHI *d'* (table 13). None of BamHI fragments from KpnI of *ts*1201 rev2 rescued the mutation and, therefore, it is likely that the second site reversion lies close to a BamHI site in *ts*1201 rev2.

4.2 PROCESSING OF UL26 GENE PRODUCT

4.2.1 Isolation and detection of antibodies against UL26 gene products

In immunoblotting experiments, six UL26 gene product have been identified in HSV-1 infected cell extracts, using a monoclonal antibody specific to UL26 gene product (Braun *et al.*, 1984b). When HSV-1 infected cell mRNA was translated *in vitro*, two specific gene products were detected in immunoprecipitation experiments using a monoclonal antibody to UL26 gene product. This result suggests that either there are two primary *in vitro* translational products of UL26 gene or there is some processing occurring in the *in vitro* translational assay. Analysis of the predicted sequence of UL26 gene has shown that there is a second potential AUG codon 27 residues from the first methionine amino acid and therefore, it is likely that there are two primary translational products (V. Preston, personal communication). When *wt* virus infected cells were pulse-labelled with [^{35}S]-methionine,

several gene products of higher apparant mw than *in vitro* translated products were made, suggesting that there is a rapid modification of the UL26 gene product. If the [^{35}S]-methionine was removed from the medium and incubation of virus-infected cells continued for a further 4-5h, lower mw products were formed (Preston *et al.*, 1983). It has been proposed that those low mw forms result from proteolytic cleavage (Gibson and Roizman, 1972; Braun *et al.*, 1984b). In addition evidence from the work by Braun *et al.* (1984b) suggests that some of UL26 gene products are phosphorylated. It is clear that there is multiple processing of UL26 gene product (s). To confirm that there are two translation products and to investigate the processing of UL26 gene product, three synthetic oligopeptides, specific to different regions of the UL26 gene, were synthesised for the purpose of raising antibodies against these peptides. An oligopeptide representing the first 9 amino-acids, an oligopeptide representing the following 12 amino-acids from the amino-terminus and an oligopeptide representing 14 amino-acid from the carboxy-terminus were prepared (figure 33). A tyrosine was added to the ends of the carboxy terminal peptide and to the end of the oligopeptides specific to the amino terminus to facilitate coupling to β-galactosidase. Interestingly, the two peptides which were made against the amino terminus dissolved easily in borate buffer in comparison to the carboxy terminus peptide which left traces of what appeared to be undissolved impurities, and since the amount of purity of this peptide was 66% as measured by the HPLC

(Miss Ania Owsianka, personal communication), it was decided to use twice the amount of the carboxy terminus peptide. All three peptides were coupled to β-galactosidase, emulsified in adjuvent, and injected into rabbits (table 14) as described in section 2.12.1. Sera were collected before each injection and after the final injection. Antibodies specific to the injected peptides were detected by radioimmunoassay. Figure 35 shows [^{125}I] counts obtained from radioimmune assay using sera from 6 rabbits injected with one of the three peptides. Preimmune sera gave background counts when compared to sera isolated from rabbits injected with peptides. Thus, it is clear that all the sera contained antibodies which recognised the antigen against which they were raised.

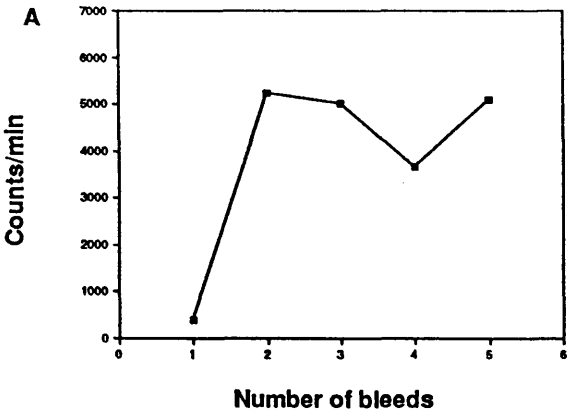
4.2.2 Detection of UL26 gene product by western blotting

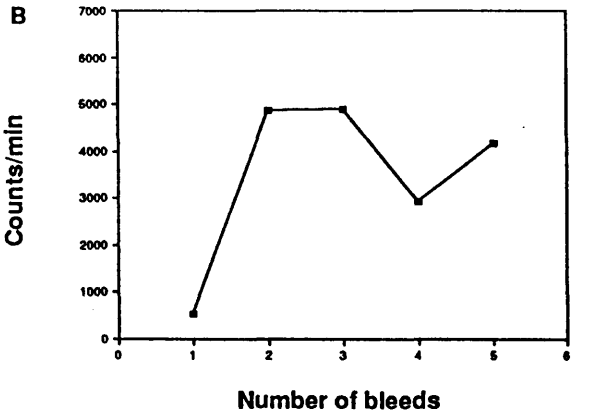
Virus-infected cell extracts, labelled with [^{35}S]-methionine, were separated by SDS PAGE, and transferred to nitrocellulose strips (refer to section, 2.12.5). The strips were incubated in different dilutions of serum, washed, dried and autoradiographs prepared as described in section (2.12.6). Analysis of autoradiographs revealed the following: Sera from rabbits injected with the two oligopeptides specific to the amino terminal end of UL26 gene product gave too weak a reaction to be of any use, while sera from the two rabbits injected with carboxy-terminus oligopeptide reacted strongly with an HSV-1 17*syn*$^+$ specific polypeptide band (figure 36). No polypeptide of this mw was detected in radiolabelled mock-infected cell

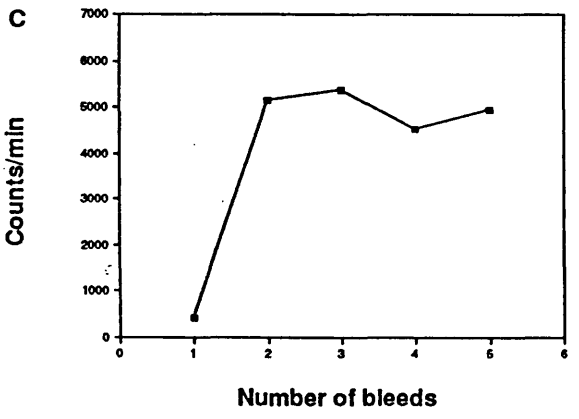
TABLE 14

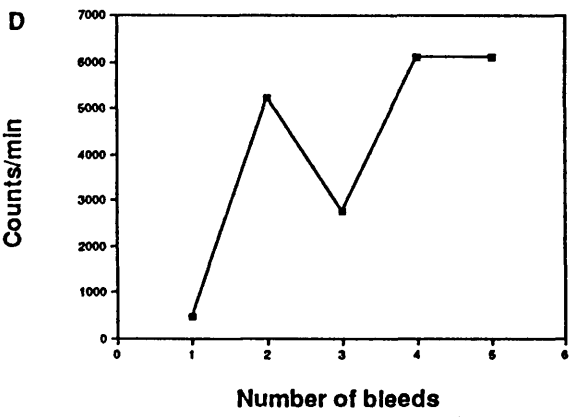
The injection protocol of UL26 peptide coupled to β-galactosidase

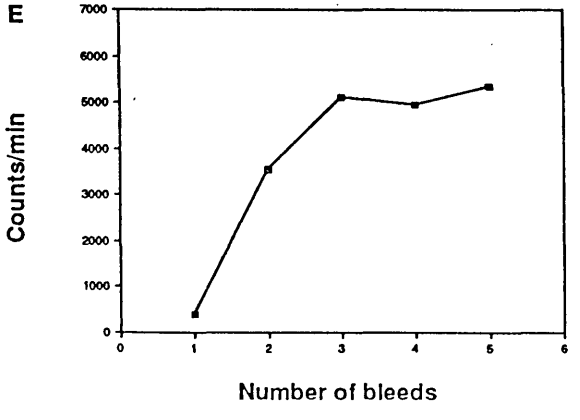
Peptide code	Peptide representing	no. of injection	purity%	peptide/ml
147	9 A.A from the First AUG methionine at NH_2-terminus	5	86%	150µg
148	12 A.A from the second AUG methionine at NH_2-terminus	"	89%	150µg
149	14 A.A from the COOH-terminus	"	66%	300µg


A
Counts/min
Number of bleeds


B
Counts/min
Number of bleeds


C
Counts/min
Number of bleeds


D
Counts/min
Number of bleeds


E
Counts/min
Number of bleeds

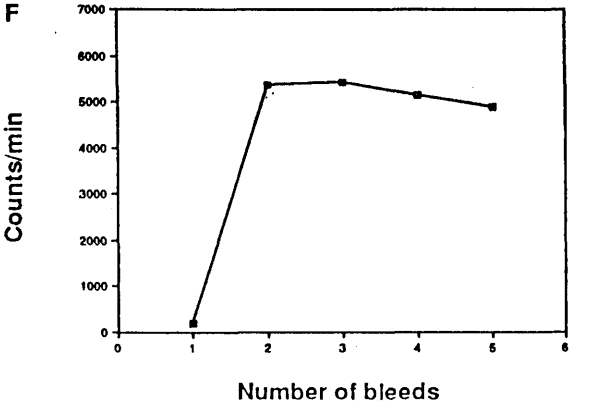

F
Counts/min
Number of bleeds

Figure 35

The graphs shows the results obtained from radio-immunoassay experiment using sera from animals injected with either the first amino terminus (A and B) or second amino terminus (C and D) or the carboxy terminus (E and F) peptides coupled to β-galactosidase. The pre-immune serum was the first bleed. Post immune bleeds were obtained prior to booster injection with the peptide.

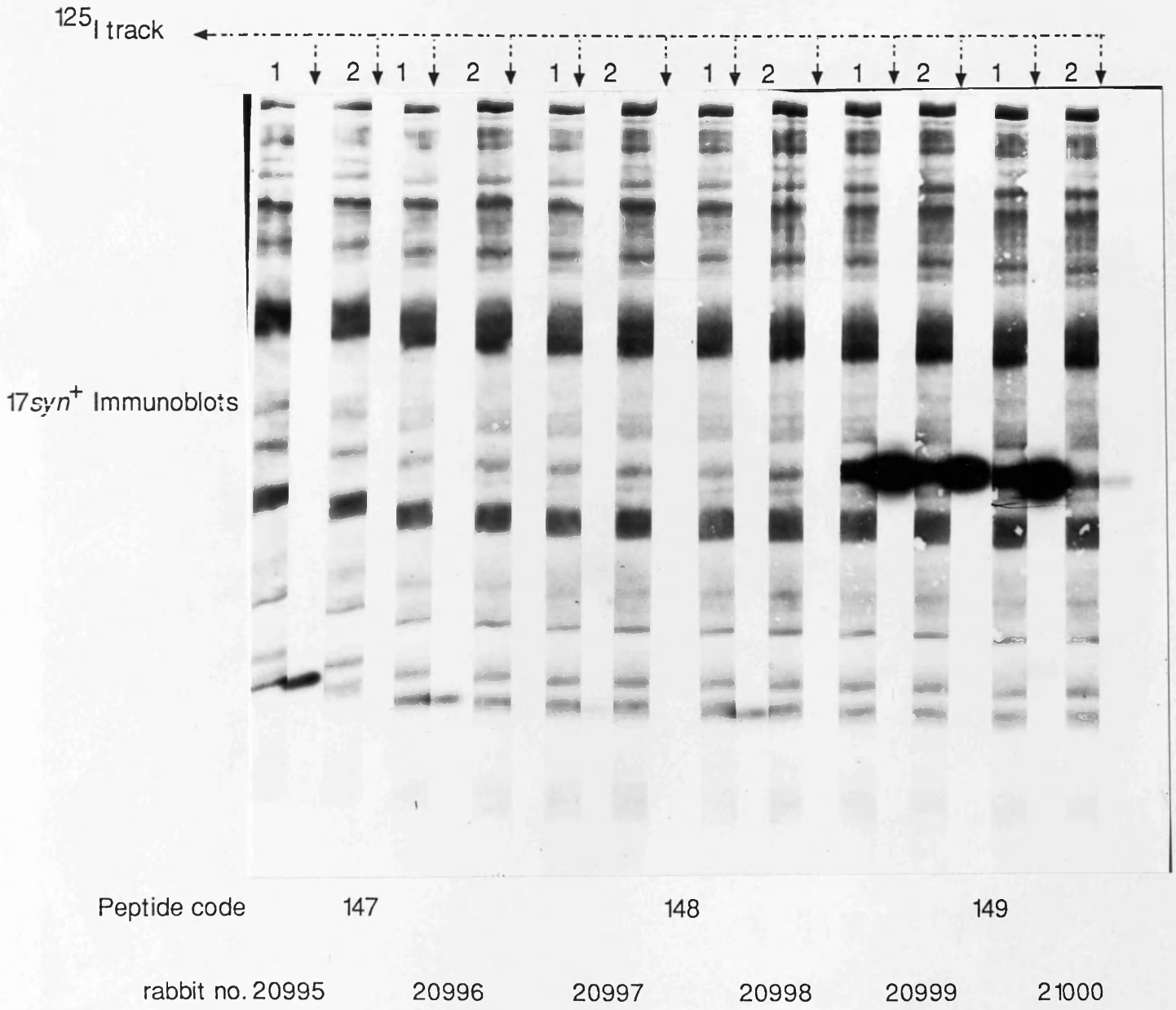

^{125}I track
1 2 1 2 1 2 1 2 1 2 1 2
17*syn*$^{+}$ Immunoblots
Peptide code
147
148
149
rabbit no. 20995
20996
20997
20998
20999
21000

Figure 36

Immunoblots of HSV-infected cell polypeptides labelled with [^{35}S]-methionine at 9hpi and harvested at 20hpi. Polypeptides were separated on 12.5% polyacrylamide SDS gel and transferred to nitrocellulose strips. Different dilutions were used (1: 1/10 and 2: 1/100) for each antisera. Sera taken from six rabbits were tested as described in sections 2.12.5 and 2.12.6. [^{125}I] autoradiographs were aligned with [^{35}S]-methionine autoradiographs.

Rabbits, 20995 and 20996, were injected with peptide 147 representing the first 9 amino acids at the amino terminus.

Rabbits, 20997 and 20998, were injected with peptide 148 representing 12 amino acids from the amino terminus following peptide 147.

Rabbits, 20999 and 21000, were injected with peptide 149 representing the carboxy terminus. The strong bands in the [^{125}I] track represent a specific reaction with a protein of 40,000mw.

extracts (figure 37). Sera taken from one rabbit at different time intervals after injection with the carboxy-terminus peptide were compared to the monoclonal antibody 5010B, specific to p40. The results showed (figure 38) that antibodies in preimmune serum gave no specific reaction whereas all sera obtained after the first injection contained antibodies which recognised a specific polypeptide band with a mw of approximately 40,000, similar to that detected by the monoclonal antibody. These findings are also supported by the results obtained from radioimmune assay for the detection of antibodies after each injection (fig 35, E and F). Thus, the polyclonal antibody against the carboxy-terminus of UL26 gene product appeared to be recognising UL26 gene products.

4.2.3 Evidence that the antibody is specific to UL26 gene product (P40)

To confirm that the polyclonal antibodies, raised against the carboxy terminus of the predicted primary UL26 gene product were reacting specifically with UL26 gene product, high concentrations of the carboxy terminus peptide were included in the immune precipitation reaction. If the antipeptide antibody was recognising UL26 gene products then the peptide should competitively inhibit immunoprecipitation of UL26 gene products. When [^{35}S]-methionine labelled virus-infected cell extracts were incubated with the antibody, a band of a around 40,000 mw was immuno-precipitated. The addition of 10µg of peptide to 50µl (10^6 cpm) of virus-infected cell extract caused a

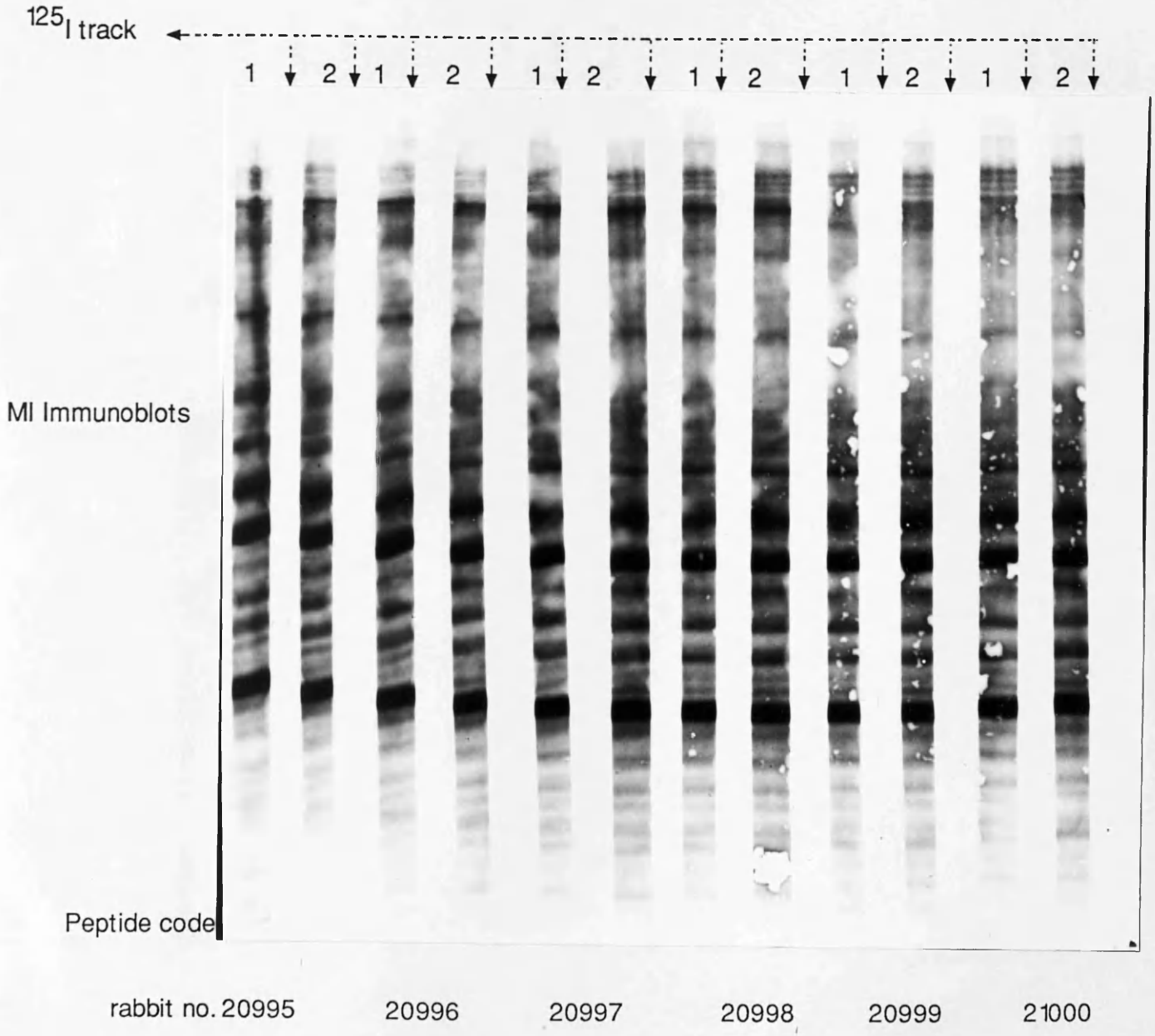

125I track
1 2 1 2 1 2 1 2 1 2 1 2
MI Immunoblots
Peptide code
rabbit no. 20995
20996
20997
20998
20999
21000

Figure 37

Immunoblots of mock-infected cells treated as described in the legend to figure 36.

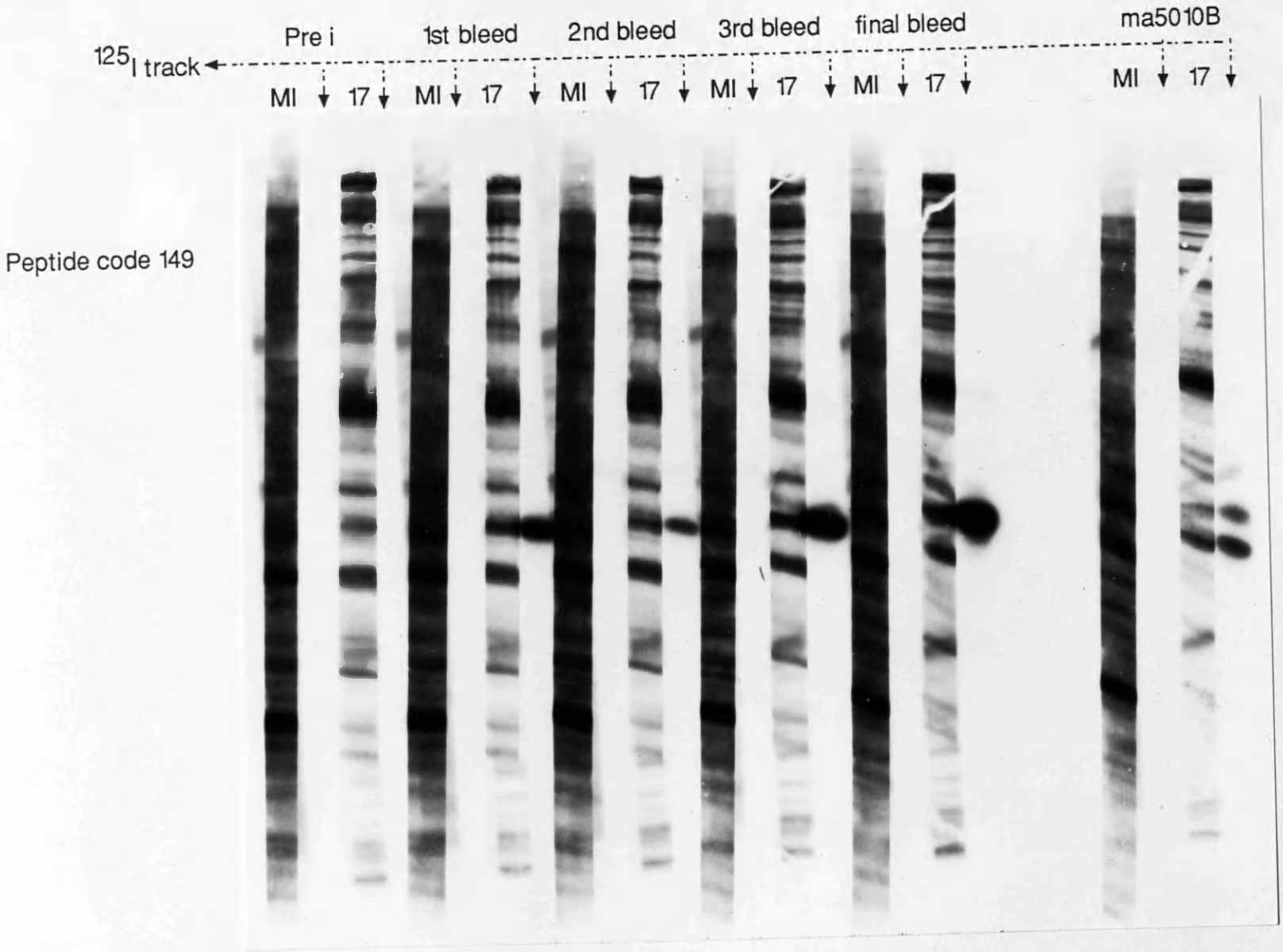

125I track
Pre i
1st bleed
2nd bleed
3rd bleed
final bleed
ma5010B
MI
17
MI
17
MI
17
MI
17
MI
17
MI
17
Peptide code 149

Figure 38

Immunoblot experiment using antisera, taken at different time intervals after the initial injection with the carboxy terminal peptide 149, specific to p40. Mock-infected (MI) and 17*syn*$^{+}$-infected (17) cell polypeptides labelled with [^{35}S]-methionine, were separated on a 12.5% polyacrylamide SDS gel, transferred to nitocellulose strips and incubated either with a monoclonal antibody 5010B, specific to p40 or with anticarboxy terminus antisera. Tracks indicated by the arrows represent reaction of antisera, bound to [^{125}I] protein A, to p40. The remaining tracks show either mock-infected (MI), or 17*syn*$^{+}$-infected cell polypeptide labelled with [^{35}S]-methionine.

reduction in the intensity of the immunoprecipitated polypeptide band detected on SDS polyacrylamide gel (figure 38), while the addition of 100µg of peptide completely inhibited immunoprecipitation of 40,000 mw polypeptide by the antipeptide antibody (figure 39). Therefore, it was concluded that the antibodies raised against the carboxy-terminus were specific for UL26 gene product.

4.2.4 UL26 gene product is processed at the carboxy terminus

To investigate whether the UL26 gene product is processed at the carboxy-terminus, *wt* virus- and *ts*1201 virus-infected cells, grown at the NPT or PT, were labelled with [^{35}S]-methionine for 15 min at 5hpi and either harvested or incubated for a further 5h in culture medium lacking [^{35}S]-methionine prior to harvesting. Cell extracts were prepared and incubated either with monoclonal antibody (ma) specific for p40 (5010B) or with polyclonal antibody directed against the carboxy-terminus of p40. Antibody-antigen complexes were bound to protein A sepharose, washed, eluted and analysed by SDS PAGE. Autoradiographs of immune precipitates (figure 40 and 41) revealed that in the 31° and 39° pulse-labelled virus-infected cell extracts, the polyclonal antibody recognised similar high mw species of UL26 gene product to those immuno-precipitated by ma5010B. In the 31° virus-infected cell samples, which had been incubated for 5h after being labelled with [^{35}S]-methionine, the polyclonal antibody

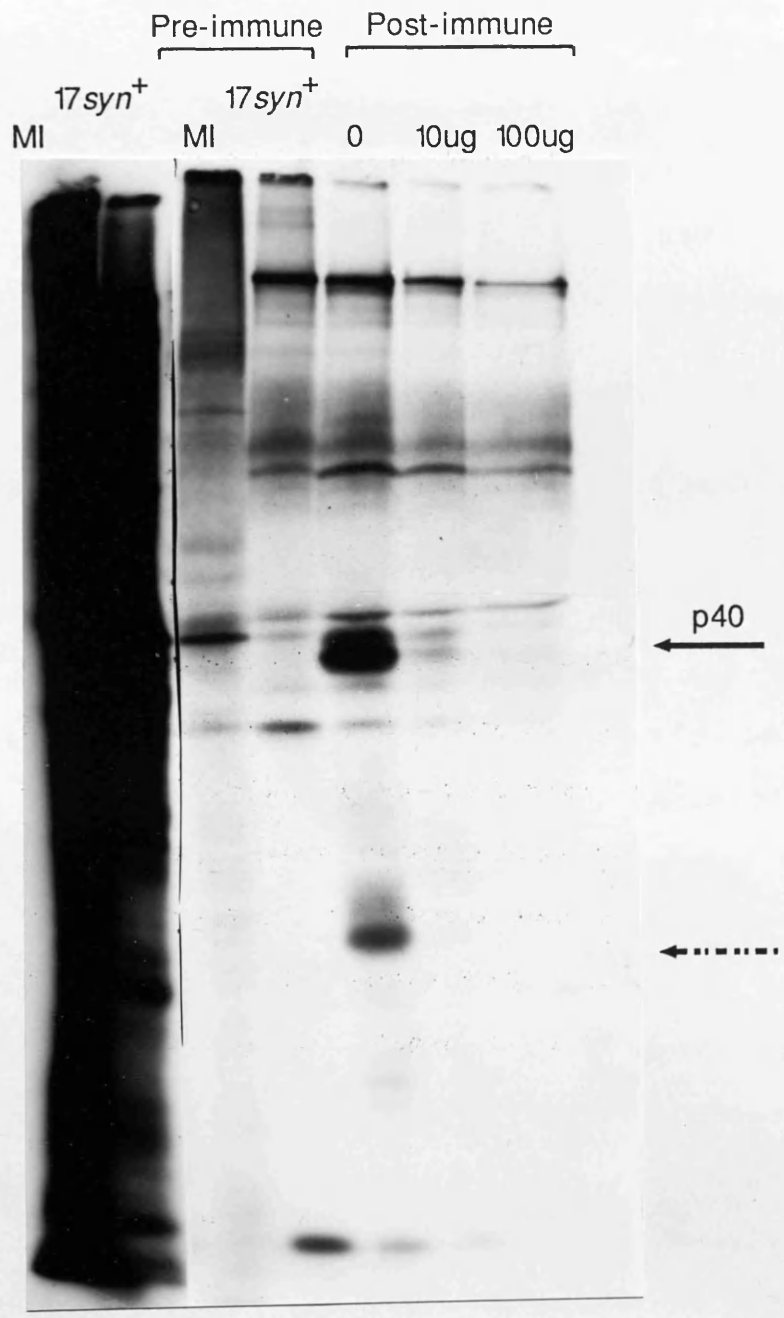

Pre-immune
Post-immune
17syn+
17syn+
MI
MI
0
10ug
100ug
p40

Figure 39

Immunoprecipitation with an antipeptide antisera. Proteins labelled with [^{35}S]-methionine were precipitated from MI (lane 1) and HSV-1 infected cell extracts with either preimmune (lane 2) or post-immune sera (lane 3-5). Anti carboxy terminus serum was incubated in the presence of 0, 10ug and 100ug of peptide 149 (lane 3-5). Arrows represent p40. Dotted arrow indicates break down products of p40.

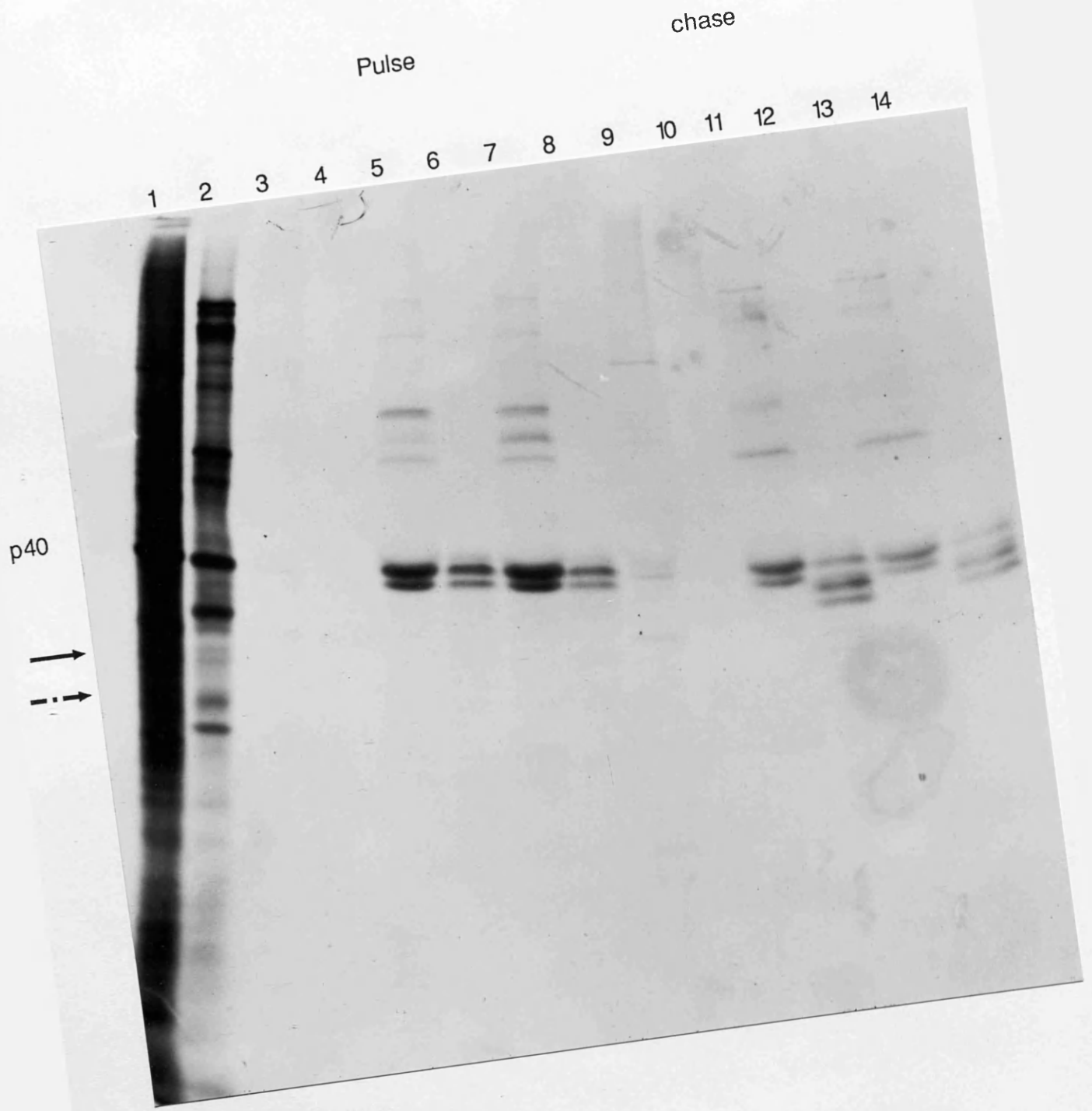

Pulse
chase
1 2 3 4 5 6 7 8 9 10 11 12 13 14
p40

Figure 40

Autoradiograph of immuneprecipitates of p40 from BHK infected at the 31° with *wt* virus (track 4, 7, 8, 10, 13 and 14), or *ts*1201 (track 5, 6, 11, 12). Virus-infected or mock- infected cells were pulse-labelled at 6hpi with [^{35}S]-methionine and either harvested immediately (pulse) or after incubation for a further 5h in EFC_{10} (chase). Immunoprecipitation was performed as described insection 2.11.2 using a ma5010B, specific to p40 (tracks 6, 8, 12, 14) or with an antipeptide antibody against the carboxy terminus (tracks 5, 7, 11, 13). Mock-infected cell extracts (tracks 3 and 9) and *wt*-infected cell extracts (tracks 4 and 10) were incubated with control non-immune rabbit serum instead of the antisera. Tracks 1 and 2 represents whole cell extracts of mock-infected cells and 17*syn*$^+$-infected cells, respectively. The polypeptides were analysed on a 8% polyacrylamide SDS gel.

Solid arrows represent high mw forms of p40.

dotted arrows " low " " " " .

failed to recognise the low mw processed forms of UL26 gene product detected by ma5010B (fig 40). At 39° (fig 41) *ts*1201 is unable to process the UL26 gene product to its lower mw forms (Preston *et al.*, 1983) and therefore, only the high mw species of UL26 gene product were detected by ma5010B in the 39° *ts*1201-infected cell sample incubated for 5h at 39° after removal of [^{35}S]-methionine. The same high mw forms were detected by the polyclonal anti-oligopeptide antibody in this extract and in *wt* virus infected cells treated in the same manner, whereas both high and low mw species in *wt*-virus infected cell sample were recognised by ma5010B (figure 41). Information from this experiment suggests that UL26 gene product is processed at the carboxy-terminus and that this step results in the formation of lower mw forms. *Ts*1201 clearly has a block in processing at the carboxy terminus of UL26 gene product.

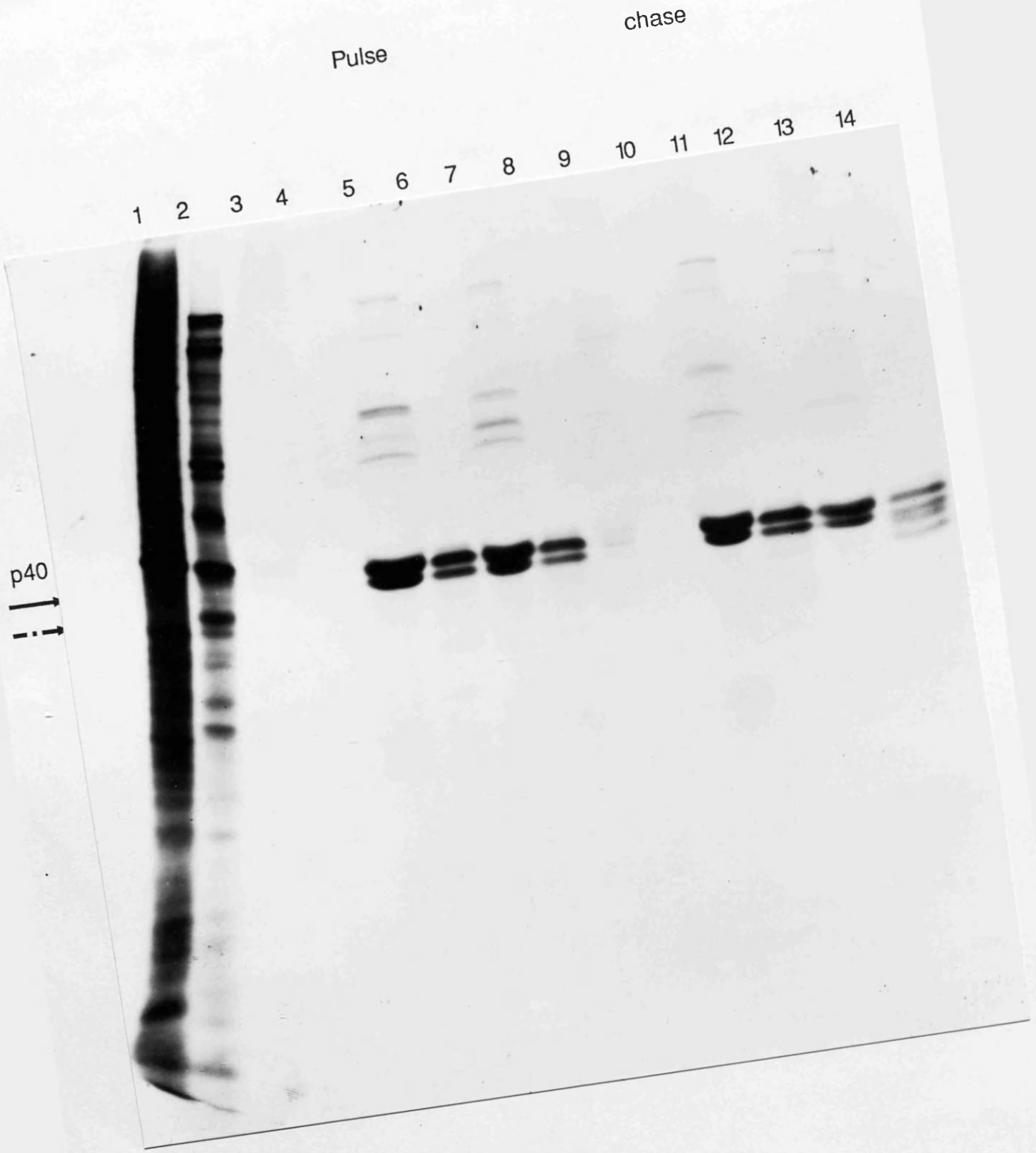

Pulse
chase
1 2 3 4 5 6 7 8 9 10 11 12 13 14
p40

Figure 41

Autoradiograph of immuneprecipitates of p40 from BHK infected at the 39° with *wt* virus (track 4, 7, 8, 10, 13 and 14), or *ts*1201 (track 5, 6, 11, 12). Virus-infected or mock- infected cells were pulse-labelled at 6hpi with [^{35}S]-methionine and either harvested immediately (pulse) or after incubation for a further 5h in EFC_{10} (chase). Immunoprecipitation was performed as described insection 2.11.2 using a ma5010B, specific to p40 (tracks 6, 8, 12, 14) or with an antipeptide antibody against the carboxy terminus (tracks 5, 7, 11, 13). Mock-infected cell extracts (tracks 3 and 9) and *wt*-infected cell extracts (tracks 4 and 10) were incubated with control non-immune rabbit serum instead of the antisera. Tracks 1 and 2 represents whole cell extracts of mock-infected cells and 17*syn*$^+$-infected cells, respectively. The polypeptides were analysed on a 8% polyacrylamide SDS gel.

Solid arrows represent high mw forms of p40.

dotted arrows " low " " " " .

4.3 DISCUSSION

In this study the UL26 gene has been further characterised together with the HSV-1 mutant *ts*1201. Sequence analysis of the lesion of *ts*1201 confirmed that the mutation mapped in gene UL26. Interestingly, both *ts*$^+$ revertants analysed retained the *ts*1201 mutation, although they had an eop$^{NPT/PT}$ similar to that of *wt* virus at the NPT of 38.5°. At 39° or 39.5°, however, these revertants showed some impairment of growth. Experiments with anti-oligopeptide antibodies, specific to UL26 carboxy-terminus, showed that the UL26 gene product undergoes processing at the carboxy terminus.

Map location of *ts*1201 lesion

Previous work located the *ts*1201 lesion to within a 673bp BamHI-SalI fragment at the right hand end of BamHI *u* (Preston *et al.*, 1983). This result has been confirmed during this study by sequencing the corresponding fragment from *ts*1201. The mutation was located 89bp downstream from the first AUG initiation codon at residue 50897. The change in the nucleotide sequence results in the substitution of a tyrosine by phenylalanine at position 30 from the first methionine amino acid in gene UL26. The nucleotide sequence of *ts*1201 rev2 was identical to that of *ts*1201 in this region. Marker rescue data showed that *ts*1201 rev3 also retained the *ts*1201 mutation. It was clear both from the temperature-sensitivity of *ts*1201 rev2 and *ts*1201 rev3 at 39° and 39.5°, and from the results of marker rescue experiments that these viruses had second

site reversions in different places within the UL26 gene. Interestingly, a low level of rescue of *ts*1201 lesion was obtained when *wt* virus BamHI *d'* fragment was used in marker rescue experiments. This result suggested that *ts*1201 may also have a mutation within BamHI *d'*. Since this work was done, cloned BamHI *u* and two independent BamHI *d'* clones from *wt* virus were screened for the ability to rescue the *ts*1201 lesion. Cells transfected with *ts*1201 and BamHI *u* yielded progeny virus with a high proportion of ts^+ virus, whereas cells transfected with the mutant and either of the BamHI *d'* clones gave progeny virus with a relatively low proportion of ts^+ virus. The resulting ts^+ progeny were analysed for temperature-sensitivity at 38.5° and 39.5°. Surprisingly, all the ts^+ virus formed plaques at 39.5° as efficiently as *wt* virus (V. Preston, personal communication). This result indicates that *ts*1201 probably has a single *ts* mutation but it will be necessary to sequence the entire UL26 gene of the mutant to confirm this.

The nature of *ts*1201 mutation

The mutant, *ts*1201 (Preston *et al.*, 1983), was derived from the multiple mutant 17*ts*JC116 (Coates, 1982) which was isolated from a UV-mutagenised *wt* HSV-1 17*syn*. As indicated in this study the transversion occurred at a single pyrimidine (thymine on the complementary strand) (figure. 33). Studies on the mechanism of UV-induced mutations in *E.coli lacI* gene has shown that one third of the mutations occured at single pyrimidines, and their

origin cannot be explained by a targeted event at a cyclobutane dimer or a [6-4] dipyrimidine photoproduct. These mutations therefore could have resulted from other, less frequent, photodamages (Schaaper *et al.*, 1987). The *ts*1201 mutation could also have arisen as a consequence of a similar untargeted event, and in this respect, differs from the *ts*1233 mutation (chapter 3) which can be explained on the basis of a targeted photodamage event occuring at a purine-pyrimidine-pyrimidine. It is possible that the *ts*1201 mutation could have arisen in 17*ts*JC116 virus as a consequence of errors during excision-repair.

The effect of *ts*1201 mutation on protein conformation

The Chou and Fasman computer program (Chou and Fasman, 1978) was used to determine whether the mutation in *ts*1201 affected the secondary structure of gene UL26. Interestingly, the the mutation in UL26 is located in a region predicted to form a β-sheet (figure 42, 43). The mutation induces a turn in the protein. Such an effect on the protein struc ture might result in a thermolabile protein.

Use of polyclonal antibodies specific to UL26 gene product

As mentioned earlier in section (4.2.1), three peptides were selected from different regions of UL26 to raise oligopeptide antibodies. Although the antisera isolated from rabbits all contained antipeptide antibodies. Only the oligopeptide antibodies specific to the carboxy terminus gave a strong specific reaction with p40. One

PLOTSTRUCTURE of: UL26-PROTEIN.

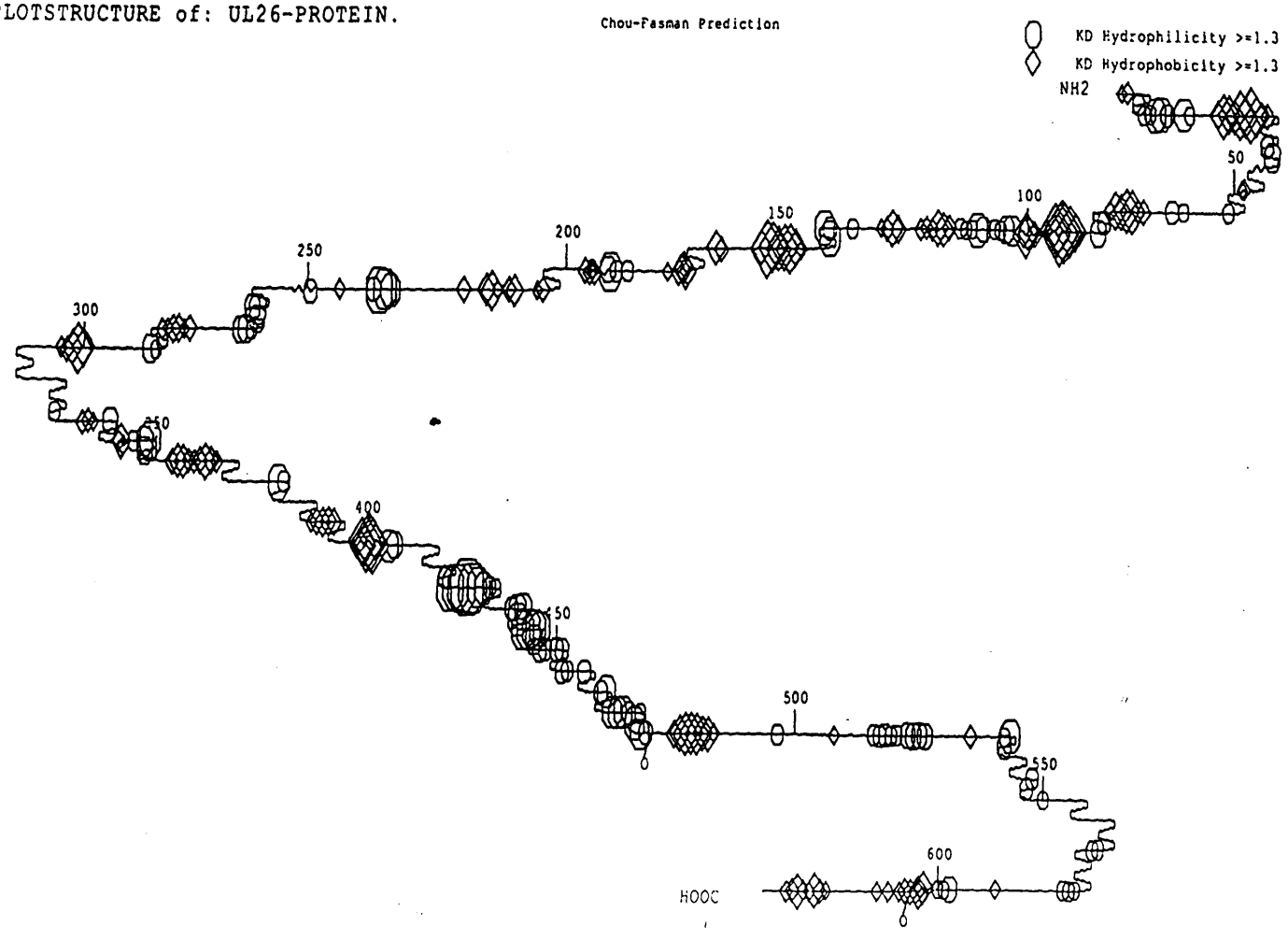


Figure 42

Predicted secondary structure of UL26 protein.

The structure is based on Chou and Fasman computer programme which predicts helices, sheets and turns according to Chou and Fasman (1978). (see figure 29)

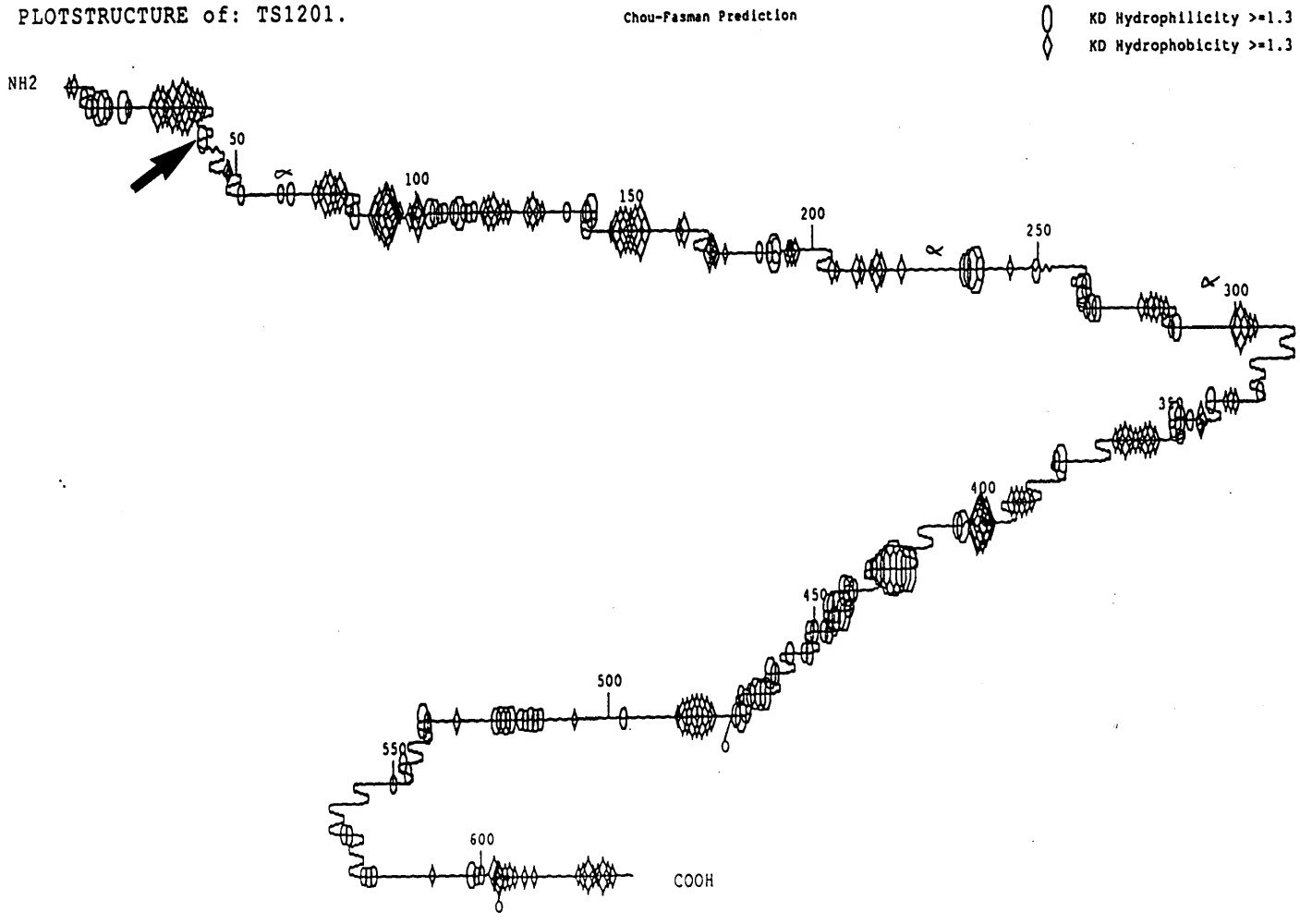

PLOTSTRUCTURE of: TS1201.
Chou-Fasman Prediction
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
200
250
300
350
400
450
500
550
600
COOH

Figure 43

Predicted secondary structure of *ts*1201 UL26 protein.
The structure is based on Chou and Fasman computer programme which predicts helices, sheets and turns according to Chou and Fasman (1978). (see figure 29).
The position of the mutation within the secondary structure is indicated by an arrow.

reason both peptides directed against the amino terminus did not show a strong specific reaction to p40 is that the length of the two peptides (9 and 12 amino acids) was not long enough to induce antibodies that react with UL26 gene product.

Processing of UL26 gene product

Previous work by Braun *et al.* (1984b) has demonstrated that the UL26 gene product (ICP35 or p40) consists of a family of polypeptides with a mw ranging between 39,000 to 55,000. Cell fractionation experiments suggested that higher mw forms are cytoplasmic precursors of the nuclear forms of the protein. Work on *ts*1201, however, has shown that in fact all forms of UL26 gene product are present in the nucleus (Rixon *et al.*, 1988). In immunoprecipitation experiments using a polyclonal antibody raised against the carboxy terminus of UL26 gene product, the antisera only recognized the high mw forms of UL26 gene product in virus-infected cells in contrast to the monoclonal antibody 5010B, which recognised all forms of UL26 gene product. This result demonstrates that the low mw forms are produced by a processing step at the carboxy terminus. Recently it has been shown that the mature lower mw forms of the homologous protein, known as the assembly protein, in CMV are produced by proteolytic cleavage at the carboxy terminal end of the protein. The precursor contained a single cysteine residue, 32 amino acid residues from the carboxy terminus of the assembly protein, while the mature form did not contain the cysteine

(Robson and Gibson, 1989). This suggests that 32 or more amino acids are removed from the carboxy terminus. It is likely on the basis of work on CMV, that the processing at the carboxy terminus of UL26 gene protein is proteolytic cleavage. It is not known whether a separate endoproteinase is required for cleavage of UL26 gene product as is the case for cleavage of structural proteins in adenovirus (G.Kemp, p.c., in press), or whether the UL26 gene product itself is responsible for this step. It is interesting to note that the lesion in *ts*1201 is located within the amino terminal end of the protein yet clearly has an effect on processing at the carboxy terminus. Certain glycoproteins in HSV including gD and gG have been shown to undergo proteolytic cleavage at the amino terminus. Removal of the signal peptide is important for transfer of the glycoprotein through the membrane (Eisenberg *et al.*, 1984; Balachandran and Hutt-Fletcher, 1985). To date, there is no evidence that any structural protein other than UL26 gene product is cleaved.

Comparison of UL26 gene product with homologues of EBV, VZV and CMV

The bestfit computer programme was used to compare the amino acid sequence of UL26 gene with the amino acid sequence of the homologous proteins of EBV, VZV and CMV. The results showed that the HSV protein is more related to the VZV and EBV homologues than to the assembly protein (table 15). Interestingly, the HSV UL26 gene product had more amino acids in common with the VZV homologue than the EBV

TABLE 15

Comparison between UL26 gene and the counterpart genes in other viruses based on bestfit computer program

Virus	HSV-1	EBV	VZV	CMV
gene	UL26	Bvrf2.EBV	33.VZV	APcDNA-1
no. of A.A.	635	605	605	310
%Similarity HSV-1	/	39.69	52.79	32.48
EBV		/	35.38	33.98
VZV			/	33.22
CMV				/

A.A. : amino acid
APcDNA-1 : assembly protein (Robson and Gibson, 1989)

counterpart protein. One striking difference between HSV, EBV, VZV and CMV proteins is that the CMV protein is almost half the size of the HSV, EBV and VZV proteins and the best matched sequences of CMV assembly protein are predominantly in the carboxy-terminal portion of the protein (data at the end of the thesis). It is interesting to note that the cysteine at position 134 in the UL26 amino acid is conserved between HSV-1, EBV,and VZV. Whether this amino acid is of any functional significance, remains to be determined.

Functional role of UL26 gene products

The processing of UL26 gene product has been linked to DNA encapsidation (Preston *et al.*, 1983). However, it is not known exactly how this protein is involved in this process. Contradictory results have been obtained regarding the association of the gene products with the capsids depending on the techniques used. From the analysis of purified capsids, it was suggested that UL26 gene products were associated with full capsids but not empty (Gibson and Roizman, 1972; 1974), and results of surface iodination experiments suggested that the presence of the processed form of ICP35 on the surface of full capsids prevented the iodination of the major capsid protein (Braun *et al.*, 1984b). However, recent studies using immuno-electron microscopy clearly showed that UL26 gene products are transiently associated with partially cored capsids but not full capsids (Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988). In support of these results are findings obtained by Newcomb *et al.* (1989)

using scanning transmission electron microscopy to measure the masses of light and intermediate capsids of equine herpes virus 1. Their results suggested that a 46,000 mw protein was present in intermediate capsids and not empty capsids. Recently, a new technique known as the Ar^+ plasma etching technique has been used to locate UL26 gene product within capsids. Intact capsids are exposed to low energy which degrades the outermost polypeptide before the internal ones (Newcomb and Brown, 1989). The results suggested that UL26 gene product was localised inside the capsid cavity in partially-cored HSV-1 capsids and not on the outside. It would be interesting to expose capsids produced at the NPT by the mutant *ts*1201 to low energy to determine the exact location of unprocessed forms of UL26 gene products.

4.4 Future prospects

The UL26 gene product of *ts*1201 and *ts*1201 rev2 and rev3 have been cloned. It would be interesting to determine the second site reversions in the revertant viruses UL26 genes and to investigate by sequence analysis whether *ts*1201 has any mutations in the region of UL26 gene encoded within BamHI *d'*. Since *ts*1201 rev2 is *ts* at 39.5°, it would also be interesting to determine whether the virus processes UL26 gene products normally at 39.5° and whether it has an encapsidation defect like *ts*1201.

It is unclear from the literature whether UL26 gene products are DNA binding. It would be interesting to find out whether the products of UL26 have the ability to bind

DNA or interact with other structural proteins.

Evidence that there are two primary translational products has been obtained from the analysis of *in vitro* transltional products. Further studies are required to show that these primary translational products exist and to determine whether they are both functional.

Although work in this thesis has clearly shown that the UL26 gene product undergoes processing at the carboxy terminus, it remains to be determined that this modification is proteolytic cleavage.

A

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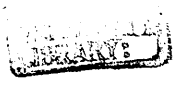

Bestfit (*) computer programme was used to compare amino acid sequence homology between different herpesvirus UL26 homologues

(*) Bestfit is a programme that makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches. A gap weight of 5 and a length weight of 0.3 were used.

BESTFIT of: **UL26** X **Bvrf2.EBV**

Gap Weight: 5.000
Length Weight: 0.300
Percent Similarity: 39.696

```
 17 RAVPIYVAGFLALYDSGDSGE.LALDPDTVRAALPPDNPLPINVDHRAGC 65
 3  QAPSVYVCGFVERPDAPPKDACLHLDPLTVKSQLPLKKPLPLTVEHLPDA 52

 66 EVGRVLAVVDDPRGPFFVGLIACVQLERVLETAASAAIFERRGPPLSREE 115
 53 PVGSVFGLYQSRAGLFSAASITSGDFLSLLDSIYHDCDIAQSQRLPLPRE 102

116 RLLYLITNYLPSVSLATKRLG....GEAHPDRTLFAHVALCAIGRRLGTI 161
103 PKVEALHAWLPSLSLASLHPDIPQTTADGGKLSFFDHVSICALGRRRGTT 152

162 VTYDTGLDAAIAPFRHLSPASREGARRLAAEAELALSGRTWAPGVEALTH 211
153 AVYGTDLAWV...LKHFSDLEPSIAAQIENDANAAKRESGCPEDHPLPLT 199

212 TLLSTAVNNMMLRDRWSLVAERRRQAGIAGHTYLQASEKFKMWGAEPVSA 261
200 KLIAKAIDAGFLRNRVETLRQDRGVANIPAESYLKASDAPDLQKPDKALQ 249

262 PARGYKNGAPESTDIPPGSIAAAPQGDRCP....IVRQRGVALSPVLPPM 307
250 SPPPASTDPATMLSGNAGEGATACGGSAAAGQDLISVPRNTFMTLLQTNL 299

308 NPVPTSGTPAPAPPGDGSYLWIPASHYNQLVAGHAAPQPQPHSAFGFPAA 357
300 DNKPPRQTPLPYAAPLPPFSHQAIATAPSYGPGAGAVAPAGGYFTSPGGY 349

358 AGSVAYGPHGAGLSQHYPPHVAHQYPGVLFSGPSPLEAQIAALVGAIAAD 407
350 YAGPAGGDPGAFLAMDAHTYHPHPHPPPAYFGLPGLFG..........  387

408 RQAGGQPAAGDPGVRGSGKRRRYEAGPSESYCDQDEPDADYPYYPGEARG 457
388 ......PPPPVPPYYGSHLRADYVPAPSRSNKRKRDPEED.....EEGGG 426

458 APRGVDSRRAARHSPGTNETITALMGAVTSLQQELAHMRARTSAPYGMYT 507
427 LFPGEDATLYRKDIAGLSKSVNELQHTLQALRRETLSYGHTGVGYCPQQG 476

508 PVAHYRPQVGEPEPTTTHPALCPPEAVYRPPPHSAPYGPPQGPASHAPTP 557
477 PCYTHSGPYGFQPHQSYEVPRYVPHPPPPPTSHQAAQAQPPPPGTQAPEA 526

558 PYAPAACPPGPPPPPCPSTQTRAPLPTEPAFPPAATGSQPEASNAEAGAL 607
527 HCVAESTIPEAGAAG.NSGPREDTNPQQPTTEGHHRGKKLVQASASGVAQ 575

608 VNASSAAHVDVDTARAADLFVSQMMGAR 635
576 SKEPTTPKAKSVSAHLKSIFCEELLNKR 603
```

BESTFIT of: **UL26** X **33.VZV**

gap Weight: 5.000
Length Weight: 0.300
Percent Similarity: 52.797

```
  9 RMEEPLPDRAVPIYVAGFLALYDSGDSGELALDPDTVRAALPPDNPLPIN 58
    ||  |  ||||||||||| | | ||| | || || ||||   ||||
  1 MAAEADEENCEALYVAGYLALY.SKDEGELNITPEIVRSALPPTSKIPIN 49

 59 VDHRAGCEVGRVLAVVDDPRGPFFVGLIACVQLERVLETAASAAIFERRG 108
    |||| || || ||||||| ||||||||| | ||  ||  ||   ||| || .
 50 IDHRKDCVVGEVIAIIEDIRGPFFLGIVRCPQLHAVLFEAAHSNFFGNRD 99

109 PPLSREERLLYLITNYLPSVSLATKRLGGEAHPDRTLFAHVALCAIGRRL 158
    ||  || ||||||||||||  |||| |  ||  || ||||| |||||
100 SVLSPLERALYLVTNYLPSVSLSSKRLSPNEIPDGNFFTHVALCVVGRRV 149

159 GTIVTYDTGLDAAIAPFRHLSPASREGARRLAAEAELALSGRTWAPGVEA 208
    |||| ||   |  | ||| ||  ||  || |    | |   | |  | |
150 GTVVNYDCTPESSIEPFRVLSMESK..ARLLSLVKDYAGLNKVWKVSEDK 197

209 LTHTLLSTAVNNMMLRDRWSLVAERRRQAGIAGHTYLQASEKFKMWGAEP 258
    |  |||||||||||||| ||| ||||||| || |||||  | |
198 LAKVLLSTAVNNMLLRDRWDVVAKRRREAGIMGHVYLQASTGYGLARITN 247

259 VSAPARGYKNGAPESTDIPPG.....SIAAAPQGDRCPIVRQRGVAL 300
    | |      |||    | ||        |||   ||    | | |   |
248 VNGVESKLPNAGVINATFHPGGPIYDLALGVGESNEDCEKTVPHLKVTQL 297

301 SPVLPPMNPVPT.....SGTPAPAPPGDGSYLWIPASHYNQLVAGHAAPQ 345
    |     |  ||       |  || | | || |||||   | |||  ||
298 CRNDSDMASVAGNASNISPQPPSGVPTGGEFVLIPTAYYSQLLTGQTKNP 347

346 PQPHSAFGFPAAAGSVAYGPHGAGLSQHY......PPHVAHQYPGVLFSG 389
      ||        |   ||| |||  |       | | |  |    |
348 QVSIGAPNNGQYIVGPYGSPHPPAFPPNTGGYGCPPGHFGGPYGFPGYPP 397

390 PSPLEAQIAALVGAIAADRQAGGQPA....AGDPGVRGSGKRRRYEAGPS 435
    |  || || ||| |||||| | | |    ||  |||  |||     |
398 PNRLEMQMSAFMNALAAERGIDLQTPCVNFPDKTDVRRPGKRDFKSMDQR 447

436 E...SYCDQDEPDADYP...YYPGEA...RGAPRGVDSRRAARHSPGTNE 476
    |    |||| | |||||   ||||||       | |       ||    ||
448 ELDSFYSGESQMDGEFPSNIYFPGEPTYITHRRRRVSPSYWQRRHRVSNG 497

477 TITALMGAVTSLQQELAHMRARTSAPYGMYTPVAHYRPQVGEPEPTTTHP 526
      | | |  ||||  ||   |    |
498 QHEELAGVVAKLQQEVTELKSQNGTQMPL..................... 526

527 ALCPPEAVYRPPPHSAPYGPPQGPASHAPTPPYAPAACPPGPPPPPCPST 576
                                                  | |
527 ............................SHHTNIPEGTRDPRISILL 545

577 QTRAPLPTEPAFPPAATGSQPEASNAEAGALVNASSAAHVDVDTARAADL 626
      |              | |    |  ||   | ||  ||  ||  |||
546 KQLQSVSGLCSSQNTTSTPHTDTVGQDVNAVEASSKAPLIQGSTADDADM 595

627 FVSQMMGAR 635
    |  ||| ||
596 FANQMMVGR 604
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BESTFIT of: **33.Vzv** X **Bvrf2.EBV**

Gap Weight: 5.000
Length Weight: 0.300
Percent Similarity: 35.385

```
  9 NCEALYVAGYL..ALYSKDEGELNITPEIVRSALPPTSKIPINIDHRKDC 56
    |||  |||   |      ||  |||  |   |||  ||    |||  |||   |
  3 QAPSVYVCGFVERPDAPPKDACLHLDPLTVKSQLPLKKPLPLTVEHLPDA 52

 57 VVGEVIAIIEDIRGPFFLGIVRCPQLHAVLFEAAHSNFFGNRDSVLSPLE 106
     ||  ||||  |    | |   | | |  ||   ||      |    ||   | |   | |
 53 PVGSVFGLYQSRAGLFSAASITSGDFLSLLDSIYHDCDIAQSQRLPLPRE 102

107 RALYLVTNYLPSVSLSSKRLSPNEIPDGN....FFTHVALCVVGRRVGTV 152
      |   |  |||||||  | |     |    |       ||  ||  ||  ||||  ||
103 PKVEALHAWLPSLSLASLHPDIPQTTADGGKLSFFDHVSICALGRRRGTT 152

153 VNYDCTPESSIEPFRVLSMESKARLLSLVKDYAGLNKVWKVSEDKLAKVL 202
      ||        |   |   |       | |                        | | |
153 AVYGTDLAWVLKHFSDLEPSIAAQIENDANAAKRESGCPEDHPLPLTK.L 201

203 LSTAVNNMLLRDRWDVVAKRRREAGIMGHVYLQASTGYGLARITNV.... 248
     |  |||   |||||  | |    |  | | |   || || | || |
202 IAKAIDAGFLRNRVETLRQDRGVANIPAESYLKASDAPDLQKPDKALQSP 251

249 ....NGVESKLPNAGVINATFHPGGPIYDLALGVGESNEDCEKTVPHLKV 294
          |    |    |    ||    |||   |  |      |          ||
252 PPASTDPATMLSGNAGEGATACGGSAAAGQDLISVPRNTFMTLLQTNLDN 301

295 TQLCRNDSDMASVAGNASNISPQPPSGVPTGGEFVLIPTAYY...SQLLT 341
                |       ||      | |    |||  |   | |||    |
302 KPPRQTPLPYAAPLPPFSHQAIATAPSYGPGAGAVAPAGGYFTSPGGYYA 351

342 GQTKNPQVSIGAPNNGQYIVGPYGSPHPPAFPPNTGGYGCPPGHFGGPYG 391
     |       | | |    |    |    |||    |       |   |||
352 GPAGGDPGAFLAMDAHTYHPHPHPPPAYFGLPGLFGPPPPVPPYYGSHLR 401

392 FPGYPPPNRLEMQMSAFMNALAAERGIDLQTPCVNFPDKTDVRRPGKRDF 441
      |||  |  |        |    ||   |   | | |    |   ||  |
402 ADYVPAPSRSNKRKRDPEEDEEGGGLFPGEDATLYRKDIAGLSKSVNELQ 451

442 KSMDQRELDSFYSGESQMDGEFPSNIYFPGEPTYITHRRRRVSPSYWQRR 491
      ||     |  |   |   ||         |  |  |   |  ||      ||   ||  |
452 HTLQALRRETLSYGHTGVG.......YCPQQGPCYTHS....GPYGFQPH 490

492 HRVSNGQHEELAGVVAKLQQEVTELKSQNGTQMPLSHHTNIPEGTRDPRI 541
     |            |    |   ||          |||  |   |
491 QSYEVPRYVPHPPPPPTSHQAAQAQPPPPGTQAPEAHCVAESTIPEAGAA 540

542 SILLKQLQSVSGLCSSQNTTSTPHTDTVGQDVNAVEASSKAPLIQGSTAD 591
     |       |          |                |     |       |    |   |
541 GNSGPREDTNPQQPTTEGHHRGKKLVQASASGVAQSKEPTTPKAKSVSAH 590

592 DADMFANQMMVGRC 605
      ||  ||||   |
591 LKSIFCEELLNKRV 604
```

BESTFIT of: **UL26** X **CMV (APcDNA-1)**

```
Gap Weight:  5.000
Length Weight:  0.300
Percent Similarity: 32.468

316 PAPAPPGDGSYLWIPASHYNQLVAGHAAPQPQPHSAFGFPAAAGSVAYGP 365
  2 SHPMSAVATPAASTVAPSQAPLALAHDGVYLPKDAFFSLIGASRPLAEAA 51

366 HGAGLSQHYPPHVAHQYPGVLFSGPSPLEAQIAALVGAIAADRQAGGQPA 415
 52 GARAAYPAVPPPPA..YPVMNYEDPSSRHFDYSAWLRRPAYDAVPPLPPP 99

416 AGDPGVRGSGKRRRYEAGPSESYCDQDEPDADYPYYPGEARGAPRGVDSR 465
100 PVMP.MPYRRRDPMMEEAERAAWERGYAPSAYDHYVNNGSWSRSRSGALK 148

466 RAARHSPGTNETITALMGAVTSLQQELAHMRARTSAPYGMYTPVAHYRPQ 515
149 RRRERDASSDEEEDMSFPGEADHGKARKRLKAHHGRDNNNSGSDAKGDRY 198

516 VGEPEPTTTHPALCPPEAVYRPPPHSAPYGPPQGPASHAPTPPYAPAACP 565
199 DDIREALQELKREMLAVRQIAPRALLAPAQLATPVASPTTTTSHQAEASE 248

566 PGPPPPPCPSTQTRAPLPTEPAFPPAATGSQPEASNAEAGALVNASSAAH 615
249 PQASTAAAASPSTASSHGSKSAERGVVNASCRVAPPLEAVNPPKDMVDLN 298

616 VDVDTARAADL 626
299 RRLFVAALNKM 309
```

BESTFIT of:**CMV (APcDNA-1)** X **Bvrf2.EBV**

```
Gap Weight:  5.000
Length Weight:  0.300
Percent Similarity: 33.981

  1 MSHPMSAVATPAASTVAPSQAPLALAHDGVYL.........PKDAFFSL 40
262 LSGNAGEGATACGGSAAAGQDLISVPRNTFMTLLQTNLDNKPPRQTPLPY 311

 41 IGASRPLAEAAGARAAYPAVPPPPAYPVMNYEDPSSRHFDYSAWLRRPAY 90
312 AAPLPPFSHQAIATAPSYGPGAGAVAPAGGYFTSPGGYYAGPAGGDPGAF 361

 91 DAVPPLPPPPVMPMPYRRRDPMMEEAERAAWERGYAPSAYDHYVNNGSWS 140
362 LAMDAHTYHPHPHPPPAYFGLPGLFGPPPPVPPYYGSHLRADYVPAPSRS 411

141 RSRSGALKRRRERDASSDEEEDMSFPGE.ADHGKARKRLK......... 179
412 NKRKRDPEEDEEGGGLFPGEDATLYRKDIAGLSKSVNELQHTLQALRRET 461

180 ...AHHGRDNNNSGSDAKGDRYDDIREALQELKREMLAVRQIAPRALLAP 226
462 LSYGHTGVGYCPQQGPCYTHSGPYGFQPHQSYEVPRYVPHPPPPPTSHQA 511

227 AQLATPVASPTTTTSHQAEASEPQASTAAAASPSTASSHGSKSAERGVVN 276
512 AQAQPPPPGTQAPEAHCVAESTIPEAGAAGNSGPREDTNPQQPTTEGHHR 561

277 ASCRVAPPLEAVNPPKDMVDLNRRLFVAALNKM 309
562 GKKLVQASASGVAQSKEPTTPKAKSVSAHLKSI 594
```

BESTFIT of: **33.Vzv X CMV (APcDNA-1)**

Gap Weight: 5.000
Length Weight: 0.300
Percent Similarity: 33.221

```
299 RNDSDMASVAGNASNISPQPPSGVPTGGEFVLIPTAYYSQLLTGQTKNPQ 348
          |      || | |  |       ||   |   ||||  | |  |
  1 MSHPMSAVATPAASTVAPSQAPLALAHDGVYLPKDAFFS..LIGASRPLA 48

349 VSIGAPNNGQYIVGPYGSPHPPAFPPNTGGYGCPPGHFGGPY.GFPGYPP 397
     ||       |  | | |       |    ||| |     || | |  ||
 49 EAAGARAAYPAVPPPPAYPVMNYEDPSSRHFDYSAWLRRPAYDAVPPLPP 98

398 PNRLEMQMSAFMNALAAERGIDLQTPCVNFPDKTDVRRPGKRDFKSMDQR 447
    |  | |          |       ||  |   |    |   |    ||   |
 99 PPVMPMPYRRRDPMMEEAERAAWERGYAPSAYDHYVNNGSWSRSRSGALK 148

448 ELDSFYSGESQMDGEFPSNIYFPGEPTYITHRRRRVSPSYWQRRHRVSNG 497
          | |  ||   || |||||     |||       ||  |  |||
149 R.....RRERDASSDEEEDMSFPGEADHGKARKRLKAHHGRDNNNSGSDA 193

498 QHEELAGVVAKLQQEVTEL.....KSQNGTQMPLSHHTNIPEGTRDPRIS 542
    |   ||   |||   ||          |   |    | ||  |
194 KGDRYDDIREALQELKREMLAVRQIAPRALLAPAQLATPVASPTTTTSHQ 243

543 ILLKQLQSVSGLCSSQNTTSTPHTDTVGQDVNAVEASSKAPLIQGSTADD 592
     | |   |   | | |       | ||        |||     | |
244 AEASEPQASTAAAASPSTASSHGSKSAERGVVNASCRVAPPLEAVNPPKD 293

593 ADMFANQMMVG 603
     |   ||||
294 MVDLNRRLFVA 304
```