Functional Analysis of the Herpes Simplex Virus Polypeptide Vmw65

by

Fiona Helen Ramsay

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Institute of Virology
Church Street
Glasgow
G11 5JR

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SUMMARY

Functional Analysis of the Herpes Simplex Virus Structural Polypeptide, Vmw65

The aim of this project was to study the role of Vmw65, a virion tegument polypeptide, which is important not only as a structural protein, but also as the trans-inducing factor (TIF) of HSV immediate-early (IE) gene transcription.

To elucidate the role of Vmw65 in the assembly of infectious virions, the HSV-2 mutant, ts2203, derived from the multiple mutant, ts13, was characterised. Previous work strongly suggested that ts13 has a temperature sensitive (ts) lesion within the Vmw65 gene, since Vmw65 produced by ts13 failed to react with the monoclonal antibody, MA1044, directed against this polypeptide. In contrast, the wild type (WT) HG52 HSV-2 Vmw65, or the gene product synthesised by ts^{+} revertants of ts13, which retained the defect within the alkaline exonuclease gene, was immunoprecipitated by the antibody (H.M. Moss and J.W. Palfreyman, personal communication). Preliminary studies on ts13 indicated that the virus has a late defect and therefore it was likely that the lesion affected the structural function of Vmw65. To obtain a mutant with a single ts lesion, a cloned fragment, BglII i, containing the ts13 Vmw65 gene, was recombined into WT HSV-2 strain HG52, and the mutant, ts2203, isolated. The ability of the plasmid pMC1, which contains the HSV-1 Vmw65 gene, to rescue ts2203 supported the idea that ts2203 had a lesion within Vmw65. Results from marker rescue experiments using HSV-2 HG52 cloned DNA fragments, together with

information from cross-hybridisation studies with HSV-1 pMC1 and subfragments of HSV-2 BglII i, suggested that the mutation was present at the 5' end of the coding sequences. The isolation of ts$^+$ revertants which formed plaques at the NPT with the same efficiency as WT virus confirmed that this mutant had a single ts lesion.

Polypeptide analysis of virus-infected cell extracts showed that ts2203 synthesised at both the PT and the NPT, a Vmw65 which had an altered electrophoretic mobility on SDS polyacrylamide gels. Two of five ts$^+$ revertants of ts2203 also specified a polypeptide with this property whilst the remainder induced Vmw65 which had a similar mobility to WT virus. This information suggests that the ts lesion in the Vmw65 gene is responsible for the alteration in the electrophoretic mobility of Vmw65 encoded by ts2203. Since ts2203 BglII i cloned DNA retained the ability to stimulate IE transcription to the same extent as WT HG52 BglII i, the lesion in ts2203 must only affect the structural function of Vmw65.

To determine which stage of virion assembly was affected by the lesion, thin section preparations of ts2203- or WT HG52-infected cells grown at the PT and NPT were examined under the electron microscope. Similar numbers of capsids were present in the nuclei of cells infected by either virus at the NPT, suggesting that ts2203 did not have a defect in the assembly of capsids. At this temperature, however, WT virus produced low numbers of dense cored capsids, making it difficult to ascertain whether there was any difference in

the number of DNA-containing capsids in the mutant-infected cells compared with those cells infected with WT virus. Analysis of viral DNA synthesised at the NPT revealed that most of the viral DNA was endless, supporting the electron microscopic observations that both viruses packaged DNA inefficiently at this temperature. These results together with the finding that cells infected with HG52 at the NPT contained very few enveloped particles, presumably because little of the replicated concatemeric viral DNA was cleaved and encapsidated, meant that it was impossible to determine whether the ts2203 defect was affecting packaging of viral DNA or envelopment of DNA-containing capsids. To overcome this problem, the BglII i fragment of ts13 was recombined into another HSV-2 strain, HVD25766, which grows well at the NPT and, in contrast to strain HG52, produces large amounts of cell-released enveloped virus. The resulting HVD mutant, ts2204, was compared with WT HVD for the ability to encapsidate virus DNA at the PT and NPT, and initial results indicated that the mutant had a packaging defect. However, a ts^+ virus, produced by recombining cloned HG52 BglII i into ts2204 DNA, also failed to package significant amounts of viral DNA at the NPT. It was, therefore, concluded that ts2204 had more than one ts mutation.

A second HVD mutant with a ts lesion in Vmw65, ts2205, isolated in the same way as ts2204, and a ts^+ virus, ts^+ 2205 (MR-2), produced by recombining cloned HG52 BglII i into ts2205 DNA, were examined for the ability to package viral

DNA. In these experiments ts 2205 (MR-2) behaved as WT HVD, indicating that ts2205, unlike ts2204, was not a multiple ts mutant. Ts2205, however, failed to package significant amounts of DNA either at the NPT, or on temperature shiftdown from the NPT to the PT in the absence of further protein synthesis. That is, ts2205 appears to have a non-reversible ts defect in encapsidation of viral DNA. This was confirmed by electron microscopic analysis of thin section preparations of ts2205-, ts 2205 (MR-2)- and HVD-infected cells. WT HVD and ts 2205 (MR-2) virus produced large quantities of dense-cored capsids and enveloped virus particles at the PT and NPT, whereas in ts2205-infected cells, only partially-cored and empty capsids were evident following incubation at the NPT, or on a temperature shiftdown to the PT in the presence of cycloheximide to prevent any further protein synthesis. Polypeptide analysis of virus-infected cell extracts showed that ts2205 retained the altered electrophoretic mobility of ts2203 Vmw65 at the PT and at the NPT, whilst WT HVD and ts 2205 (MR-2) synthesised a Vmw65 with a mobility similar to that of WT HG52. The evidence suggests, therefore, that Vmw65 is required for encapsidation of viral DNA.

Further experiments were concerned with the analysis of the functional domains of HSV-1 Vmw65 gene. This was determined by site-specific mutagenesis of the plasmid pMC1, which contains HSV-1 Vmw65 gene. Mutations were obtained by the insertion of a 6 base pair (bp) XbaI oligonucleotide linker

(5'-TCTAGA-3') into HaeIII-digested linear plasmid DNA. The mutated plasmids were compared with pMC1 for the ability to stimulate thymidine kinase (TK) activity in a transient expression assay in which cloned TK coding sequences were placed under IE control. In this way, regions of the gene affected by the insertion of two amino acids could be directly located. The results indicated that a small central domain of between 50 and 200bp was required for transcriptional activity of Vmw65, since plasmids with a 6bp insertion in this region failed to stimulate expression of an IE-TK gene chimaera.

Interestingly, the insertion of a termination codon 59bp upstream from the normal transcriptional termination codon did not affect the ability of Vmw65 to stimulate IE gene expression. However, a similar nonsense codon insertion at a distance of 240bp upstream from the normal termination site disrupted the integrity of the polypeptide such that the transcriptional stimulatory ability was lost.

Using ts2203, the structural domains of the Vmw65 polypeptide could also be located from the mutagenised pMC1 plasmids. The failure of a construct to produce significant numbers of ts^+ progeny virus when cotransfected with ts2203 into cells indicated that the insertional mutation in pMC1 was present in the region of Vmw65 essential for its structural function. The results of these experiments suggested that the central region of the polypeptide, important for transcriptional activity, may also be required for assembly of virus particles.

Abbreviations

A	adenine
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
BUdR	5-bromo-2'-deoxyridine
C	cytosine
CCV	channel catfish virus
Ci	Curies
cm	centimetre
CMV	cytomegalovirus
CO_2	carbon dioxide
CPE	cytopathic effect
cpm	counts per minute
CsCl	caesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphoshate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
EDTA	sodium ethylene-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
g	grams
h	hour
HA	hydroxylamine
HCl	hydrochloric acid
Hfl	human foetal lung
HSV	herpes simplex virus
HU	human serum
HVS	herpesvirus saimiri
ICP	infected cell polypeptide
IE	immediate early
IgG	immunoglobulin G
IP	immunoprecipitation
K	kilo
Kb	kilobase
l	litre
M	molar
MI	mock-infected
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
moi	multiplicity of infection
mRNA	messenger ribonucleic acid

mu	map units
MW	molecular weight
NA	nitrous acid
ng	nanograms
NP40	nonidet p40
NPC	nucleoprotein complex
NPT	non-permissive temperature
OD	optical density
ori	origin of viral DNA replication
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
pi	post infection
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	seconds
syn	syncytical
syn^+	non-syncytial
T	thymidine
TEMED	N,N,N'-N'-tetramethylethylenediamine
TK^-	thymidine kinase-minus
TK^+	thymidine kinase-positive

ts	temperature-sensitive
ts^+	wild-type for temperature-sensitivity
UV	ultra-violet
V	volts
v/v	volume per volume
Vmw	molecular weight of virus-induced polypeptide
VP	virion protein
VZV	varicella zoster virus
WT	wild type
w/v	weight per volume
w/w	weight per weight
uCi	microcuries
ug	microgram
ul	microlitre
%	percentage

U = units

CHAPTER 1

AIMS OF THE PROJECT

Very little is known about the assembly of the herpesvirus particle and the role of individual structural polypeptides, especially the components of the virus tegument. It is therefore interesting that a tegument protein, Vmw65, has recently been identified as a transcriptional *trans*-inducer of HSV IE genes (Campbell *et al.*, 1984). In view of this information, and the availability of the HSV-2 multiple mutant *ts*13, thought to have a *ts* lesion in the HSV-2 gene equivalent to HSV-1 Vmw65 (Moss *et al.*, 1979), this project was undertaken:

(1) to determine the structural role of Vmw65 by recombining the *ts*13 mutation in the Vmw65 gene into WT HG52 DNA, and analysing the resulting mutant containing a single *ts* lesion,

(2) to make a preliminary study of functional domains in the HSV-1 protein.

These aspects of the project are described in Chapters 3 and 4 respectively.

The following Chapter (1) is an introduction to the biology of herpes simplex virus, with particular emphasis on the assembly of the virus particle and encapsidation of virus DNA.

1. CLASSIFICATION AND PROPERTIES OF HERPESVIRUSES

Herpesviruses are a heterogeneous group of viruses which have been isolated from a wide range of vertebrates and invertebrates (for a comprehensive list refer to Roizman, 1982). The herpesvirion is composed of a double-stranded (ds), deoxyribonucleic acid (DNA) genome enclosed within an icosahedral capsid, which is surrounded by a lipid envelope (Wildy et al, 1960; Epstein, 1962; Roizman and Furlong, 1974)(Figure 1). Characteristically, herpesviruses replicate in the cell nucleus, and acquire their envelope by budding through the nuclear membrane (Morgan et al, 1954; Wildy et al, 1960).

Although there is wide diversity both in the biological characteristics of herpesviruses and in the composition of the viral genome (Figure 2), a hierarchical system determined by the biological characteristics of herpesviruses has been devised. This scheme divides the herpesvirus family into three subfamilies on the basis of host range, duration of the lytic cycle, cytopathology and the properties of latent infection (Roizman et al, 1981; Matthews, 1982).

1.1 Subfamily Alphaherpesvirinae

Herpes simplex virus type 1 (HSV-1), a human pathogen, is the prototype for this group. Members of this subfamily are rapidly multiplying cytotoxic viruses

Figure 1

The Structural Components of the HSV Virion

A section through a herpesvirion particle (Perdue et al., 1976).

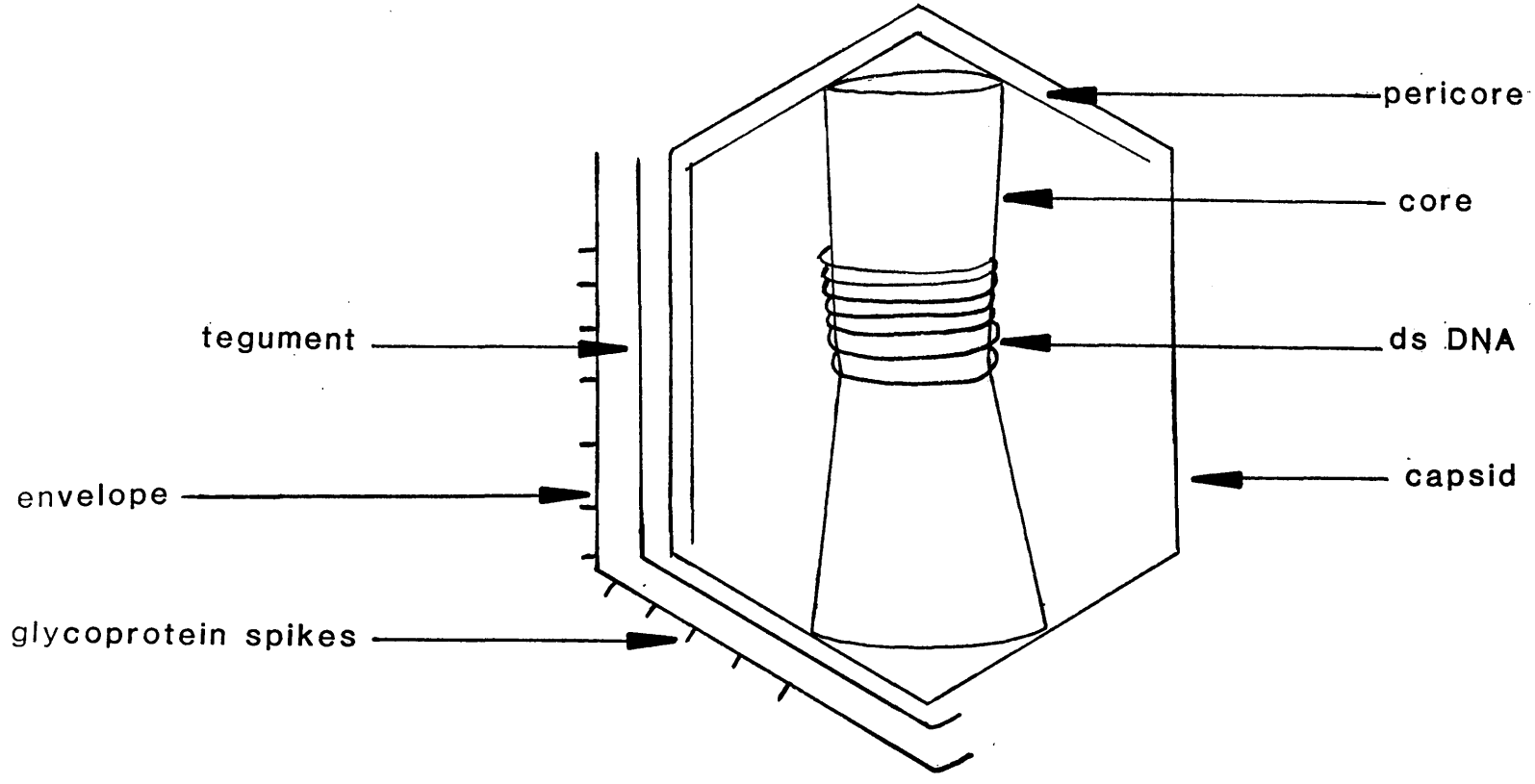

pericore
core
tegument
ds DNA
envelope
capsid
glycoprotein spikes

Figure 2

A schematic representation of the known genome structures of the herpesviruses

Herpes simplex virus (HSV-1 or HSV-2) (See also Figure 3).

Varizella zoster virus (VZV)

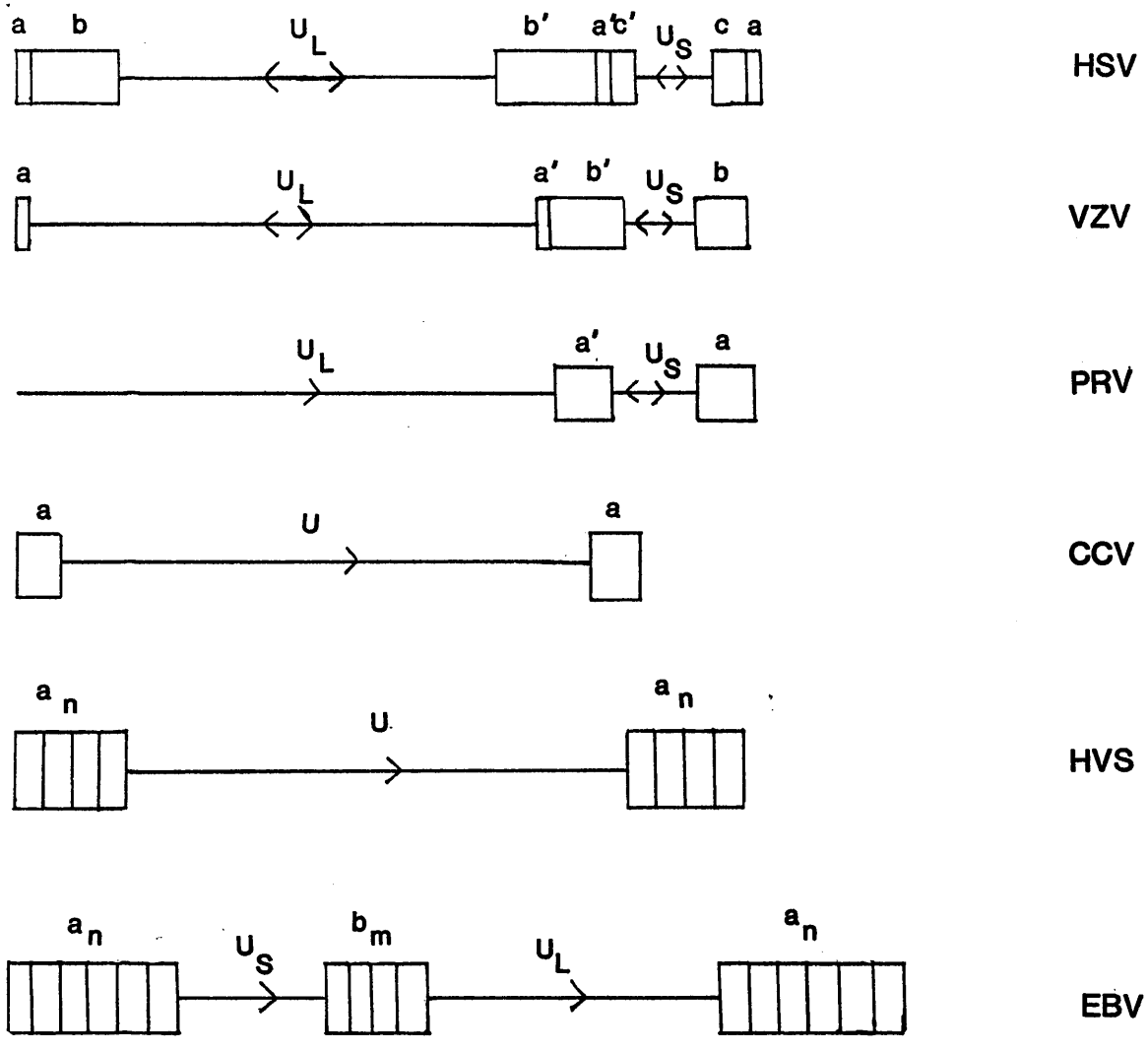
Pseudorabies virus (PRV)

Channel Catfish virus (CCV)

Herpesvirus Saimiri (HVS)

Epstein Barr Virus (EBV)

Unique sequences (U_S or U_L) are flanked by repeats (open boxes: a,b',c,a',b',c'), and these may be in different orientations relative to the repeats (arrows < > represent possible orientations of U_S or U_L).


a b
U_L
b' a' c'
U_S
c a
HSV
a
U_L
a' b'
U_S
b
VZV
U_L
a'
U_S
a
PRV
a
U
a
CCV
a_n
U
a_n
HVS
a_n
U_S
b_m
U_L
a_n
EBV

in experimental animals which have a variable host range both *in vivo* and *in vitro*. The reproductive cycle is short, usually 24 hours (h) or less, and latency generally occurs in the ganglia (Stevens and Cook, 1971, 1973).

Other alphaherpesvirinae include pseudorabies virus (PRV), which infects pigs (Gustafsohn, 1970), and varicella zoster virus (VZV) which infects man. The primary infection of VZV, which is normally a childhood disease, is known as chicken pox or varicella, whilst the recurrent disease, known as shingles, generally occurs in the elderly (Weller *et al*, 1958; Richards *et al*., 1979) and in immunocompromised people.

1.2 Subfamily Betaherpesvirinae

This group comprises the cytomegaloviruses (CMV) which can be isolated from a wide variety of mammals including man (Human CMV, HCMV). The host range of these viruses is narrow and the growth cycle slow. Lytic foci are produced in cell culture, and latency can be established in a variety of tissues, including the secretory glands, lymphoreticular cells and the kidneys. HCMV normally causes asymptomatic infections in immunocompetent adults. However, it is an important cause of infection, particularly in immunocompromised adults, for example, organ transplant patients and in particular, kidney transplant patients (Alford and Britt, 1985). This virus is also responsible for certain congenital deformities in babies.

1.3 Subfamily Gammaherpesvirinae

These are the lymphocyte associated viruses and include the human herpesvirus, Epstein Barr Virus (EBV). This virus is the causative agent of infectious mononucleosis, and has been strongly implicated in Burkitt's lymphoma and nasopharyngeal carcinoma (Henle et al., 1968, 1973; DeThe et al., 1978; Raab-Traub et al., 1983). Other viruses in this group are herpesvirus saimiri (HVS) which infects primates, and Marek's disease virus (MDV) which infects chickens.

The reproductive cycle of the gammaherpesviruses varies in length, and the host range is generally limited. In vitro they can replicate in lymphoblastoid cells, but some members of this group undergo lytic infections in cell types such as the T or B lympocytes. For example, EBV is specific for B lymphocytes (Koide et al., 1981). Frequently, infection in lymphocytes is arrested, and no virus is produced.

Latent virus is normally present in lymphoid tissue.

1.4 Herpes Simplex Virus Types 1 and 2

These viruses have similar serological and biological properties, as well as very similar genome structures. Both HSV-1 and HSV-2 infect man, but in general have different sites of infection.

HSV-1 is mainly responsible for lesions about the mouth and lips, but can also infect the eye, causing a disease known as keratoconjunctivitis, a major infective cause of blindness in the western world (Whitley, 1985). On rare occasions, encephalitis can result from HSV-1 infection (Olson et al., 1967; Hammer et al., 1980). HSV-1 is normally spread by direct contact or by droplets from an infected person.

HSV-2 is usually transmitted venereally and causes genital lesions. This can produce a severe and often fatal disease in neonates, which acquire their infections during birth from genital lesions of the mother.

HSV-2 can also infect other regions of the body, for example, the eye and mouth, although these infections are uncommon. Likewise, HSV-1 can also cause genital lesions, but these infections are generally less severe than cases where HSV-2 is the causative agent (Corey et al., 1983).

2. HSV CELL TRANSFORMATION AND ONCOGENESIS

Seroepidemiological research during the past 30 years has implicated HSV-2 in the aetiology of cervical carcinoma. HSV-2 is normally transmitted venereally and readily infects the cervix and vulva; antibody titres of the virus are generally higher in women with pre-invasive carcinoma or cervical cancer than in matched control women (Nahmias et al., 1980).

Attempts to show that HSV-2 is a tumour virus have concentrated on examining the ability of the virus to transform cells in vitro. Initial work, using virus under conditions in which lytic infection was suppressed (Duff and Rapp, 1971; Darai and Munk, 1973; Macnab, 1974) failed to detect viral DNA sequences or proteins consistently associated with the transformed phenotype. Subsequent studies, using defined fragments of HSV DNA, identified two transforming regions of the HSV-2 genome, the BglII n region (0.58-0.63mu) (Reyes et al., 1979; Galloway and McDougall, 1981; Galloway et al., 1984; Jones et al., 1986), and the BglII c fragment (0.41-0.58mu) (Jariwalla et al., 1980, 1983) and one region of HSV-1, XbaI f (0.30-0.45mu) (Camacho and Spear, 1978; Reyes et al., 1979).

Although HSV-transformed cells are generally oncogenic when transplanted into the syngeneic host animal, HSV DNA is progressively lost from the transformed cells during passaging in vitro (Minson et al., 1976; Galloway and McDougall, 1983; Cameron et al., 1985). In addition, no viral oncogenes of HSV have been identified (Galloway and McDougall, 1983; Macnab et al., 1985b). This has led to a revival of the "hit and run" hypothesis (Skinner, 1976; Minson et al., 1976) since the continuous expression of an HSV DNA sequence does not appear to be required for the maintenance of cellular transformation (Galloway and McDougall, 1983).

Transformation by HSV-2 is, therefore, a complex process. There is considerable evidence that HSV can act as a mutagen causing:

1. Gene amplification (Lavi, 1981; Brandt et al., 1987).

2. Chromosomal re-arrangements (Hampar and Ellison, 1961; O'Neill and Rapp, 1971).

3. The demonstration of mutants at a specific cell locus, for example, the HGPT locus (Schlehofer and Zur Hausen, 1982), or alternatively,

4. The enhancement of expression of certain cellular genes (either by mutation or by altered regulation eg. demethylation (Macnab et al., 1985a).

HSV DNA has been detected in some cervical tumours (Park et al., 1983) and generally these sequences span regions which correspond to those identified in in vitro transformation studies. However, it should be noted that HSV DNA sequences are present in only a small percentage of cervical carcinomas (Park et al., 1983; Galloway and McDougall, 1983; Macnab et al., 1985b), whilst human papilloma virus (HPV) DNA can be detected in up to 80% of cervical carcinomas examined (Macnab et al., 1986).

3. HSV LATENCY

The establishment of a latent infection by HSV was first postulated by Goodpasture (1929), who also put forward the hypothesis that the latent virus resided in the neurons of the sensory ganglia. It was, however, not until 1971 that Stevens and Cook (1971) first demonstrated that virus could be isolated from spinal ganglia explants of mice which were latently infected with HSV-1. Shortly afterwards it was shown that HSV-1 could be recovered from explants of trigeminal ganglia of humans (Bastian et al., 1972; Baringer and Swoveland, 1973), and since then HSV-2 has been reactivated from sacral ganglia explants cultured in vitro (Baringer, 1974). Latent virus can be reactivated by a variety of stimuli such as sunlight, the common cold, or menstruation, and the available evidence suggests that the latent virus is harboured in the neuronal cells of the sensory ganglia (Cook et al., 1974; McLennan and Darby, 1980).

Although it has been shown that TK viruses and several ts mutants of HSV-1 fail to establish latent infections in various animal models (Lofgren et al., 1977; Field and Wildy, 1978; Watson et al., 1980; Price and Kahn, 1981), the virus gene(s) that are directly required for the initiation and maintenance of the latent state have not yet been identified with any certainty. Recently, a mutation, which is thought to affect reactivation of latent virus, has been identified in the HSV-1 strain

17 mutant tsI. The gene in which the lesion lies, however, has not been located (Cook et al., 1986).

In both the mouse model and in humans, the physical state of the latent viral DNA appears to be in the form of "endless" DNA molecules (Rock and Fraser, 1983, 1985; Efstathiou et al., 1986). It has not yet been determined whether the viral DNA exists in an extrachromosomal state or is integrated into the host cell genome. It is also uncertain how much of the latent virus genome is transcribed, but recent evidence suggests that very little of the virus DNA is transcribed. Interestingly, an anti-sense mRNA to Vmw110 has been identified in cells harbouring latent HSV in the mouse model (Stevens et al., 1987).

4. VIRION STRUCTURE

The herpesvirion is composed of four major structural components: the core, capsid, tegument and envelope (Wildy et al., 1960; Roizman and Furlong, 1974) Figure 1.

The core is thought to consist of viral DNA tightly spooled in the form of a toroid around a cylindrical mass which is probably composed of protein (Furlong et al., 1972). Since the core is centrally located within the capsid, it has been proposed that it is maintained in this position by interaction with inner capsid

material known as the pericore although the presence of the pericore has not been demonstrated in HSV (Roizman and Furlong, 1974). Electron microscope studies on MDV and CMV suggest that the core is attached at its poles to the capsid (Nazerian, 1974; Haguenau and Michelson-Fiske, 1975).

The icosahedral shaped capsid is approximately 100nm in diameter and is calculated to be composed of 150 hexameric and 12 pentameric units (Wildy et al., 1960). EM examination of partially disrupted capsids suggests that the hexamers may be linked by intercapsomeric fibrils (Palmer et al., 1975; Vernon et al., 1974).

The amorphous layer between the capsid and envelope is known as the tegument or matrix (Morgan et al., 1968; Wildy et al., 1960; Schwartz and Roizman, 1969). Its thickness is genetically determined (McCombs et al., 1971) and varies considerably between the different herpesviruses (McCombs et al., 1971), but it is not particularly distinct in HSV (Nii et al., 1968; McCombs et al., 1971). The tegument may play a role in envelopment of the virus (Vernon et al., 1982).

The herpesvirus envelope surrounds the virion (Darlington and Moss, 1968) and adheres closely to the capsid (Nii et al., 1968; Nii 1971). It consists of a trilaminar membrane and has spikes of about 8nm in

length projecting from its surface (Wildy et al., 1960).

4.1 Structural Polypeptides of HSV

Most work has concentrated on HSV-1, but other herpesviruses have a similar number, and range of sizes, of structural polypeptides (Spear and Roizman, 1972; Gibson and Roizman, 1972; Heine et al., 1974; Perdue et al., 1975, 1976; Cohen et al., 1980).

Approximately 30 structural polypeptides have been identified in HSV-1 and HSV-2 purified virions (Marsden et al., 1976; Heine et al., 1974; Cassai et al., 1975), and 7 different polypeptides have been identified in HSV-1 capsids which contain DNA (Gibson and Roizman, 1972; Cohen et al., 1980) (Figure 3). Of these, Vmw55 (VP19C) was shown to be located in an internal position and to have DNA-binding properties (Braun et al., 1984a), suggesting that it may be a core or pericore protein.

The core has also been shown to contain the polyamine, spermine (Gibson and Roizman, 1971), which is thought to bind to the narrow groove of DNA and neutralise phosphate charges.

The ICP35 family of immunologically related proteins (p40, NC3) (Cohen et al., 1980; Heilman et al., 1979;

Figure 3

The map location of genes encoding

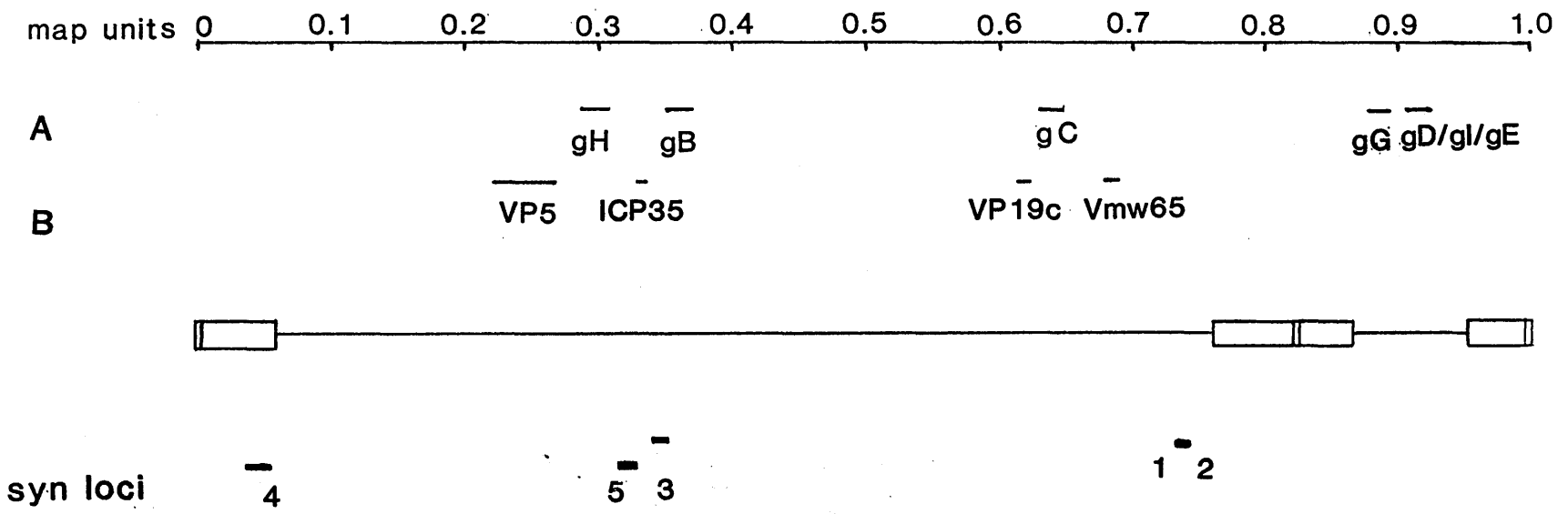
A. Major glycoproteins: gB, gC, gD, gE, gG, gH, gI

B. Components of the capsid and tegument

C. Map location of HSV-1 syncytial (syn) loci:

Syn 1, syn 2; syn 3; syn 4; syn 5.

References are given in the text.


map units
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
A
gH
gB
gC
gG
gD/gI/gE
B
VP5
ICP35
VP19c
Vmw65
syn loci
4
5
3
1
2

Gibson and Roizman 1972) includes VP21, which is a minor unprocessed form, and VP22a (p40) which is a low MW processed form of the polypeptide (Braun et al., 1984b). Recent work with immune-gold electron microscope techniques has indicated that this form of the protein is located on the surface of empty but not full capsids (F J Rixon, personal communication). The function of p40 is uncertain but it has been termed an "assembly" protein since its presence on the capsid is a transient one during maturation (Gibson and Roizman, 1972). It does not appear to be necessary for maintenance of the capsid and therefore cannot be termed a capsid structural polypeptide.

This leaves four polypeptides which are most likely to be true components of the capsid. The major capsid protein, Vmw155 (ICP5,NC1), coats the capsid and is thought to be the major constituent of the hexamers.

The other minor capsid proteins may be associated with the intercapsomeric fibrils, with the hexamers or alternatively they may be present at the exterior surface of the pericore (Vernon et al., 1981).

4.2 HSV Tegument Polypeptides

Polypeptides which remain associated with the capsid when the virus is de-enveloped, but which are absent from capsids isolated from infected cell nuclei, are

classified as tegument proteins (Roizman and Furlong, 1974; Lemaster and Roizman, 1980). This definition, however, does not allow the inclusion of nonglycosylated polypeptides which are closely associated with the envelope and are solubilised by detergent (Spear, 1980; Johnson et al., 1984).

Although a large number of structural polypeptides have been identified as components of the tegument, their precise function has not yet been determined. A major component of the tegument is the high MW protein, Vmw273, which may have a role in uncoating of the viral genome and in envelopment (V G Preston, personal communication). Another major constituent is Vmw65, which has been identified both as an essential structural polypeptide of the virion (Moss et al., 1979), and as the virion component responsible for trans-activation of immediate-early (IE) gene transcription (Campbell et al., 1984) [Section 7.5.3]. The HSV U_S9 gene also specifies a tegument polypeptide of 10,000MW (McGeoch et al., 1985) which can be precipitated from NP40 extracts of HSV-1 virions using an oligopeptide antiserum (Frame et al., 1986b). This phosphoprotein is located around the perimeters of the capsid and probably becomes associated with capsids soon after their assembly (Frame et al., 1986b).

It has been proposed that HSV encodes at least one protein kinase species (Lemaster and Roizman 1980;

Purves et al., 1986), and that this is located in the tegument. However, although a gene homologous to eukaryotic protein kinase genes has been identified in the U_S, U_S 3 (McGeoch and Davidson, 1986), it remains unclear as to whether the enzyme isolated by Lemaster and Roizman (1980) is virus- or host-specified (Stevely et al., 1985).

4.3 HSV Envelope Glycoproteins

It should be noted that the polyamine spermidine is present in the HSV envelope (Gibson and Roizman, 1971), but the major constituents of the envelope are the virus glycoproteins. These polypeptides are important determinants of pathogenicity, and mediate the entry of virus into the host cell and the spread of the virus between cells. The precise role of individual glycoproteins in these processes is not well understood.

Seven major HSV-1 glycoproteins (Figure 3) with homologues in HSV-2 have been identified in the virus envelope and their functions are summarised in Table 1. In addition, one minor glycoprotein has recently been discovered through analysis of the HSV-1 DNA sequence (McGeoch et al., 1985), but it is not yet known whether these polypeptides are components of the virus envelope.

Table 1

Functions of the Major HSV Glycoproteins

+	Function demonstrated; positive role
-	Negative role
___	Role unknown at present, or uncertain

References

gB	Sarmiento et al., 1979; Little et al., 1981
gC	Friedmann et al., 1984; Smiley and Friedmann 1985
gD	Noble et al., 1983; Para et al., 1985
gE	Baucke and Spear 1979; Para et al., 1980, 1982
gG	Roizman et al., 1985; McGeoch et al., 1985, 1986; Frame et al., 1986; Richman et al., 1986
gH	Buckmaster et al., 1984; Gompels and Minson, 1986
gI	Johnson and Feenstra (1987)

Table 1

HSV Glycoproteins

Function	gB	gC	gD	gE	gG	gH	gI
Neutralisation without complement	+	+	+	+	+	+	
Adsorption	+	+	+	+			
Penetration	+	+					
Cell fusion (syn)	+	+					
Fc receptor	-	-	-	*	-	-	*
C3b receptor	-	gC-1	-	-	-	-	-
Virus exit						+	

*gE/gI may form a functional complex (M.C.Frame, personal communication).

The map location of genes encoding major components of the virus capsid, tegument and envelope are given in Figure 3.

5. GENOME STRUCTURE OF HSV

Detailed analysis of the DNA of HSV-1 and HSV-2 has shown that a very close relationship exists between these viruses. The genomes of both HSV-1 and HSV-2 are linear, ds DNA molecules (Becker et al., 1968; Graham et al., 1972) with a mw of 95-100 x 10^6, representing approximately 155kbp (Kieff et al., 1971). A striking feature of HSV DNA is its very high guanosine and cytosine (G+C) content : 67% overall for HSV-1 and 69% for HSV-2 (Goodheart et al., 1968), although the distribution of G+C % residues is not uniform. For example, within the DNA sequence encoding the immediate-early (IE) polypeptide Vmw175 there is an especially high G+C content of 81.5% (McGeoch et al., 1986a).

The HSV genome (Figure 2) is composed of a long unique (U_L) and a short unique (U_S) segment, each bounded by inverted repeat sequences (Sheldrick and Berthelot, 1974). At each terminus there is a direct repeat known as the a sequence, which is present in an inverted orientation at the L-S junction (Grafstrom et al., 1974, 1975; Sheldrick and Berthelot, 1974; Wadsworth et al., 1975; Davison and Wilkie, 1981). As a consequence of this genome structure, the U_L and U_S components

invert relative to the repeat sequences, producing a population of virus genomes containing equimolar amounts of four isomeric forms, referred to as P (prototype), I_S (U_S inversion), I_L (U_L inversion) and I_{SL} (U_S/U_L inversion) (Sheldrick and Berthelot, 1974; Hayward et al., 1975; Clements et al., 1976; Delius and Clements, 1976)(Figure 4).

All the isomeric forms can replicate and produce viable progeny (Jenkins and Roizman, 1986). However, the ability to invert is not essential for virus growth, and non-inverting viable populations of HSV have been isolated (Preston et al., 1978; Davison and Wilkie, 1981; Poffenberger et al., 1983; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986).

A cis-acting signal for inversion has been identified within the a sequence of HSV. Evidence for this was derived from the observation that the insertion of an a sequence into U_L caused additional inversions, and, consequently, the generation of novel genomic termini (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981). Inversion of the U_L fragment did not occur if it was flanked by a sequences present in the same orientation (Smiley et al., 1981), or if the a sequences on either side were heterotypic (Davison and Wilkie, 1983a)(See also Section 1.10.1).

Figure 4

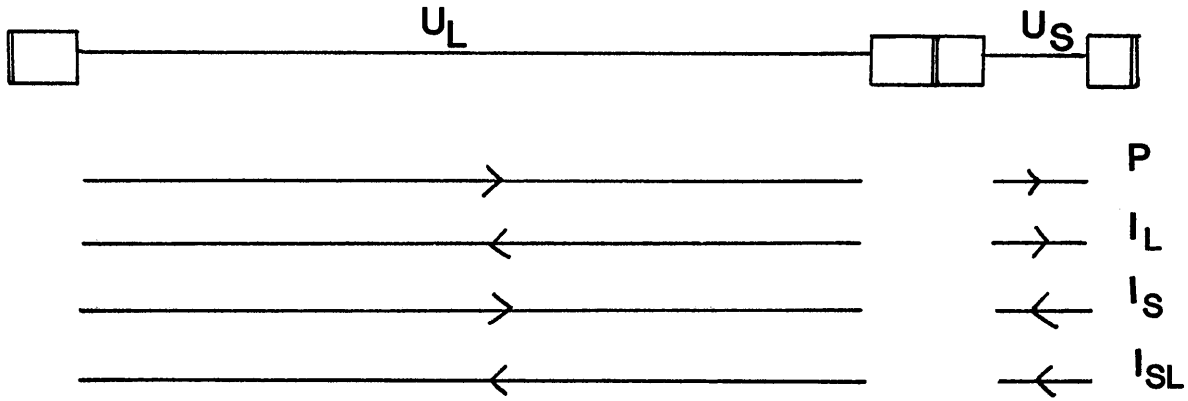
Genome isomers of HSV

P = prototype orientation (details are given in Figure 2)

I_S = inversion of long region (U_S)

I_L = inversion of short region (U_L)

I_{SL} = inversion of short and long regions (U_S and U_L)


U_L
U_S
P
I_L
I_S
I_{SL}

5.1 Gene Layout in HSV

The complete DNA sequence of the HSV genome is now available (McGeoch, 1985; McGeoch et al, 1985, 1986a., 1986b., 1987, D. McGeoch, personal communication) and is estimated to be 152260 bp. The largest contiguous published HSV sequence is the U_S region which is almost 13kbp long, and contains 12 genes (McGeoch et al., 1985; Rixon and McGeoch, 1985). The polypeptide coding sequences are compactly arranged and comprise 79% of the sequence.

All the U_S genes have separate promoters, but several share 3'termination sites, and one pair, U_S10 and U_S11, have overlapping coding sequences in different reading frames. The promoter sequences of U_S10 are also present within coding sequences of U_S11 (Rixon and McGeoch, 1984; McGeoch et al., 1985). Whilst none of these gene arrangements are restricted to U_S, overlapping coding sequences are rare.

The overall (G+C%) content of U_S is 64.3% (McGeoch et al., 1985), which is considerably lower than that of the adjacent TR_S/IR_S sequence, which has an especially high (G+C) content of 79.5% (McGeoch et al., 1986a). The TR_S/IR_S region is 6.6kbp in length and encodes a single gene, IE175. The a sequence is present at the 3' end of this gene, and at the 5' end there is an origin of replication, termed ori_S, plus promoter

sequences which are shared by the genes, IE68 and IE12, whose coding sequences are contained entirely within U_S (Murchie and McGeoch, 1982; McGeoch et al., 1985, 1986a).

The presence of short, tandemly repeated sequences is a widespread feature of herpesvirus genomes (McGeoch , 1985). Repeats of 12-54bp in length have been detected in the non-coding sequences of HSV-1 strain 17 (Murchie and McGeoch, 1982; Davison and Wilkie, 1981; Rixon et al., 1984; Perry et al., 1986; Chou and Roizman, 1986), and their copy number can vary considerably between different virus plaque isolates. Other short tandem repeats have been located in the polypeptide coding sequences of genes, for example, U_S7, U_S10 and U_S11 (Rixon and McGeoch, 1984; McGeoch et al., 1985).

5.2 Relatedness of HSV-1 and HSV-2

Hybridisation experiments by Kieff et al (1972) showed that there was a close relationship between the genomes of HSV-1 and HSV-2 since about 50% of the DNA sequences had strong homology. Subsequent electron microscope analysis of hybrid molecules of DNA (Kudler et al., 1983) and cross-hybridisation studies (Davison and Wilkie, 1983b) revealed that the homologous regions of HSV-1 and HSV-2 were distributed throughout the genome. This conclusion was supported by genetic studies which showed that recombination (Timbury and Subak-Sharpe,

1973; Halliburton et al., 1977; Morse et al., 1977; Preston et al., 1978), and complementation (Timbury and Subak-Sharpe, 1973; Esparza et al., 1976; Schaffer et al., 1978) between the serotypes can occur. Cross-over sites in the recombinants were detected across the whole of the genome.

The colinearity of HSV-1 and HSV-2 has also been confirmed through the sequence analysis of large regions of HSV DNA. Although the sequence data on HSV-2 is limited, the sequence spanning most of the HSV-2 U_S has been determined (Whitton and Clements, 1984; McGeoch et al., 1987). A comparison of HSV-1 and HSV-2 U_S regions confirmed that they possessed equivalent genes, and that homology at the amino acid level is distributed throughout U_S. One noteable exception, however, is the gene U_S4.

In HSV-1, U_S4 encodes a glycoprotein, designated gG-1, with a MW of 25,000 (McGeoch et al., 1985; Frame et al., 1986a; Richman et al., 1986). In HSV-2, near the N-terminus, the U_S4 coding sequence has an extra 1,460 residues (McGeoch et al., 1987), and this gene specifies a 72,000 MW polypeptide. The finding that an oligopeptide antiserum raised against the HSV-2 gG amino acid sequences near to the C terminus (which are homologous to the HSV-1 sequence with the exception of a single amino acid), could immunoprecipitate gG-1,

confirmed that the HSV-2 U_S4-encoded polypeptide, designated gG-2, was the HSV-2 counterpart to gG-1 (McGeoch et al., 1987).

5.3 Evolution of HSV-1 and HSV-2

The sequence analysis of the HSV-1 and HSV-2 genomes has enabled their divergent evolution to be studied. Within the U_S sequence many single nucleotide differences have arisen between the viruses, presumably through point mutations occurring after divergence of HSV-1 and HSV-2 from a common ancestor (McGeoch et al., 1987). These base pair changes are more extensive in non-coding than coding sequences. In coding regions the redundant third codon position has diverged most, whilst the second codon position is the more conserved (McGeoch et al., 1987). Nevertheless, many amino acid alterations are tolerated in the U_S sequence, and this characteristic is also apparent in genes located outside U_S, for example, the HSV thymidine kinase (TK) gene (Swain and Galloway, 1983).

Duplication and deletion differences between HSV-1 and HSV-2 genomes have also been identified. Some of these differences have occurred in reiterated sequences, resulting in different copy numbers of the repeated elements. Others have been found in non-reiterated sequences. One particularly interesting example, mentioned earlier, is gene U_S4 of HSV-2. The N

terminal region of this gene shares amino acid homology with PRV gX (McGeoch et al., 1987), and it may, therefore, represent an ancient alphaherpesvirus gene, present before divergence of PRV and HSV. It is proposed that the HSV-1 U_S4 gene lost amino acid sequences after divergence of HSV-1 and HSV-2. Another remarkable feature of HSV-2 U_S4 is that it has homology at the amino acid level to gD of both HSV-1 and HSV-2, again at the N terminal region, indicating that gene duplication has occurred during the evolution of HSV.

6. HSV LYTIC CYCLE

The early stages of HSV infection can be divided into three distinct phases: attachment of the virion to the cell, penetration of the cell, and uncoating of the viral genome.

6.1 Attachment

Attachment of HSV to the cell surface occurs very rapidly after addition of the virus inoculum to the cells (Hochberg and Becker, 1968) and can take place at temperatures ranging from 4°C to 40°C (Farnham and Newton, 1959). The action of heparin, a negatively charged polysaccharide which prevents the adsorption of virus to cells and causes the release of virus already attached to the cell surface suggests that initially the virus is bound to the cell by weak electrostatic forces (Hochberg and Becker, 1968). The subsequent

reduction in cell surface protein mobility indicates that there is an increase in the strength of binding (Rosenthal et al., 1984). This restriction in mobility is thought to be due to the cross-linking of cellular receptor proteins and cytoskeletal elements, and is similar to the effects of attachment of multivalent ligands to cell surface receptors (Gall and Edelman, 1981; Henis and Elson, 1981). However, this is a temporary feature and protein mobility returns to normal upon viral penetration of the cell.

Several virus glycoproteins have been implicated in attachment of the virus to the cell. A study using virosomes, lipid vesicles enriched with HSV-1 glycoproteins, which bind to cells and may fuse with them, showed that gB and gD were important for this process (Johnson et al., 1984). When virosomes were depleted of gB or gD, there was a reduction in the number of virosomes binding to cells. Subsequent work by Fuller and Spear (1985) using a virion adsorption assay demonstrated that HSV-1 adsorption to cells was inhibited by antibodies to both gD and gC, or by the addition of high concentrations of the Fc portion of immunoglobulin G (IgG). This suggested that gD, gC and possibly gI (Johnson and Feenstra, 1987; M. Frame, personal communication) which is an Fc receptor protein complexing to gE (Baucke and Spear, 1979), participate in viral adsorption (Fuller and Spear, 1985). Although gD is one of the major targets for virus

neutralising antibodies (Para et al., 1985), strongly neutralising anti-gD antibodies had little effect on adsorption even at high concentrations (Fuller and Spear, 1985). This implies that whilst viral neutralisation may prevent adsorption of virions to cells, it normally affects a later stage of infection, possibly virus penetration.

6.2 Penetration of the Cell Membrane

Penetration is a temperature dependent event (Farnham and Newton, 1959), during which the virus is thought to enter the cell by fusion of the virus envelope with the cellular plasma membrane (Morgan et al., 1968; Abodeely et al., 1970) rather than by viral phagoctyosis or viropexis (Dales and Silverberg, 1969). The results of Para et al. (1980) which demonstrated the movement of Fc binding receptors from the viral envelope to the cell membrane in the presence of clycoheximide supports the fusion model of virus entry, as does genetic evidence from HSV-1 mutants which are ts for production of gB. These viruses do not contain mature forms of gB in the virus envelope when grown at the NPT (Manservigi et al., 1977; Sarmiento et al., 1979; Little et al., 1981), and although the virions produced at this temperature can bind to cells they are unable to penetrate the cell membrane. Exposure of virus-cell complexes to a fusion promoter, polyethylene glycol (PEG), enhanced infection at the NPT, suggesting that

the envelope glycoprotein, gB, is essential for infection, and that penetration occurs by fusion of virus with the cell membrane (Sarmiento et al., 1979, Little et al., 1981).

The importance of gB has been examined further by means of tsB5, an HSV-1 mutant with a syn mutation, responsible for syncytial plaque morphology of this virus, and a ts lesion in the gene encoding gB (Haffey and Spear, 1980). The finding that tsB5 also has an accelerated rate of entry into the host cell in comparison with the WT HSV-1 KOS virus (DeLuca et al., 1982) supported the idea that gB played a role in virus entry. From the analysis of tsB5-KOS5 recombinants DeLuca et al. (1982) demonstrated that the DNA sequences responsible for rate of entry of adsorbed virus were located between the syn and ts loci on the genome of tsB5, and that the three functions were encoded within the gene specifying gB (Figure 3). They may, therefore, represent functional domains of the gB gene. Subsequent work on ts1204, an HSV-1 mutant which has a ts lesion in a non-glycosylated polypeptide with a MW of 65,000, suggested that this gene product may also be involved in virus penetration, since this virus adsorbed to cells at the NPT, but failed to penetrate the cell membrane (Addison et al., 1984).

6.3 Uncoating of the Viral Genome

Early work indicated that degradation of the virus

capsid occurred in the cytoplasm in the perinuclear region (Morgan et al., 1968; Miyamoto and Morgan, 1971), and that the viral DNA was rapidly transported to the nucleus (Hochberg and Becker, 1968; Hummeler et al., 1969; Miyamoto and Morgan, 1971).

Hochberg and Becker (1968) found that transport of the viral DNA from cytoplasm to nucleus was unaffected by RNA or protein synthesis inhibitors, suggesting that the virus utilises pre-existing cellular enzymes or virion proteins to release the viral DNA from the capsid. Miyamoto and Morgan (1971) on the other hand, found that treatment of cells with protein synthesis inhibitors prior to infection did have an effect, and concluded that protein synthesis was required for transport of viral DNA from the capsid to the cell nucleus. This contradiction has not yet been resolved.

Vernon et al. (1974) noted in electron microscopic studies that preparations of flattened capsid sheets had lost their pentamers, and Dargan (1986) has suggested that this might allow the exit of viral DNA from the capsid. An HSV-1 ts mutant, tsB7, which fails to uncoat at the NPT was used to analyse the uncoating process. At the NPT full virus capsids accumulated outside the nucleus next to the nuclear pore structures (Knipe et al., 1981; Batterson et al., 1983). On a temperature shift from the NPT to the PT, the viral DNA

was released from the capsids. The proximity of capsids to the nuclear pores suggests that the viral DNA is actively transported through the nuclear pores into the nucleus.

6.4 The Effect of Virus Infection on the Host Cell Macromolecular Metabolism

In a productive lytic infection by HSV, the synthesis of host cell macromolecules is inhibited; the synthesis of both 45s and 4s rRNA, as well as the subsequent processing of 45s rRNA to 28s and 18s rRNA is severely reduced (Wagner and Roizman, 1969). Host tRNA synthesis, however, is unaffected. Although synthesis of most cellular genes transcribed by cellular polymerase II declines during virus infection, a few are transcriptionally "upregulated" (La Thangue et al., 1984; Kemp et al., 1986).

Stenberg and Pizer (1982) studied the inhibition of cellular gene transcription in an HSV infection of an adenovirus-transformed cell line, and proposed that HSV IE genes were responsible for the decrease in host RNA synthesis. This reduction in host RNA, together with the inhibition of cellular protein synthesis in the infected cell, then contributes to the decline in the synthesis of cellular DNA. However, even at late times in infection, some cellular DNA synthesis can still be detected (Roizman and Roane, 1964).

The inhibition of host polypeptide synthesis is a multistep process (Nishioka and Silverstein, 1977, 1978b) and can be divided into two phases termed "early" and "delayed" shut off.

"Early" shut-off begins soon after infection, and is mediated by one or more structural components of the virion (Fenwick and Walker, 1978; Nishioka and Silverstein, 1978; Fenwick et al., 1979). Disaggregation of host polyribosomes is responsible for the early suppression of cellular protein synthesis (Sydiskis and Roizman, 1966, 1967; Silverstein and Engelhardt, 1979). The polyribosomes are subsequently reassembled, and the majority of mRNAs associated with these ribosomes are virus coded (Stringer et al., 1977). In addition, inactivation of mRNAs (Fenwick and McMenamin, 1984) by degradation (Nishioka and Sliverstein, 1977, 1978a., 1978b; Inglis, 1982) also contributes to early shut-off of host protein synthesis. Whether or not the same structural protein is responsible for both processes is not known.

The rate of early shut-off of cellular protein synthesis mediated by HSV-2 is faster than that of HSV-1, although some strains of HSV-2, including HG52, are relatively poor at this function (Pereira et al., 1977; Fenwick and Clarke, 1982; Fenwick and McMenamin, 1984; Schek and Bachenheimer, 1985). This difference between the two types of viruses has enabled the early

host shut-off function to be located on the virus genome by analysis of HSV-1/HSV-2 recombinants. The locus was mapped within 0.52-0.59mu (Fenwick et al., 1979). The host shut-off function has proved to be a non-essential property for virus growth in tissue culture since non-lethal mutants of HSV-1 with defects in the virion-associated suppression of protein synthesis have been isolated. These mutants are deficient in the ability to degrade mRNA (Stromm and Frenkel, 1987). It is not known, however, if these mutants are impaired in the ability to disaggregate ribosomes.

Complete inhibition of host polypeptide synthesis (delayed shut-off) requires the expression of an early or late viral gene (Honess and Roizman, 1975b; Hill et al., 1983; Read and Frenkel, 1983; Schek and Bachenheimer, 1985). Since inhibition of protein synthesis is greater than might be accounted for by the effect of RNA metabolism, there may be additional factors required for full suppression. Fenwick and Walker (1979) have reported that a 48,000MW protein associated with ribosomes, was phosphorylated during virion infection, and that the process required virus gene expression. Subsequently, Kennedy et al. (1981) showed that the S6 protein of the small ribosomal subunit was phosphorylated, but it is not known whether this is the same protein described by Fenwick and

Walker (1979). It is intriguing to speculate that this modification to a ribosomal protein may affect translation of host proteins.

7. HSV TRANSCRIPTION

Introduction

HSV transcription occurs in the infected cell nucleus (Wagner and Roizman, 1969) and is thought to be carried out by a cellular RNA polymerase II (polII), since at all stages of the lytic cycle HSV transcription is sensitive to inhibition by α-amanitin (Alwine et al., 1974; Ben Ze'ev et al., 1976; Costanzo et al., 1977). The fact that the first viral gene transcripts, the immediate early (IE) mRNAs, are made in the absence of viral protein synthesis (Honess and Roizman, 1974, 1975; Clements et al., 1977; Watson et al., 1979), together with the observation that naked viral DNA is infectious (Graham et al., 1973), strongly suggests that the very early stages of transcription are probably carried out by an unmodified host polII. It is possible, though unlikely, that modification of the host polII takes place later in infection (Ben Ze'ev et al., 1976), or that a new RNA polymerase might be synthesised.

7.1 Transcript Processing

Cellular and viral genes share a number of properties

1. Polyadenylation at the mRNA 3' termini which is

dependent on the consensus signal 'AATAAA' (Bachenheimer and Roizman, 1972; Silverstein et al., 1976; Proudfoot and Brownlee, 1976; Cole and Santangelo, 1983).

2. A 'G-T'-rich sequence (Consenus YGTGTTYY-3') approximately 30bp downstream from the poly-A signal, which is necessary for the efficient formation of 3' termini of gene transcripts (Taya et al., 1982; McLauchlan et al., 1985; Whitton et al, 1983; Cole and Stacey, 1985).

3. The 'TATA-box' homology located close to the 5' end of most eukaryotic genes (Gannon et al., 1979), and all the HSV genes so far sequenced (McGeoch et al., 1985, 1986). This is important for the efficient and accurate initiation of transcription.

4. A 5' terminus mRNA 'cap', consisting of 7-methyl guanosine triphosphate residues (7MeG), added by a 5'-5' linkage. The cap structure is important for efficient translation (Shatkin, 1976) and may protect the mRNA against phosphatase and nuclease action (Moss et al., 1977). Methylation of internal adenine residues (6MeA) also takes place in both viral and cellular mRNA although late viral mRNAs are reported to be deficient in 6MeA (Bartkoski and Roizman, 1976; Moss et al., 1977).

7.2 Splicing

HSV has very few spliced genes. These include IE68 and

IE12 genes (Watson et al., 1981; Watson and Vaude-Woude, 1982; Rixon and Clements, 1982; Murchie and McGeoch, 1982) (Figure 5) which have single introns in the 5' noncoding sequences. In contrast, IE110 contains two introns located in the coding sequences of the gene (Watson et al., 1979; Perry et al., 1986). There is also a spliced late viral transcript of 2.7kb (at approximately 0.2mu) which has a particularly large intron of 4kb (Costa et al., 1985).

7.3 Temporal Regulation of HSV Transcription

In a normal lytic cycle, HSV transcription is a temporally regulated process which can be separated into three stages known as the IE, early (E) and late (L) phases, alternatively the α, β and γ phases (Swanstrom and Wagner, 1974; Honess and Roizman, 1974, 1975; Clements et al., 1977; Jones and Roizman, 1979). On the basis of inhibitor and kinetic studies a "cascade model" of gene regulation was proposed by Honess and Roizman (1974, 1975), however, more recent work has demonstrated that the process and control of sequential HSV gene regulation is far more complex than was originally envisaged.

7.4 Nomenclature of IE Genes and IE Polypeptides

Several different nomenclatures have been proposed for the IE genes and their protein products (see also Figure 5). However, in this thesis they will be

Figure 5

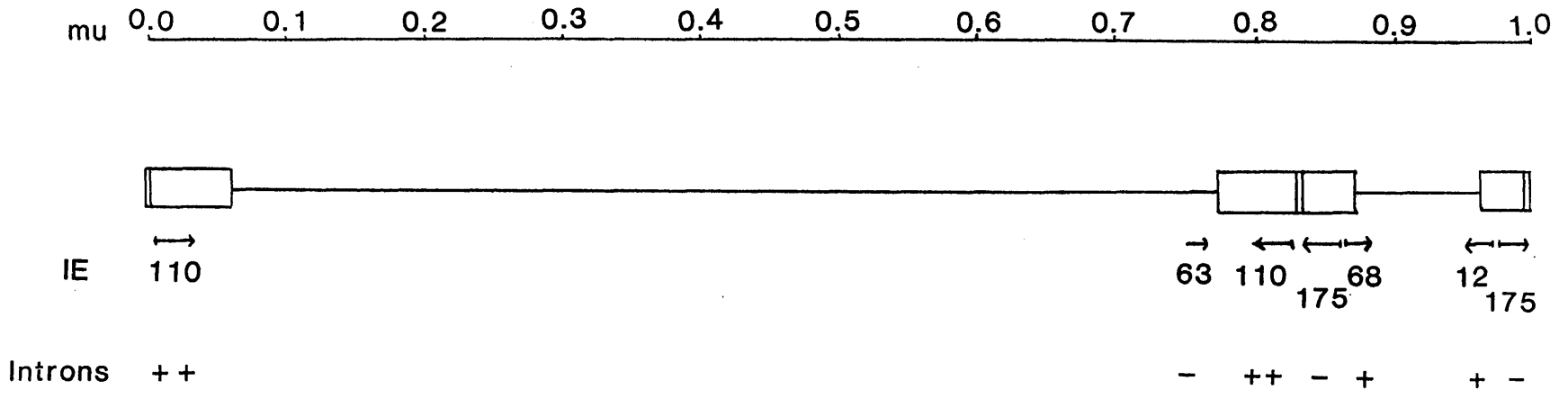
The map locations of HSV-1 IE mRNA transcripts

IE genes are termed IE110, IE63, IE175, IE68 and IE12 or alternatively the nomenclature is IE1, 2, 3, 4 and 5 respectively.

The sizes of protein products by SDS-PAGE and sequencing are:

IE gene	SDS-PAGE(mw×000)	Sequencing Data(mw)
110	110	78,452
63	63	55,376
175	175	132,835
68	68	46,522
12	12	9,792

References are given in the text.


mu
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
IE
110
63
110
175
68
12
175
Introns
+ +
−
++
−
+
+
−

referred to solely in terms of their sizes on SDS-PAGE. Thus, for example, the gene IE175, and its protein product, Vmw175.

7.5 Immediate Early Genes

The IE genes are defined as those which are transcribed very early in a normal lytic infection, and in the absence of de novo viral protein synthesis as demonstrated by the addition of inhibitors such as cycloheximide or anisomycin from the time of infection (Honess and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979; Harris Hamilton and Bachenheimer, 1985).

The IE genes are located within or close to the repeat sequences of HSV (Figure 5). The IE polypeptides range in predicted size from 12,000 to 175,000 MW as estimated by electrophoresis on SDS-polyacrylamide gels and this correlates roughly with the predicted MW as determined by DNA sequencing of the IE genes. This information is summarised in Figure 5 (Clements et al., 1979; Watson et al., 1979, 1981; Watson and Vande-Woude, 1982; Whitton et al., 1983; McGeoch et al., 1985, 1986; Perry et al., 1986). Apart from Vmw12, all the IE polypeptides are phosphorylated and bind to DNA (Marsden et al., 1976; Pereira et al., 1977; Hay and Hay, 1980).

IE175 is present as two copies on the genome (Rixon et al, 1982; McGeoch et al., 1986a.), and the gene product (Vmw175) is essential for the transcription of early and late genes. The DNA binding properties of Vmw175 were lost on extensive purification of this polypeptide, but could be restored by the addition of uninfected cell extracts, indicating that direct binding of Vmw175 to DNA was unlikely (Freeman and Powell, 1982). In further work, Vwm175 which had been only partially purified, was found to form a complex with DNA at sequences upstream of the gD promoter and in pBR322 (Faber and Wilcox, 1986; Beard et al., 1986). This work has led to the description of a consensus sequence for Vmw175-DNA binding (5' ATCGTCNNNYCGRC-3': N=any base; Y=pyrimidine R=purine)(Faber and Wilcox, 1986). Recently, Muller (1987) showed that extracts of HSV-1 infected cells contain proteins which form a complex with DNA fragments that contain the IE175 cap site and sequences fitting the consensus. Furthermore, Vmw175 is a component of this complex. However, Kristie and Roizman (1986b.) have demonstrated the binding of Vmw175 to different DNA sequences in IE promoters, and at upstream sequences in IE175. The reason for these differences is unclear at the present time.

IE110, like IE175, is a diploid gene, but is not essential for virus growth in tissue culture (Stow and

Stow, 1986). Vmw110 and the products of the unique genes IE63 and IE12, Vmw63 and Vmw12, have been implicated in the trans-activation of early and late genes (O'Hare and Hayward, 1985a., 1985b.; Sacks et al., 1985; Everett, 1986). However, IE12, as well as IE68, is a non-essential gene (Post and Roizman, 1981; Sears et al., 1985; Longnecker and Roizman, 1986) in cells

7.5.1 IE Promoters and Regulation

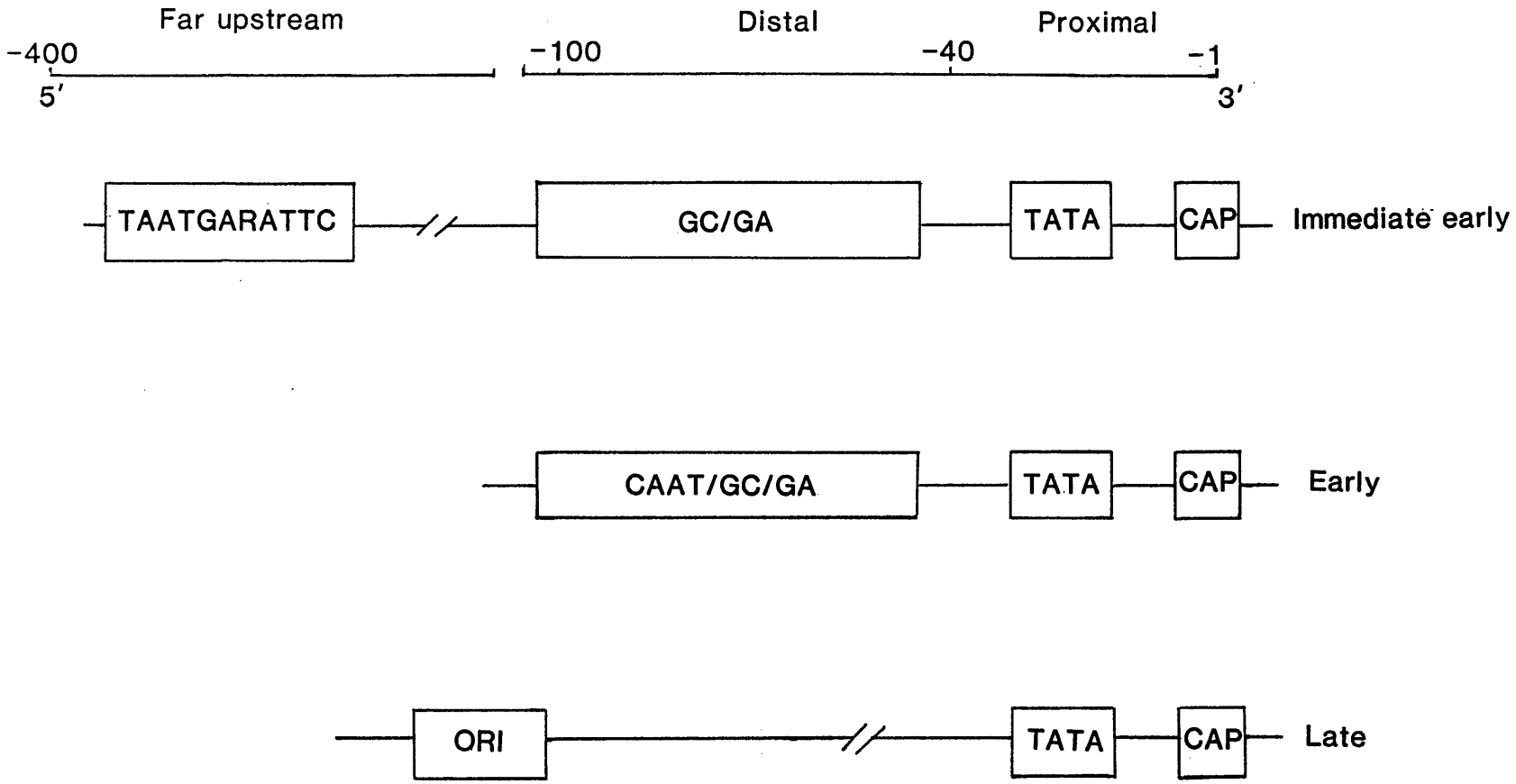
The basal level of transcription from IE gene promoters is detectable even in the absence of trans-activation. Using a recombinant plasmid where the coding sequence of a gene with an easily assayed product is placed under IE control, the basal levels of transcription have been studied by cell transformation assays and in transient expression assay systems. The virus TK and the bacterial plasmid chloramphenicol acetyl transferase genes have been particularly useful in this work (Wigler et al., 1977; Kit et al., 1978; Post et al., 1981; Mackem and Roizman, 1982a.; Cordingley et al., 1983). Analysis of IE gene promoter and regulatory regions has thus been carried out by deletions of the promoter sequences, revealing a number of different upstream sequence elements (Figure 6). In the case of the IE175 gene, the TATA box homology is present at approximately 30bp from the mRNA start site. A proximal promoter region which contains Sp1 binding sites is located between -38 and -108bp (Jones and Tjian, 1985) and within this region is an element whose

Figure 6

The upstream noncoding promoter sequences of immediate early, early and late genes

Details are given in the text

From Johnson and Everett (1986b).


Far upstream
Distal
Proximal
-400
-100
-40
-1
5′
3′
TAATGARATTC
GC/GA
TATA
CAP
Immediate early
CAAT/GC/GA
TATA
CAP
Early
ORI
TATA
CAP
Late

deletion abolishes transcription of the IE gene (Cordingley et al., 1983). Enhancer-like sequences have been identified in IE175, so termed because they can function in either orientation at a distance of up to 1,300bp from the promoter, and because they exhibit some host specifity (Cordingley et al., 1983; Preston and Tannahill, 1984; Lang et al., 1984; Bzik and Preston, 1986). In addition, there is a regulatory element with a conserved 'AT'-rich sequence (consensus TAATGARATTC, where R is a purine) present far upstream of all HSV-1 and HSV-2 IE genes (Mackem and Roizman, 1982a, 1982b; Cordingley et al., 1983; Whitton et al., 1983; Whitton and Clements, 1984). This region is responsive to trans-activation of IE transcription.

7.5.2 Negative Regulation of IE Gene Expression

All IE polypeptides are made very early in infection and their synthesis declines at early and late times (Honess and Roizman, 1974, 1975; Clements et al., 1977). Initially it was proposed that early gene products were responsible for the "switch-off" of IE synthesis (Honess and Roizman, 1974), and this theory was supported by Read and Frenkel (1983) who identified a structural protein which both inhibited host protein synthesis and affected IE mRNA stability. However, studies on tsK, which is an HSV-1 mutant which has a ts lesion in IE175 gene (Preston, 1979a, 1979b), suggested that IE synthesis was autoregulated at the level of

transcription. When tsK-infected cells were transferred from the PT to the NPT in the presence of cycloheximide, early and late mRNA synthesis declined, and IE transcripts were overproduced, implying that functional Vmw175 plays a role in the suppression of IE mRNA synthesis (Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980).

DeLuca and Schaffer (1985) also demonstrated Vmw175 involvement in IE gene regulation with a transient expression assay system where low levels of Vmw175 were found to stimulate expression from the IE68/12 promoter, whilst at high concentrations of Vmw175, negative regulation of transcription was detected. The effect of Vmw175 on transcription from an IE175-CAT gene construct was studied by O'Hare and Hayward (1985b), and the polypeptide was shown to have a consistently negative effect. These workers also demonstrated that the stimulation induced in the IE175-CAT plasmid by Vmw110 (another IE polypeptide), could be inhibited by the addition of Vmw175, and proposed that this was due to the dominant negative regulation by Vmw175 over Vmw110-stimulation of the plasmid construct. However, this result may also be explained as the negative regulation of IE110 by Vmw175 (O'Hare and Hayward, 1985b).

The autoregulation of IE175 could be mediated by the binding of Vmw175 to negatively regulating sequences at the IE175 mRNA cap site, as suggested in recent work by Muller (1987), or alternatively by the removal, inhibition or inactivation of a cellular factor essential for activation of the IE promoter, in which case an apparent "down-regulation" of IE175 transcription would result. It should also be noted that whilst Vmw110 has only been implicated in a stimulatory role, IE polypeptides apart from Vmw175 may be involved in some way in the repression of IE gene expression.

Sacks et al (1985) have shown that viruses which contain ts mutants within IE63 overproduce IE polypeptides at the NPT, and it is possible that a late gene product required for IE gene regulation is not synthesised at the NPT by these viruses. This suggests that Vmw63 may be involved, indirectly, in the regulation of IE gene transcription.

7.5.3 Trans-activation of IE Genes

Trans-activation of IE genes was first demonstrated by Post et al (1981). These workers converted the early gene for TK into an IE-regulated gene by inserting IE175 promoter sequences upstream of the TK coding sequence. Transfection of TK or IE-TK constructs into LTK^- cells resulted in the expression of TK at similar

basal levels. On superinfection of the transformed cells with a TK virus, expression of normal TK was stimulated 2-3 fold by 6-8h pi. However, expression of IE-TK was enhanced within 2-3h pi, and by 6-8h pi expression was 5-10 fold greater than the basal level.

Further experiments demonstrated that stimulation of IE-TK by superinfecting virus was unaffected by the presence of cycloheximide, and this suggested, therefore, that a component affecting IE *trans*-activation was present in the HSV particle (Post *et al*., 1981). This phenomenon has been demonstrated *in vitro* using a transient expression assay system (Mackem and Roizman, 1982b; Cordingley *et al*., 1983; Preston *et al*., 1984), and the virion component has since been identified as a 65,000 MW structural polypeptide, located in the virus tegument (Campbell *et al*., 1984).

The process of IE gene *trans*-activation is thought to be required by the virus as a means of increasing the efficiency of the round of virus infection and replication. Thus, this represents an important field of study, and it has been the basis of much of the work in this thesis. The subject of IE *trans*-activation and the studies resulting from the work of Post *et al* (1981) will, therefore, be discussed in more detail in Section 4.

7.6 Early Gene Transcription and Regulation

Early genes are transcribed after the IE genes and in the presence of inhibitors of viral DNA synthesis, such as cytosine arabinoside (araC) or phosphonoacetic acid (PAA) (Honess and Roizman, 1974, 1975), but require the presence of functional IE polypeptides for their transcription (Swanstrom and Wagner, 1974; Swanstrom et al., 1975; Clements et al., 1977; Harris-Hamilton and Bachenheimer, 1985). The early class of polypeptides includes many enzymes, and also some structural proteins, for example, gD (Watson et al., 1983).

Early gene transcripts can be detected in HSV-1 infected BHK cells, at 37°, from 2h pi, and increase in abundance until 6-8h pi. At this point the level of most early mRNAs declines, although some individual mRNAs continue to be synthesised until much later in infection (McLauchlan and Clements, 1982; Sharp et al., 1983; Johnson and Spear, 1984; Harris-Hamilton and Bachenheimer, 1985).

7.6.1 Cis-acting Regulatory Sequences of Early Genes

Early genes such as the viral TK exhibit a basal promoter activity which can be detected both in cells where the HSV TK gene is integrated into the host chromosom (Wigler et al., 1977; Maitland and McDougall, 1977; Minson et al., 1978) and on the microinjection of Xenopus Laevis oocytes, where functional TK is produced

(McKnight and Gavis, 1980; Cordingley and Preston, 1981). Thus, it is possible to define the control elements responsible for constitutive transcription of a typical early gene such as TK. The results from the analysis of the TK gene have located at least three important regulatory domains in the 5' noncoding sequences of this gene (McKnight et al., 1981; McKnight and Kingsbury, 1982). These domains were identified through the production of linker scanning (L-S) mutants (McKnight and Kingsbury, 1982). Mutations were engineered into the TK gene by the recombination of 5' and 3' deletion mutants of the gene, whose termini were separated by 10 nucleotide residues. The 10 nucleotides were substituted by linker sequences and clusters of base substitutions were therefore inserted across the gene.

Three promoter regulatory regions were identified by this means: (1) the proximal signal or TATA box homology located between -16 and -37bp; (2) a distal signal, dsI, at -47 to -79bp; (3), a distal signal dsII, located between -84 and -104bp upstream from the start of the coding sequences (McKnight, 1980, 1982). The distal signals each have a GC-rich hexanucleotide sequence (McKnight and Kingsbury, 1982; McKnight et al., 1984) which are functionally related and operate in a mutually dependent manner (McKnight et al., 1981; McKnight, 1982). In addition, dsII contains an

inverted 'CCAAT' pentanucleotide, which, like the GC-sequences, is responsive to transcriptional stimulation by cellular transcription factors (see also Section 8.5).

7.6.2 Early Gene Trans-activation

A number of viral gene products have been identified as trans-activators of early gene transcription.

Vmw175 is essential throughout infection for the transcription of both E and L genes, and the basis of this dependence has been investigated in several ways. It has been demonstrated that ts mutants of HSV-1, such as tsK,' which have a lesion in IE175, fail to process functional Vmw175 at the NPT, or to transcribe E genes, but overproduce IE transcripts (Preston, 1979a, 1979b; Watson and Clements, 1980). On temperature shiftdown to the PT in the presence of cycloheximide, E gene transcription proceeds, whilst a shift back to the NPT causes E gene transcription to cease, and IE transcription to recommence (Preston, 1979a., 1979b; Watson and Clements, 1980).

Alternatively, stably transformed cell lines which constitutively express Vmw175 have been produced. These stimulate early gene expression from superinfecting virus in the presence of CHX (Davidson and Stow, 1985; Persson et al., 1985). In addition,

early genes resident in transformed cells can be trans-activated by IE polypeptides expressed from a superinfecting virus (Leiden et al., 1976; Sandri-Goldin et al., 1983).

Another approach to the study of E gene promoter trans-activation is the use of a transient expression assay system, by which the promoters of the early genes, gD (Everett, 1983, 1984a, 1984b), TK (O'Hare and Hayward, 1985a, 1985b; Gelman and Silverstein, 1985, 1986), and the major DNA binding protein (MDBP) of HSV-1 (Quinlan and Knipe, 1985) have been analysed. In all cases, expression of the early genes was stimulated by cotransfection with plasmids which expressed functional Vmw175.

Transfection studies have also demonstrated that an independent role exists for Vmw110 in the trans-activation of early genes (O'Hare and Hayward, 1985a, 1985b; Everett, 1984a, 1985; Gelman and Silverstein, 1985; Quinlan and Knipe, 1985). The effect of this trans-activation varied considerably in different experiments, and this is thought to be due to differences between gene promoters, the various cell types and the assay systems which have been used (Everett, 1986).

Interestingly, the cotransfection of plasmids

expressing Vmw175 and Vmw110 resulted in an enhancement of the trans-activation of early promoters to a level considerably greater than the sum of trans-activation by Vmw175 or Vmw110 alone (Everett, 1985, 1986; Gelman and Silverstein, 1985, 1986). In some cases, this stimulation appears to be augmented further by the addition of Vmw12 (O'Hare and Hayward, 1985b).

Gelman and Silverstein (1986) have suggested that the synergistic effect of Vmw175/Vmw110 co-stimulation may be the result of an interaction between the two proteins to regulate early gene expression. The existence of a tight protein complex has been excluded, since antisera to Vmw175 or Vmw110 will only immunoprecipitate their respective proteins (Metzler and Wilcox, 1985), however, this does not rule out a weak association between these polypeptides. Alternatively, either or both IE proteins might modify the host polII, or interact with cellular transcription factors to stimulate E gene expression.

A number of workers have studied E gene promoter mutants, but have been unable to identify any specific promoter region which is essential for trans-activation (Everett, 1984b; Eisenberg et al., 1985). However, using L-S mutants of the TK promoter, Coen et al., (1986) have shown that the upstream TK sequences which are sensitive to base substitutions are those which are

important for recognition by cellular transcription factors. Thus, the evidence suggests that trans-activation of E genes by IE gene products may occur through an interaction with cellular factors which recognise promoter elements.

7.7 Late Gene Transcription and Regulation

Late genes can be broadly defined as those whose expression is significantly reduced in the presence of inhibitors of viral DNA synthesis such as PAA and ara-C (Honess and Roizman, 1974, 1975; Swanstrom et al., 1975; Clements et al., 1977). Two groups of late genes have been identified within this general description; these are known as leaky-late (γ_1, or $\beta\gamma$) and true late (γ_2 or γ) genes (Holland et al., 1980; Johnson and Everett, 1986a.).

γ_1, gene transcripts are easily detectable prior to DNA synthesis, whereas γ_2 mRNAs are present only in very small amounts early in infection, at about 5% of the levels detectable after viral DNA synthesis (Holland et al., 1980; Johnson and Everett, 1986b.; Johnson et al., 1986).

Recent work on the promoter regions of a true late gene, U_S11, which encodes a polypeptide Vmw21 (Rixon and McGeoch, 1984; Johnson and Everett, 1986a., 1986b.; Johnson et al., 1986) has shown that late genes do not

possess any detectable upstream sequences which are responsible for the late pattern of regulation (Johnson and Everett, 1986b). In these experiments, the plasmid-borne U_S11 gene promoter was first shown to be regulated as a late gene in a transient expression assay in the presence of origin of replication (ori_S) sequences (Johnson and Everett, 1986a). Deletion analysis of the promoter sequences then showed that all the required DNA sequence elements were located within 31bp of the mRNA cap site, that is, the cap site and TATA box homology alone (Johnson and Everett, 1986b) (Figure 6). These results were confirmed by the observation that the early gene gD, was converted to a gene with late-regulation by the removal of promoter sequences upstream from the TATA box (Johnson and Everett, 1986b).

In addition, Homa et al (1986) have shown that the late gene, gC, has similar structural requirements to U_S11, suggesting that late genes are characterised by the absence of promoter elements upstream from the TATA box. These findings support the theory that late genes have weak promoters, and that viral DNA replication enhances the copy number of the genes, as has been proposed for SV40 late gene regulation (Rio et al., 1980; Tjian, 1981; Keller and Alwine, 1984). However, if this were solely the case, the expression of E genes would also be expected to increase dramatically on

viral DNA replication. This anomaly may be explained by:

1. A requirement by early genes for a transcription factor which is present in the cell in rate limiting amounts, or which is unstable at late times in infection.
2. An E mRNA instability at late times.
3. The negative regulation of E gene transcription at late times in infection.

Studies on late gene expression using transient assay systems have shown that Vmw175 and Vmw110 play an important role in late gene trans-activation (Mavromara-Nazos et al., 1986b; DeLuca and Schaffer, 1985; Silver and Roizman, 1985). These experiments, together with the work of Johnson and Everett (1986b) implies that the TATA box and cap site alone may fulfil the requirement for trans-activation of late genes by Vmw175 and Vmw110. Other gene products which have been implicated in the regulation of gene expression include Vmw63, since ts mutants with lesions in this gene were found to make reduced amounts of L proteins at the NPT (Sacks et al., 1985), although there was no block to viral DNA synthesis in these mutants.

The HSV major DNA binding protein (ICP8) (Powell and Purifoy, 1976, 1977; Knipe et al., 1982) has been shown to repress expression of the late gene gC, in the

absence of DNA replication (Godowski and Knipe, 1985). Mutants of HSV-1 with a ts lesion in ICP8 made increased amounts of gC at the NPT compared with WT virus which was treated with PAA. However, the amount of gC synthesised in the ts mutant-infected cells under these conditions was still approximately 10-fold less than that produced late in infection in WT virus cells in the absence of a viral DNA synthesis inhibitor (Godowski and Knipe, 1985).

8. CELLULAR TRANSCRIPTION FACTORS

The analysis of eukaryotic gene promoters has led to the identification of a number of sequence elements located upstream from the normal transcriptional start site which have important roles in the initiation of polII transcription.

a. The TATA box (Benoist and Chambon, 1981).

b. Promoter motifs, which may be gene specific, for example for the metallothionein metal response element (Karin et al., 1984) and the heat shock regulatory sequence (Pelham, 1982), or general, such as the GC hexanucleotide, or 'CCAAT'-box (Benoist et al., 1980; Efstratiadis et al., 1980).

c. Enhancers, which vary in their specificity (Banerji et al., 1981; Gruss et al., 1981; Moreau et al., 1981; Fromm and Berg, 1982).

Recent studies on transcriptional control have focused on polypeptides which bind to DNA sequences.

8.1 The Identification of Cellular Factors

In vitro initiation of transcription by polII was first demonstrated with crude cell extracts (Manley et al., 1980; Handa et al., 1981; Dignam et al., 1983), and the subsequent fractionation of these extracts resulted in the identification of a number of cellular transcription factors. Certain factors were found to be required for transcription of all genes tested (Matsui et al., 1980; Samuels et al., 1982) whilst others exhibited promoter specificity (Dynan and Tjian, 1983a; Sawadogo and Roeder, 1985b; Jones et al., 1985). Using dimethylsulphate methylation and DNaseI protection studies (Dynan and Tjian, 1983b; Gidoni et al., 1985) several of these proteins were shown to bind specifically to discrete DNA sequences, such as the GC-hexanucleotide box.

8.2 Sp1

Cellular transcription factor Sp1 was first identified in HeLa cells (Dynan and Tjian, 1983a), and found to be essential for SV40 early gene transcription. Its recognition sequence, 5'GGGCGG3', is present in the SV40 early gene promoter as six tandem repeats within three 21bp repeat sequences (Figure 7), and up to 5 Sp1 subunits can bind to these at any one time (Dynan and Tjian, 1983b., 1985; Gidoni et al., 1985).

Figure 7

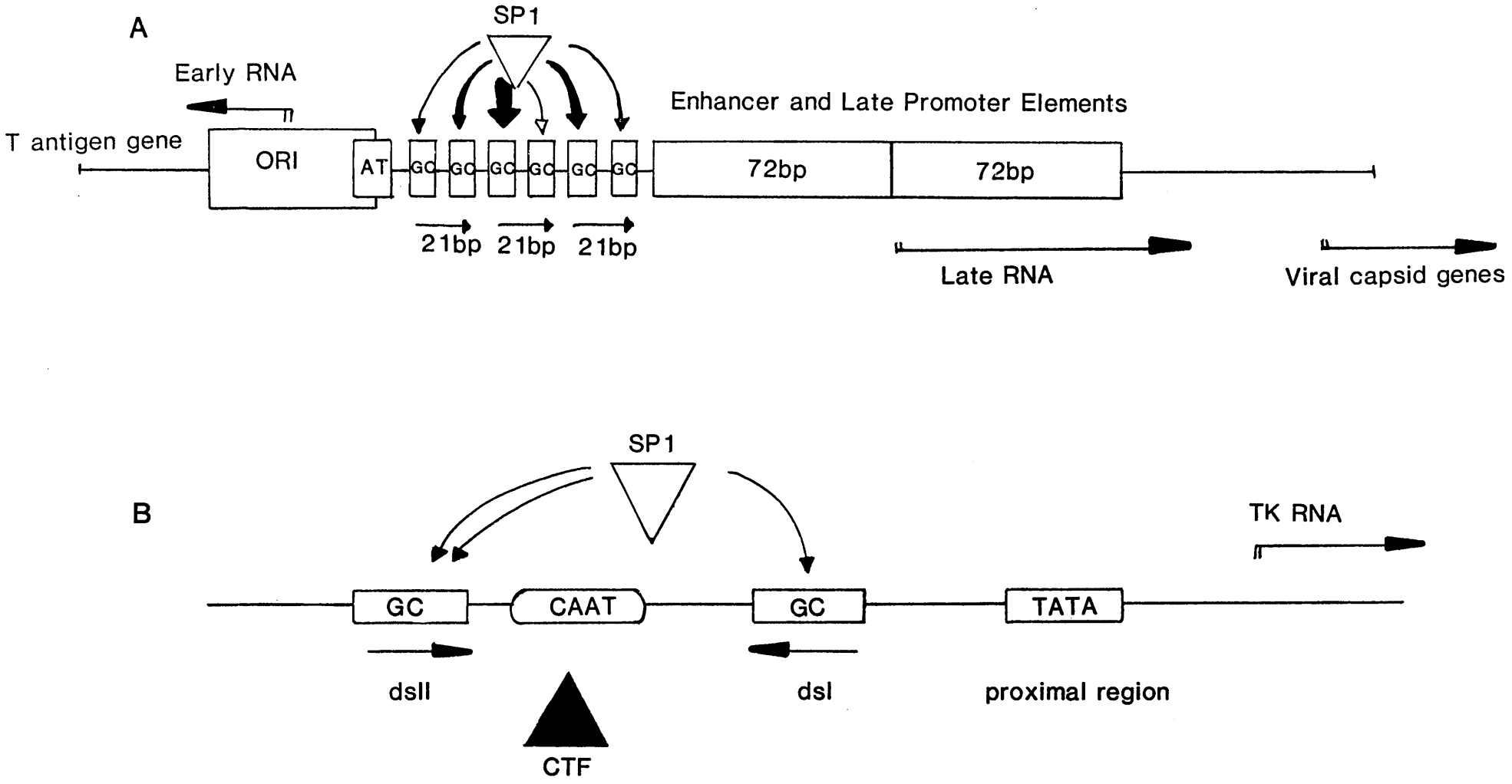
Viral promoter elements and cellular transcription factors

A. SV40 Control region

ORI represents SV40 origin of replication sequences. Open rectangles represent ; (AT) the TATA-like element, and (GC), six 'GC' hexanucleotides (Sp1 binding sites). GC-boxes are underscored by horizontal arrows. Two open boxes represent 72bp repeats which contain cis-acting elements for enhancers and late promoter activity. The direction of mRNA transcription is represented by arrows. Open triangles represent a protomer of Sp1. The thickness of curved arrows from Sp1 represent different affinities of individual 'GC' hexanucleotides for Sp1 binding.

B. HSV: cis-acting control elements in the TK promoter

These include the proximal region (TATA box), distal signal I (dsI) and distal signal II (dsII). Transcription is represented by an arrow. dsI and dsII contain a 'GC' hexanucleotide represented by an open box and inverted horizontal arrows. dsII also contains 'CCAAT' homology represented by an open ellipse. An Sp1 protomer is represented by an open triangle, and the number of arrows to the dsII 'GC' box represents greater affinity of binding to the dsII 'GC' hexanucleotide. A protomer of CTF is represented by a closed triangle which is bound to a functional 'CCAAT'-box in dsII.


A
SP1
Early RNA
Enhancer and Late Promoter Elements
T antigen gene
ORI
AT
GC
GC
GC
GC
GC
GC
72bp
72bp
21bp
21bp
21bp
Late RNA
Viral capsid genes
B
SP1
TK RNA
GC
CAAT
GC
TATA
dsII
dsI
proximal region
CTF

Since its initial detection in the SV40 early gene promoter the Sp1 recognition sequence has been identified in a variety of other gene promoters. These include, for example, the mouse hypoxanthine phosporibosyl transferase (Melton et al., 1984), human adenosine deaminase (Valerio et al., 1985) the HIV terminal repeat (Ratner et al., 1985; Wain Hobson et al., 1985; Jones et al., 1986) and the HSV IE and TK promoters (see also Section 1.8.5) (Jones et al., 1985; Jones and Tjian, 1985; Kadonaga et al., 1986). The hexanucleotide core is present at all binding sites, however further analysis has shown that binding of the promoter to DNA is not dependent on the orientation of the hexanucleotide (Everett et al., 1983; Gidoni et al., 1985), and the affinity of the Sp1 molecule is affected by sequences adjacent to the GC core. A 10bp consensus sequence which is probably required for competent binding of an Sp1 protomer, has therefore been derived from the known Sp1-binding sequences as 5'GGGGCGGGGG3'(Kadonaga et al., 1986). Efficient binding of Sp1 to this sequence can increase the rate of gene transcription by 10 to 50 fold.

T AAT

8.3 CCAAT Transcription Factor (CTF)/CCAAT Binding Protein (CBP)

Two similar proteins, CTF (Jones et al., 1985) and CBP (Graves et al., 1986) which bind to a domain specified by 5'CCAAT 3', have been isolated from HeLa cells and rat liver nuclei, respectively. It is not yet clear if

these are the equivalent proteins of different cell systems, since whilst both bind to the 5'CCAAT 3' sequence, they differ in a number of properties. For example, CBP is heat stable in comparison with CTF (McKnight and Tjian, 1986), and can bind to altered CCAAT sequences. Furthermore, their DNase footprinting patterns in the HSV TK gene are slightly different. They may, therefore, be two different gene products which are simply related in their ability to bind specific DNA sequences (McKnight and Tjian, 1986).

CTF has been compared with nuclear factor-1 (NF-1), a cellular polypeptide required for the initiation of adenovirus DNA replication (Nagata et al., 1982; Hay, 1985). On purification, NF-1 and CTF (Rosenfeld and Kelly, 1986) appeared to be identical, and it is now thought that there may be a single protein factor with the ability to function both as a replication and transcription factor (Jones et al., 1987).

8.4 Upstream Stimulatory Factor (USF), TFIIB, TFIID, TFIIE

At least three different protein factors, TFIIB, TFIID and TFIIE plus RNA polII have been shown to be required for initiation of transcription of the adenovirus major late (ML) promoter in vitro (Sawadogo and Roeder, 1985a). A further protein, the upstream stimulatory factor (USF), has also been identified in uninfected

HeLa cells, and this appears to increase transcription efficiency by simultaneous interaction with TFIID on the adenovirus ML promoter upstream sequences (Sawadogo and Roeder, 1985b).

8.5 The Action of Cellular Protein Factors on HSV Gene Transcription

Sp1 binds to at least eight different regions within the HSV TR_S, and can stimulate transcription from IE175, IE68 and IE12 genes by up to twenty-five fold (Jones and Tjian, 1985; Jones et al., 1985), although the individual Sp1 sites in the IE175 promoter stimulate transcription to quite varying degrees. The IE175 upstream region also contains sequences which respond specifically to trans-activation by Vmw65 (Bzik and Preston, 1986). Vmw65, which does not bind directly to DNA (Marsden et al., 1987) is believed to affect transcriptional stimulation by means of a cellular protein-Vmw65 sequence-specific DNA binding complex, and this will be discussed in more detail in Section 4.

Cellular transcription factor binding sequences have also been identified in the HSV TK promoter. This early gene promoter is composed of 3 elements which affect transcription; the TATA box, and two distal elements, dsI and dsII (Figure 7), both of which have a single GC box which is inverted in dsI (McKnight et

al., 1981; McKnight and Kingsbury, 1982; McKnight et al., 1984). Both GC-rich hexanucleotides function as Sp1 binding sites (Jones et al., 1985), but dsI is much weaker than dsII (McKnight and Kingsbury, 1982). This difference in affinity for Sp1 may be explained by the change of a single nucleotide in dsI which disrupts the consensus outside the basic GC box: 5'GGGGCGGCGC3', whilst dsII maintains the consensus sequence (Kadonaga et al., 1986; Jones et al., 1985).

In addition to the Sp1 site, dsII, unlike dsI, has 2 possible, adjacent 'CCAAT'-boxes (Figure 7). One of these is in the usual orientation at -81 to -77bp upstream, and contains a single base mismatch (CGAAT). However, the disruption of this element has little effect on TK promoter activity. The second site is a perfect 'CCAAT'-box (-82 to -86bp), but is in an inverted orientation (Graves et al., 1986). Mutation of this CCAAT-box results in a reduced efficiency of TK transcription both in vitro and in vivo (Jones et al., 1985). Transcription of TK, therefore, may be dependent on Sp1 and CTF/CBP acting separately or in concert; however, it appears that independently neither factor may be entirely sufficient for efficient expression of TK (McKnight and Tjian, 1986).

9. VIRAL DNA SYNTHESIS

Semiconservative viral DNA synthesis takes place in the infected cell nucleus (Munk and Sauer, 1964; Roizman,

1969). Replication of HSV-2 strain HG52 at 37°C can be detected in BHK cells by 3h pi, reaching a maximal rate within 9-11h pi (Rixon, 1977), and similar results have been reported for HSV-1 strain ∝(Wilkie, 1973). Only a small proportion (<5%) of input HSV genomes are replicated, whereas 70-80% of parental PRV genomes participate in DNA synthesis (Jacob and Roizman, 1977; Ben-Porat et al., 1976a.). During replication nicks and gaps of unknown function which are present within the DNA are repaired (Wilkie, 1973; Frenkel and Roizman, 1972; Ben Porat et al., 1976b.). Viral DNA also contains short stretches of ribonucleotides (Biswal et al., 1974), however, it is not known whether such ribonucleotides can act as primers for discontinuous synthesis of the DNA.

The precise details concerning viral DNA replication have not yet been fully elucidated. Electron microscope studies on viral DNA suggested that the linear genome circularises early in infection (Jean and Ben-Porat, 1976; Jacob and Roizman, 1977; Ben-Porat and Veach, 1980) and the restriction endonuclease analysis of a population of a non-inverting HSV-1 mutant with lacked an internal a sequence and most of the IR_L/IR_S confirmed and extended these observations (Poffenberger et al., 1983; Poffenberger and Roizman, 1985). In these experiments, it was found that the viral genome circularised in the absence of viral protein synthesis,

indicating that a host enzyme or a virion protein was responsible for this process. In addition, the discovery that the circularisation process occurred very rapidly, together with the finding that a small proportion of virion genomes were circular, suggested that HSV genome termini are available for immediate ligation (Poffenberger and Roizman, 1985).

Although HSV DNA has terminally redundant ends (Grafstrom et al., 1974; Sheldrick and Berthelot, 1974; Wadsworth et al., 1975), sequence analysis of the termini and joint regions indicates that the DNA probably circularises by direct ligation of its termini (Davison and Wilkie, 1983) rather than by the base pairing of exposed complementary sequences as was originally proposed (Jacob and Roizman, 1977). The PRV genome, which has unique ends, is also thought to circularise by direct ligation of its termini.

9.1 The Mechanism of Viral DNA Synthesis

At early times in the virus reproductive cycle, "lariats" of DNA and genomes containing "eyes" or branches can be visualised under the electron microscope, and these are believed to represent replicative intermediates (Shlomai et al., 1976; Ben-Porat et al., 1976; Hirsch et al., 1977). In view of the observations that most viral genomes circularise

early in infection, it is likely that initially, PRV and HSV replicate by a theta type mechanism (Figure 8). Late in infection, however, replicating PRV and HSV DNA form rapidly sedimenting "tangles" of DNA, indicative of high MW intermediates (Ben-Porat and Tokazewski, 1977; Jacob et al., 1979). Restriction endonuclease analysis of this DNA revealed a low concentration of terminal fragments relative to the joint-spanning fragments (Ben-Porat and Rixon, 1979; Jacob et al., 1979). This suggested that the replicated DNA was present as head-to-tail concatemers, and, on the basis of this information, Jacob et al (1979) proposed that at this stage, HSV replicates by a rolling-circle mechanism, whereby head-to-tail concatemers of DNA are formed from the continuous synthesis of one strand and discontinuous synthesis of the other strand (Figure 8).

9.2 Viral Origins of Replication

Electron microscope analysis of replicating wild type HSV DNA suggested that DNA synthesis was initiated at multiple sites on the virus genome (Friedmann et al., 1977). This approach, however, was unsuitable for determining the precise locations of the viral origins of replication. Indirect evidence for the presence of cis-acting sequences required for viral DNA replication was obtained from the analysis of defective genomes of both HSV and PRV (Frenkel et al., 1975, 1976; Rixon and Ben-Porat, 1979; Graham et al., 1978; Locker and Frenkel, 1979).

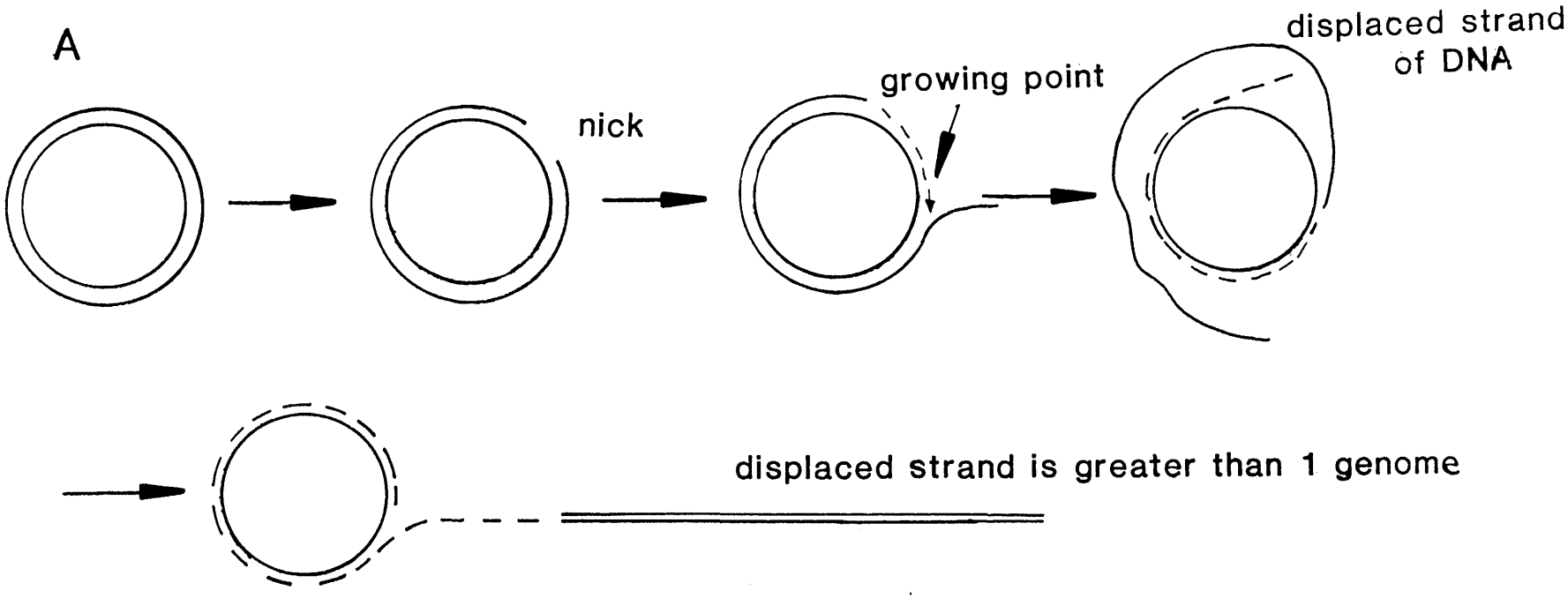
Figure 8

The replicative forms of HSV

A. The rolling-circle mechanism

A DNA strand is displaced from nicked circular DNA and DNA is synthesised using the opposite strand as a template, forming a concatemer.

B. Early in replication viral DNA appears to replicate by a Theta (Θ) mechanism involving the continuous synthesis of one strand and discontinuous synthesis of the other strand.


A
displaced strand
of DNA
growing point
nick
displaced strand is greater than 1 genome
B
Theta form of replicating DNA

Defective virus particles are produced by the serial passage of a virus stock at a high moi (Bronson et al., 1973) and contain genomes consisting of tandem reiterations of a portion of the parental genome. Two classes of HSV defective DNA molecules have been identified: Class 1 genomes consist of sequences derived from U_S and the entire short repeat (Vlazny and Frenkel, 1981) whereas Class 2 genomes have sequences from U_L linked to sequences from a small part of the short repeat (Schroder et al., 1975/1976; Kaerner et al., 1979). It was deduced from these studies that there were three origins of replication (ori) within the HSV genome; two copies of ori_S, one in each short repeat, and one copy of ori_L present in U_L.

Direct evidence for the presence of origins of replication within Class 1 and 2 defective molecules was obtained by Vlazny and Frenkel (1981) and Spaete and Frenkel (1982). These workers showed that tandemly reiterated DNA sequences in a head-to-tail arrangement were generated from defective monomer DNA molecules in cells cotransfected with helper virus DNA, which supplied trans-acting replication functions, and the defective DNA.

The precise map location of ori_S was achieved by screening cloned HSV DNA fragments for the ability to

replicate in the presence of helper virus, using a transfection assay for replication (Stow, 1982; Mocarski and Roizman, 1982b; Stow and McMonagle, 1983). A 90bp element, which contained all the cis-acting sequences essential for replication, was identified.

An almost perfect 45bp palindrome was found within this sequence (Murchie and McGeoch, 1982). Comparison of the HSV-2 strain HG52 DNA sequence between IE175, and IE genes 68 and 12 with that of the HSV-1 strain 17 ori_S region revealed the presence of a strongly conserved sequence containing a tandem duplication (Whitton and Clements, 1984b). The ori_S of HSV-2 is 137bp long and the homology to HSV-1 extends just beyond the limit of the HSV-1 90bp ori_S region.

The identification of ori_L sequences has proved to be a more difficult task because the essential sequences were deleted when fragments containing the putative ori_L were cloned into bacterial plasmids. HSV-1 ori sequences were determined from defective viral DNA (Gray and Kaerner, 1984) and also from virion DNA (Quinn and McGeoch, 1985). The problem of deletion was overcome by cloning the HSV DNA sequence into a yeast vector (Weller et al., 1985) and subsequent analysis of the cloned DNA identified a 144bp palindrome in which the central region of was highly homologous to the HSV-1 ori_S (Weller et al., 1985).

An HSV-2 ori_L was identified in an analogous position on the genome to that of the HSV-1 ori_L (Spaete and Frenkel, 1985) and cloned in an undeleted form into a bacterial vector by minimising the growth of recombination-deficient host bacteria (Lockshon and Galloway, 1986). This ori_L contains an AT-rich 136bp palindrome and has strong homology with the HSV-1 ori_L. The AT-rich palindrome is present in all the HSV origins of replication and is a feature shared with other origins of replication such as those of SV40, polyoma virus and VZV (Jay et al., 1976; Soeda et al., 1979; Davison and Scott, 1985).

A second feature common to the origins of replication of HSV-1 and HSV-2, is their location at the 5' ends of divergently transcribed genes. HSV-1 and HSV-2 ori_S is flanked on one side by the gene encoding Vmw175 and on the other side by the IE genes specifying Vmw12 or Vmw68 (Stow and Davison, 1986), whilst the HSV-1 and HSV-2 ori_L lies between the genes encoding the DNA polymerase and the major DNA binding protein (Quinn and McGeoch, 1985), which are essential for viral DNA replication. The significance of the positioning of ori_S and ori_L is not known, but it is interesting to note that VZV, which has sequences in the short repeat homologous to HSV-1 ori_S, does not have any sequences resembling HSV-1 ori_L between genes encoding the DNA polymerase and major DNA binding protein (Stow and Davison, 1986).

10. MATURATION OF VIRAL DNA

Late in infection, the replicated DNA forms a large pool of concatemers from which viral DNA molecules are randomly withdrawn for cleavage and packaging. Analyses of *ts* mutants of HSV and PRV have shown that cleavage of concatemeric viral DNA into monomeric units is closely linked to the encapsidation of full length viral genomes (Ladin *et al.*, 1980, 1982; C.Addison, 1986). In addition, sequence determination of the termini of virion and defective genomes has demonstrated that the mechanism of cleavage is site-specific (Mocarski and Roizman, 1982a; Vlazny *et al.*, 1982; Deiss and Frenkel, 1986).

There is little information on the proteins involved in DNA maturation and, to date, most work has concentrated on determining the *cis*-acting sequences which are important for cleavage and packaging. Analysis of the structure of defective genomes of HSV-1 suggested that these cleavage and packaging signals were located at the end of the S terminus since this sequence was common to all defective DNA molecules (Frenkel *et al.*, 1976; Kaerner *et al.*, 1979; Vlazny and Frenkel, 1981; Vlazny *et al.*, 1982). Subsequently, transfection studies, using plasmids containing the HSV-1 ori_S and the *a* sequence, confirmed that the cleavage-packaging signal mapped within the *a* sequence, since only replicated plasmids possessing this sequence were

packaged into capsids when transfected into cells together with helper virus DNA (Stow et al., 1983, 1986; Deiss and Frenkel, 1986).

10.1 The a Sequence

Sequence analyses of the joint and terminal restriction endonuclease fragments of various HSV-1 strains have shown that the a sequence is composed of unique (U) and directly repeated (DR) elements (Figure 9). A wide variation in the size of a sequences have been observed between different HSV strains and this is due to variation in the copy number of DR elements (Figure 9) (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Mocarski et al., 1985), whilst the HSV-2 a sequence appears to be a constant 251bp and no DR reiterations have been identified (Davison and Wilkie, 1981; Mocarski et al., 1985). In spite of this difference, the HSV-1 a sequence is recognised in trans by HSV-2 functions for cleavage, packaging and inversion (Mocarski and Roizman, 1982b; Spaete and Frenkel, 1985).

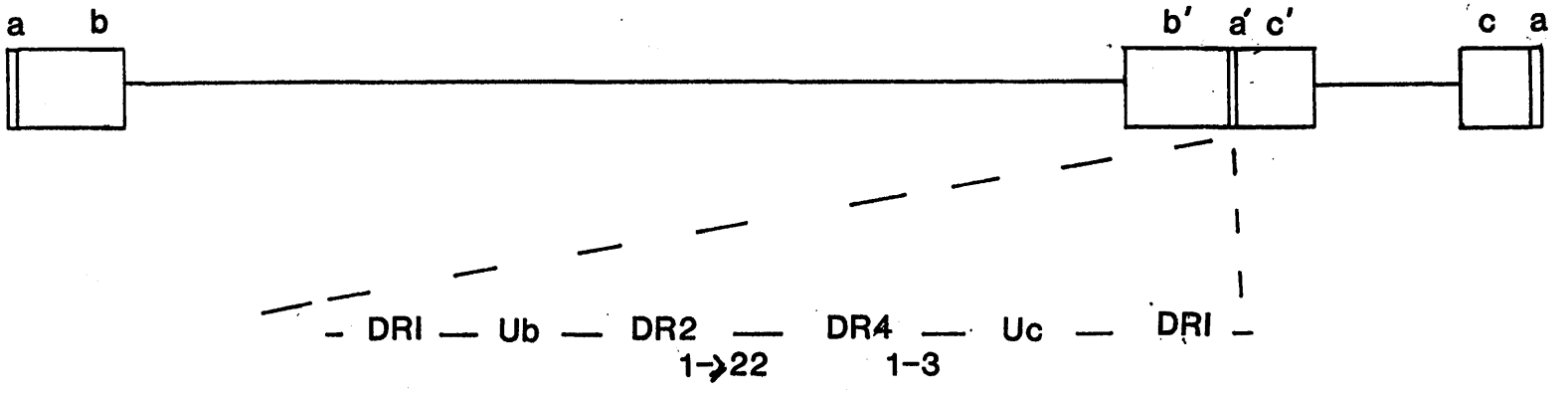
A single a sequence at the L-S junction has a direct repeat at each end known as DR1. Multiple a sequences at this junction, however, are only separated by a single DR element. TR_S and TR_L have a sequences ending in incomplete copies of DR1 such that direct ligation of HSV-1 termini reconstitutes a complete DR1 element between two a sequences, and this finding suggested

Figure 9

The a sequence of HSV-1

Unique (U_b, U_c) and directly repeated (DR1, DR2, DR4) regions are shown. The numbers of the repeats, DR2 and DR4 vary between different virus isolates.

References are given in the text.


a
b
b'
a'
c'
c
a
DRI
Ub
DR2
1→22
DR4
1-3
Uc
DRI

that the cleavage signal was located within DR1 (Mocarski and Roizman, 1982a). Subsequent work revealed that although cleavage occurred within DR1, signals specifying this process were located elsewhere in the a sequence. Furthermore, the lack of homology between the DR1 elements of different HSV-1 strains, and also between HSV-1 and HSV-2 supported the idea that the recognition signal mapped outside DR1, probably within conserved regions of the a sequence, perhaps Ub and/or Uc (Davison and Wilkie, 1981).

Experiments by Varmuza and Smiley (1985) extended the observations of Mocarski et al (1985). Various subfragments of the a sequence were inserted into the TK locus of HSV-1 and the resulting recombinant virus was tested for the ability to produce novel termini. The presence of such novel fragments was taken as evidence that the inserted a sequence could promote cleavage. A cloned fragment, SmaI f, which lacked DR1 and part of Ub at one end, and contained only a partial copy of DR1 at the other end was found to behave as a functional a sequence. This result provides strong evidence that the cis-acting cleavage-packaging signals map outside DR1 (Varmuza and Smiley, 1985). When the novel termini produced in the experiment were sequenced, the cleavage sites were discovered to lie in the HSV U_L sequences adjacent to the inserted SmaI f

sequence. Furthermore, the novel S and L termini were created by two distinct cleavage events within unrelated nucleotide sequences. Varmuza and Smiley (1985) therefore proposed that in a standard HSV genome two distinct cleavage events normally occur fortuitously within DR1, and suggested that the cleavage-packaging signals mapped within the conserved regions of the HSV-1 and HSV-2 a sequence outside DR1. This is supported by recent work in which plasmid vectors containing deleted a sequences were tested for efficient cleavage and packaging (Deiss et al., 1986). Deletions in the Ub or Uc regions abolished or severely reduced the ability of the plasmid to be propagated into defective virus stock. However, it was not possible from this data to determine whether the Ub and Uc signals act in concert as suggested by Varmuza and Smiley (1985) or separately (Deiss et al., 1986).

Whilst most viral genomes have a single a sequence at the termini and junctions, which are sufficient for cleavage and packaging to proceed (Deiss and Frenkel, 1986), tandem reiterations of the a sequence arise within the genome, but only at the L terminal and joint sequences (Wagner and Summers, 1978). Mocarski and Roizman (1981) proposed that amplification of the a sequence resulted from multiple rounds of unequal recombination through DR1. However, subsequent work by Varmuza and Smiley (1985) excluded this mechanism.

From an analysis of SmaI f reiterations present at novel termini generated by the insertion of the SmaI f (a subfragment of the a sequence) into the HSV-1 U_L sequences, Varmuza and Smiley (1985) concluded that in the majority of cases, amplification of the a sequence occurred through rounds of cleavage and religation of the termini, that is, the cleavage-packaging system was responsible for a sequence duplication.

10.2 Cleavage and Packaging of Viral DNA

A number of models, incorporating the known details of viral DNA maturation, have been proposed to explain the mechanism by which concatemeric viral DNA is processed to unit length genomes and packaged within the viral capsid.

In the "theft mechanism" (Varmuza and Smiley, 1985) (Figure 10) a packaging complex recognises cleavage signals located within the DR1 element in the a sequence of a standard HSV genome, and makes a ds cleavage at an L-S junction, producing, at least in some cases, ends with a protruding 3' nucleotide (Mocarski and Roizman, 1982a). This cleavage event creates two termini, one of which lacks an a sequence. The DNA carrying an a sequence is packaged into a preformed capsid until a second L-S junction in the same orientation (ie. at full genome's length) is encountered, and a second ds cleavage event then

Figure 10

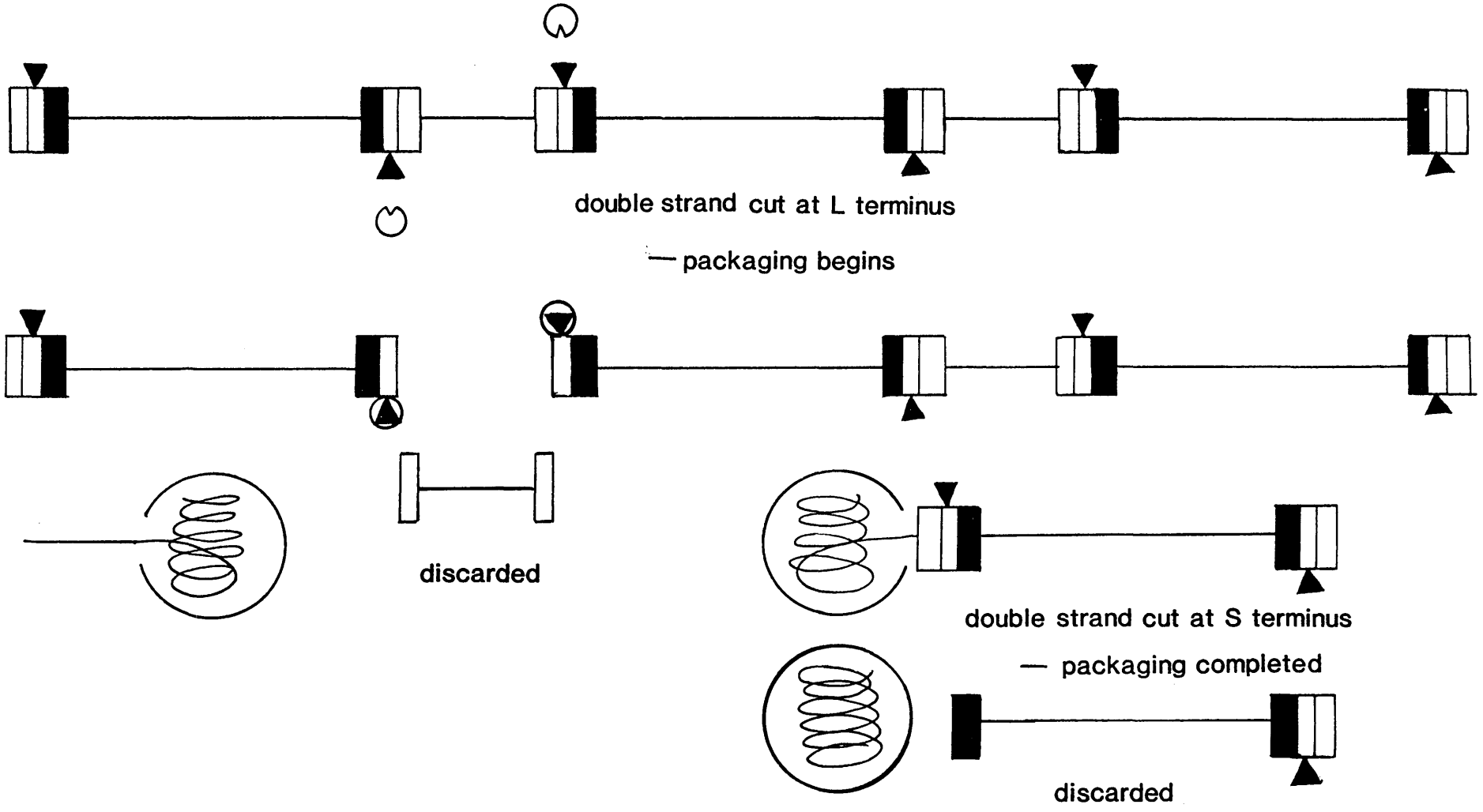
"Theft" mechanism

ds cleavage at one of two cleavage sites at an L-S junction containing a single a sequence produces L and S termini, one of which lacks an a sequence. DNA fragments lacking an a sequence are discarded and degraded. Termini containing an a sequence are packaged (in this example the L terminus) until the next L-S junction in the same orientation is reached and an S terminal cut is made.

Packaging signals - ▼ ▲

Cleavage-packaging complex - ⌒

from Varmuza and Smiley (1985).


double strand cut at L terminus
— packaging begins
discarded
double strand cut at S terminus
— packaging completed
discarded

creates 2 termini as before (Figure 10). Thus, the termini of the packaged molecules all contain an a sequence. This mechanism, however, does not explain the asymmetric distribution of a sequences within HSV. To account for this feature of the HSV genome, a directional packaging model was proposed (Deiss et al., 1986). In this "modified theft model" the packaging complex scans the viral concatemer and cleaves the DNA in the DR element, proximal to the first Uc sequence encountered, creating an L terminus with at least one a sequence (ba or ba_n) and an S terminus lacking an a sequence (ca_{n-1} or c). Packaging then proceeds in an L-to-S direction to a repeat junction where a second ds cleavage, proximal to the first Ub sequence of the junction, creates an S terminus with a single a sequence. In genomes where multiple a sequences are present, this process also creates an L terminus with at least one a sequence which may become packaged into a separate capsid. In this model, and the basic theft mechanism, only DNA which retains an a sequence at each end of the genome is packaged. The disadvantage of both models is that they predict the formation of DNA fragments which lack a sequences. Such termini, however, have not been identified in total nuclear DNA and it must be assumed that they are rapidly degraded.

As an alternative to the theft model, Varmuza and Smiley (1985) proposed the staggered nick repair model

in which L and S termini are produced by single stranded nicks rather than by a double-strand cleavage. At an L-S junction containing a single a sequence, a cleavage-packaging complex bound to Ub, and another interacting with Uc, make single strand nicks at the future location of S and L termini, respectively. The nicked DNA then undergoes strand repair synthesis across the staggered cleavages. This process generates 2 termini, each carrying an a sequence. These L and S termini can religate to form tandem a sequences, and such junctions, containing duplicate a sequences, can be cleaved by the coincident action of two single strand nicks from adjacent a sequences, producing a ds cleavage (Figure 11).

To take account of the findings of Mocarski and Roizman (1982a), it was proposed that the ends created by the break have a 3' nucleotide overhang.

In addition to the modified theft model, Deiss et al (1986) have described a scheme known as the double strand-break and gap repair model, which is based on the mechanism proposed by Szostak et al (1983) for gene conversion. This model incorporates directional scanning and packaging of the viral genome, plus amplification of the a sequence (Szostak et al., 1983; Deiss et al., 1986).

The packaging complex scans the genome and juxtaposes

Figure 11

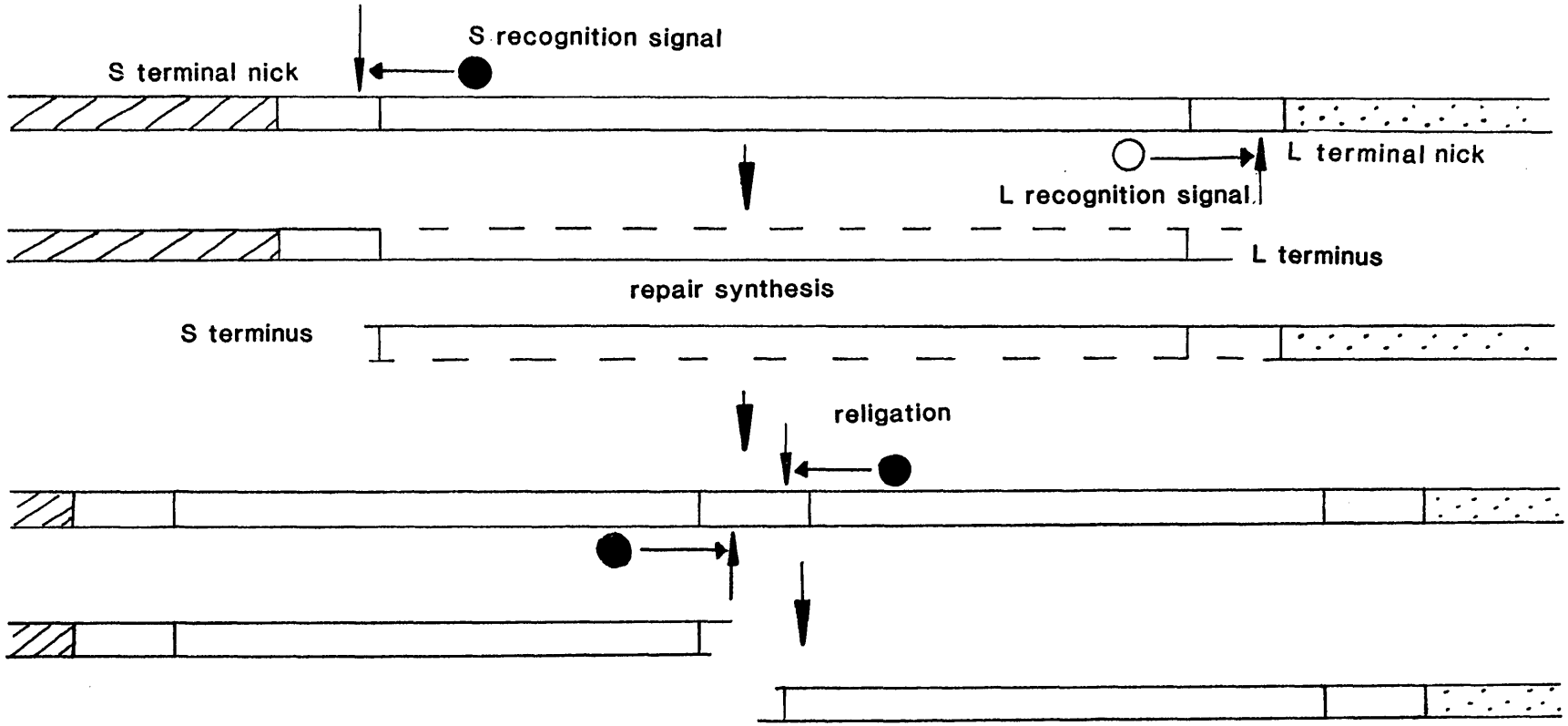
Staggered nick repair mechanism proposed by Varmuza and Smiley (1985)

L and S termini arise by

a. Staggered single strand nicks followed by repair synthesis.

b. an alternative pathway in which tandemly reiterated copies of the a sequence are processed by co-operation of L and S recognition complexes in adjacent a sequences, resulting in a single ds break.

L (O) and S (●) recognition complexes bound to signals in Ub and Uc respectively.


S recognition signal
S terminal nick
L terminal nick
L recognition signal
L terminus
repair synthesis
S terminus
religation

two directly repeated single a sequences at the L-S junctions. The a sequence is then amplified by a gene conversion mechanism, and termini are formed by a ds break within DR1. In this model, no termini devoid of a sequences are produced, and therefore all the ends can be packaged. The ds-break and gap repair model accounts for the structure of defective genomes generated from plasmids containing an a sequence with a deletion in Ub, and an origin of replication, cotransfected into cells with helper virus DNA. However, the simpler possibility that acquisition of a sequences by plasmid progeny occurred as a result of high frequency recombination between the helper viruses and plasmid DNA cannot be discounted.

11. GENE PRODUCTS INVOLVED IN VIRAL DNA SYNTHESIS

Although the available evidence indicates that the majority of polypeptides involved in viral DNA replication are virus-coded, work on ts BHK cell cycle mutants has suggested that cellular proteins may also be required (Yanagi et al., 1978). Little work, however, has beeen done on this aspect. Various approaches have been used to identify viral polypeptides involved in DNA metabolism, and these include:

1. the search for virus-infected cell enzyme activities that have different biochemical properties from the uninfected cell enzyme and

2. genetic analysis, in particular the isolation and characterisation of HSV ts mutants which fail to synthesise DNA at the NPT.

3. Recently, a plasmid replication assay has been used to identify seven genes required for viral DNA replication (Challberg, 1986).

Using this last technique, it was found that a combination of cloned HSV DNA fragments was able to supply all the functions required for amplification of a plasmid containing an HSV origin of replication, when transfected into cells. This assay implicated four U_L genes of unknown function, $U_L 5$, $U_L 8$, $U_L 9$ and $U_L 52$, encoding polypeptides with MWs of 99,000; 80,000; 94,000 and 115,000 respectively (Challberg and McGeoch, personal communication) (Figure 12). Since ts mutations which impair viral DNA synthesis at the NPT have been induced in each of these four genes (Matz et al., 1983; V.Preston, personal communication), they must be essential for viral DNA replication.

Viral polypeptides involved in DNA synthesis can be divided into two classes:

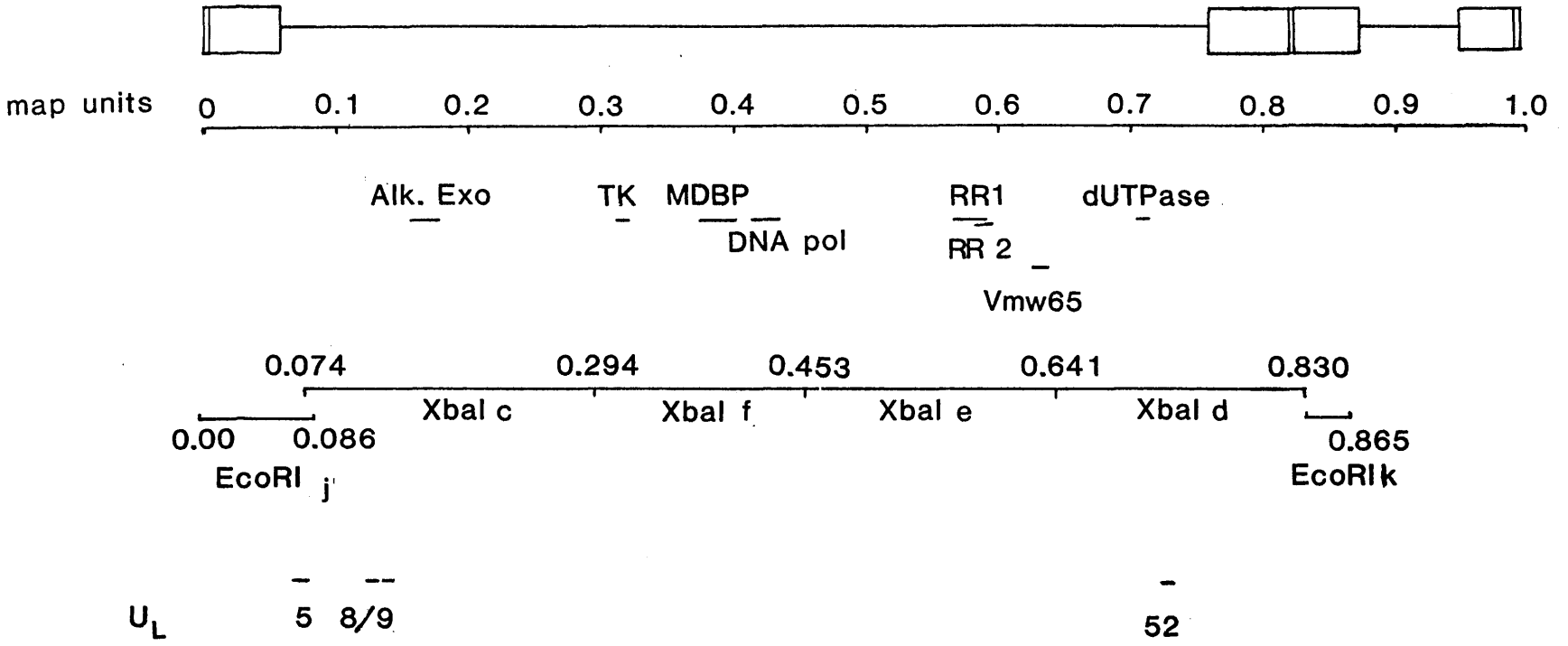
1. the gene products which are directly involved in DNA replication, for example, the major DNA binding protein (MDBP) and the DNA polymerase.

2. the enzymes which supply the precursors for viral DNA synthesis, for example, the ribonucleotide reductase and TK.

Figure 12

The map locations of genes for alkaline exonuclease (alk exo), TK, MDBP, DNA polymerase, ribonucleotide reductase large (RR1) and small (RR2) subunits, Vmw65 DNA binding protein and dUTPase, which are involved in HSV DNA metabolism.

Also shown are the map positions of U_L5, U_L8, U_L9 and U_L52, and the HSV-1 DNA fragments which can supply all replication functions in the plasmid amplification assay (Challberg, 1986).


map units
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Alk. Exo
TK
MDBP
DNA pol
RR1
RR 2
dUTPase
Vmw65
0.074
0.294
0.453
0.641
0.830
Xbal c
Xbal f
Xbal e
Xbal d
0.00
0.086
0.865
EcoRI j
EcoRI k
U_L
5
8/9
52

The map locations of genes involved in viral DNA metabolism, and the locations of the HSV-1 DNA fragments which supply all these functions (Challberg, 1986) are given in Figure 12.

11.1 Polypeptides Which are Directly Involved in DNA Synthesis

MDBP

The HSV-1 polypeptide is also referred to as ICP8 (Conley et al., 1981), and has a MW of 128,347 (Quinn and McGeoch, 1985). The HSV-2 counterpart is known as ICSP11, and has a similar MW to the HSV-1 MDBP (Powell and Purifoy, 1976).

This polypeptide binds more strongly to single-stranded DNA than to ds DNA (Bayliss et al., 1975; Powell and Purifoy, 1976), and is thought to play a major role in viral DNA synthesis, probably by maintaining the viral DNA in an extended configuration, thereby reducing hairpin-loop formation (Ruyechan, 1983), and increasing the processivity of the DNA polymerase. Evidence that the MDBP is involved in viral DNA replication was first obtained from the observation that in vitro, synthesis of viral DNA was inhibited by monospecific antiserum to the MDBP (Powell et al., 1981). The failure of ts mutants, with lesions in the MDBP gene, to synthesise viral DNA at the NPT (Conley et al., 1981; Littler et al., 1983), confirmed that this polypeptide was essential for viral DNA replication.

The presence of a ts lesion in the MDBP also caused the destabilisation of both the DNA polymerase and alkaline exonuclease at the NPT (Littler et al., 1983), suggesting that these three polypeptides are part of a replication complex.

DNA Polymerase

The viral DNA polymerase has a MW of 136,272 in HSV-1 strain 17 (Quinn and McGeoch, 1985). Its main function is to extend the replicating DNA chain in the 5'->3' direction, but it also has a 3'->5' exonuclease proofreading activity (Weissbach et al., 1973; Knopf, 1979). The viral DNA polymerase can be distinguished from the host enzyme on the basis of its requirement for high salt for maximal activity, its heat stability and its ability to use different DNA primers (Keir and Gold, 1963).

Evidence for the essential role of the DNA polymerase in viral DNA replication comes not only from the plasmid replication assay (Challberg, 1986), but also from the characterisation of HSV ts mutants which have lesions in this gene (Aron et al., 1975; Purifoy and Benyesh-Melnick, 1975; Hay and Subak-Sharpe, 1976; Purifoy and Powell, 1981), and also from the study of drugs which act on the viral DNA polymerase (Section 1.16.3).

Vmw65 DNA Binding Protein

A number of polypeptides of varying sizes copurify with the DNA polymerase. Using immunoaffinity chromatography, Vaughan et al (1985) demonstrated that a Vmw65 DNA binding protein was consistently among these. This polypeptide is distinct from the Vmw65 trans-inducing factor (Marsden et al., 1987), and may be an accessory protein required for efficient viral DNA synthesis (Knopf, 1979). The isolation of an HSV-1 ts mutant with a lesion in this gene, which fails to synthesise viral DNA at the NPT, strongly suggests that this gene is essential for viral DNA replication (V Preston, personal communication).

Alkaline Exonuclease

The virus exonuclease has an alkaline pH optimum (Keir and Gold, 1963; Keir, 1968) and is composed of a single polypeptide species, Vmw85, which has a MW of 67,503, as determined by sequence analysis (McGeoch et al., 1986). The purified enzyme also has an associated endonuclease activity (Hoffman and Cheng, 1978, 1979; Strobel Fidler and Francke, 1980).

The finding that the HSV-2 mutant, ts13, which has a lesion in the alkaline exonuclease gene, makes reduced amounts of viral DNA at the NPT (Moss, 1986), indicated that this gene may have an essential role in DNA replication. However, Challberg (1986) found that this gene was not required for plasmid replication in his

assay identifying genes required for viral DNA synthesis. This discrepancy has yet to be resolved.

11.2 Gene Products which Supply Precursors for DNA Synthesis

Ribonucleotide Reductase

The HSV-encoded ribonucleotide reductase catalyses the reduction of all four ribonucleotide diphosphates to the corresponding deoxyribonucleotides (Averett et al., 1983) and can be distinguished from the cellular enzyme activity by its insensitivity to inhibition by dTTP and dATP, and in its lack of requirement for ATP or magnesium salt for activity (Cohen, 1972; Ponce de Leon et al., 1977; Huszar and Bacchetti, 1981).

Both the HSV-1 and HSV-2 ribonucleotide reductase are composed of two non-identical subunits, the large one consisting of a polypeptide of apparent MW 136,000 in HSV-1 and 138,000 in HSV-2, and the small one consisting of a polypeptide with an apparent MW of 38,000 in HSV-1 and 36,000 in HSV-2 (Dutia, 1983; Huszar and Bacchetti, 1983; Reichard and Ehrenberg, 1983; Bacchetti et al., 1984; Preston et al., 1984).

Evidence that Vmw136 and 38 form a complex was obtained from studies on the HSV-1 mutant, ts1207 which has a lesion in Vmw136 (Preston et al., 1984), and induces a thermolabile ribonucleotide reductase activity (Dutia, 1983). Monoclonal antibodies specific for Vmw136 or Vmw38 bind to ribonucleotide reductase and precipitate

both polypeptides from wild type virus-infected cells (Bacchetti et al., 1984; Preston et al., 1984). However, these two polypeptides were not coprecipitated from extracts prepared from ts1207-infected cells grown at the NPT, either by a monoclonal antibody specific for Vmw136, or by an oligopeptide antiserum specific for Vmw38, even though both polypeptides were synthesised by ts1207 at this temperature.

This result indicates that the functional enzyme requires the association of Vmw38 with Vmw136 (Frame et al., 1985).

TK

The virus TK, or pyrimidine deoxyribonucleoside kinase, phosphorylates either of the pyrimidine deoxyribonucleosides, thymidine or deoxycytidine, but thymidine is preferentially converted to dTMP and dTTP (Kit and Dubbs, 1963; Dubbs and Kit, 1964; Klemperer et al., 1967; Cooper, 1983; Jamieson et al., 1974).

The enzyme has a MW of 40,900 in HSV-1 and 38,000 in HSV-2 (Wagner et al., 1981; Swain and Galloway, 1983) and, in comparison to the host enzyme, is relatively insensitive to thymidine triphosphate inhibition. It also has a low pH optimum and a low Km compared with the host TK, and is stable on heating at 40°C (Klemperer et al., 1967).

Virus-encoded dUTPase

The dUTPase catalyses the conversion of dUTP to dUMP and pyrophosphate. The cellular enzyme has two functions; it minimises the incorporation of dUTP into DNA by maintaining low levels of dUTP, and also supplies the cell with dUMP, the substrate for thymidylate synthetase (Ostrander and Cheng, 1980). The precise role of the virus specified dUTPase is uncertain. Since HSV does not encode a thymidylate synthetase gene and viruses deficient in dUTPase activity grow as well as WT viruses in tissue culture (Preston and Fisher, 1984) the main function of this enzyme is probably to maintain low levels of dUTP to minimise incorporation of uracil into DNA by DNA polymerase.

Two further enzymes that may be involved in virus DNA metabolism have been found in HSV-infected cells. These are:

1. a type I topoisomerase, which can relax supercoiled DNA by breaking and rejoining the backbone phosphodiester bonds of a single strand of the DNA (Muller et al., 1985). Although many DNA replication systems require a topoisomerase, it is not yet known whether HSV encodes a topoisomerase or if this enzyme is required for HSV DNA synthesis (Biswal et al., 1983; Leary and Francke, 1984).

2. a uracil DNA glycosylase. This enzyme removes uracil bases present in replicating DNA (Caradonna and Cheng, 1981), and is important in reducing the number of mutations arising from deamination of cytosine residues. To date, little information is known about this enzyme which is present in HSV-infected cells.

12. CAPSID ASSEMBLY

HSV proteins are synthesised in the cytoplasm and some proteins, including the nucleocapsid polypeptides, migrate to the nucleus where the capsids are assembled (Morgan et al., 1954; Olshevsky et al., 1967).

Recent evidence suggests that nucleocapsid assembly takes place at the nuclear matrix (Bibor-Hardy et al., 1982a, 1982b, 1985; Ben-Ze'ev, et al., 1983). This structural component of the cell is thought to represent the skeletal framework of the nucleus and is defined as the insoluble material remaining after purified nuclei have been sequentially treated with nonionic detergent, low magnesium salt buffer, high salt buffer and finally digested with nucleases (Berezney and Coffey, 1974). Examination of the matrix under the electron microscope has shown that it consists of a residual peripheral lamina with nuclear pore complexes, nucleoli, and an internal fibrogranular network (Berezney and Coffey, 1974, 1977). It has been proposed that in uninfected cells the matrix is the active site for DNA replication and RNA processing,

since newly synthesised DNA and unprocessed RNA are associated with this structure (Pardoll et al., 1980; Van Eekelen and Van Venrooji, 1982). In HSV-1 infected cells, a subset of both structural and non-structural viral proteins are present in matrix preparations and many capsids, most of which are empty, are attached to the fibrogranular network (Bibor-Hardy et al., 1982a, 1982b, 1985). Similar results have been obtained with HSV-2 infected cells (Tsutsui et al., 1983). These findings, together with the observation that herpesvirus DNA co-purifies with the nuclear matrix (Ben-Porat et al., 1984, F.J.Rixon, personal communication), led Bibor-Hardy et al. (1985) to propose that the matrix is the site of both viral DNA synthesis and nucleocapsid assembly, and that these two processes may be linked. However, there remains the possibility that viral DNA and capsids become trapped within the matrix during the extraction procedure, and this has not yet been disproved.

The analysis of DNA positive, ts mutants of PRV which failed to produce capsids at the NPT (Ladin et al., 1980, 1982) suggested that the accumulation of nucleocapsid proteins within the nucleus is linked to capsid assembly. In mutant virus-infected cells grown at the NPT, the major capsid proteins were found in the cytoplasmic fraction instead of the nuclear fraction. To explain this finding, Ladin et al. (1982) put

forward the proposal that the formation of capsids promotes the transport of structural proteins into the cell nucleus by creating a "sink", whereby the retention of capsid polypeptides within the nucleus favours the continued movement of structural polypeptides from cytoplasm to nucleus.

12.1 Viral DNA Encapsidation

There are two general methods for the packaging of virus DNA:

1. The DNA is inserted into a pre-formed capsid.
2. The capsid is assembled around the virus DNA.

The weight of evidence suggests that the capsid is assembled first, and that the viral DNA is inserted into it. A model for this mechanism was first proposed

* are rearranged to form a large cylindrical structure onto which the DNA is spooled. As the DNA is wound around the cylinder to form a densely staining toroid structure, the cylinder undergoes condensation (Perdue et al., 1976). This scheme is in agreement with the process described for HSV encapsidation by Furlong et al. (1972).

The model described by Perdue and co-workers is supported by work on DNA positive mutants of PRV and

HSV which are ts for the ability to package virus DNA (Ladin et al., 1980; Preston et al., 1983). At the NPT these mutants generally produce large numbers of partially-cored capsids which resemble the I capsid forms of EHV-1 as described by Perdue et al. (1975). When mutant virus-infected cells were transferred from the NPT to the PT in the presence of cycloheximide, an increase in the numbers of full capsids in the nucleus and enveloped virus in the cytoplasm, together with a concomitant decrease in the partially-cored (I) capsids was observed (Ladin et al., 1980; Preston et al., 1983), suggesting that like the 'I' capsids of EHV-1, the partially-cored capsids are precursors to full capsids. Analyses of the different forms of purified capsids isolated from PRV mutant virus-infected cells in "pulse-chase" experiments, confirmed that partially cored capsids were intermediates of full capsids (Ladin et al., 1980).

Pignatti and Cassai (1980) proposed an alternative theory for encapsidation, in which newly synthesised viral DNA, derived from a replication complex, is condensed and packaged into a nucleoprotein complex (NPC). They suggested that VP5, 12, 15.2, 19 and 24 were required for initiation of encapsidation since these proteins were present in the NPC, which appeared in EM analysis to be present at one end of the genome. The addition of structural proteins to the NPC would lead to the formation of nucleocapsids. However, this

theory is not generally accepted because the experimental evidence favours the previous model in which the DNA enters a preformed capsid. Furthermore, Pignatti and Cassai (1980) have not excluded the possibility that the NPCs represent disrupted nucleocapsids.

Viral DNA is cleaved as it is packaged; this relationship between capsid assembly and maturation of virus DNA has been demonstrated both by the characterisation of ts mutants and by the study of defective viral genomes. DNA-positive ts mutants of PRV and HSV which fail to package DNA at the NPT do not cleave concatemeric virus DNA into unit length molecules (Ladin et al., 1980; Addison, 1986). On shift-down of mutant virus-infected cells from the NPT to the PT in the presence of cycloheximide, the DNA is cleaved and packaged, and full capsids are produced (Ladin et al., 1980; Preston et al., 1983; Addison, 1986). Deiss and Frenkel (1986) studied the cleavage and packaging of defective genomes of HSV-1 in the presence in helper virus. Their results show that the majority of DNA termini in the nuclear fraction of infected cells are present in encapsidated viral DNA, again linking cleavage with packaging of viral DNA.

12.2 Gene Products Involved in Viral DNA Encapsidation

Several polypeptides have been implicated in the

encapsidation of viral DNA. The best studied of these is the gene, U_L26 encoding ICP35 (MW 40,000). This is present in multiple forms in the infected cell, the lower MW species of which have been termed VP22a (Gibson and Roizman, 1972), p40 (Heilman et al., 1981) and NC-3 (Cohen et al., 1980). The analogous polypeptides in PRV and CMV are referred to as the 35,000 MW structural protein (Ladin et al., 1982) and the 36,000 MW "assembly protein" (Irmiere and Gibson, 1985) respectively. Early work by Gibson and Roizman (1972) suggested that VP22a was involved in DNA encapsidation because this polypeptide was found in full but not empty capsids purified from infected cell nuclei. Subsequent work by Braun et al. (1984b) localised ICP35 to the surface of full capsids. Further evidence that ICP35 was required for DNA packaging was obtained from the characterisation of the HSV-1 mutant, ts1201. This virus, which has a lesion in the ICP35 gene, fails both to process ICP35 to its lower MW forms (Preston et al., 1983) and to package viral DNA at the NPT.

Interestingly, the empty capsids produced by ts1201 at the NPT have a larger internal core structure than those of packaging deficient ts mutants with lesions in other genes, for example ts1203 (Addison, 1986).

More recently, the distribution of ICP35 within virus-

infected cells has been determined by immuno-electron microscopy. In contrast to earlier results, F.J.Rixon and V.Preston (personal communication) found that the polypeptide was localised on the surface of empty but not full capsids from WT- and ts1201-infected cells at the NPT. This result suggests that ICP35 is associated with empty capsids, and that following packaging this association is weakened, resulting in loss of the polypeptide from full nucleocapsids. This finding is in contradiction with the results obtained by Gibson and Roizman (1972) which suggested that ICP35 is present in full capsids. A possible explanation for these conflicting data is that the method of capsid purification used by Gibson and Roizman (1972) may have resulted in the isolation of a capsid population consisting of a mixture of dense-cored, DNA-containing capsids and partially cored capsids which lacked DNA. VP19c (ICP32, U_L 41) has a MW of 50,000, and is an internal component of both full and empty capsids (Gibson and Roizman, 1972; Braun et al., 1984a). Since this polypeptide binds to DNA, it has been speculated that it has a role in DNA encapsidation or in the anchorage of newly packaged DNA within the capsid (Braun et al., 1984a). To date, however, there is no direct information about the function of this structural polypeptide.

Results from DNA binding experiments with HSV-infected

cell extracts suggested that the U_L11 gene products, Vmw21 and 22 bound specifically to the 'a' sequence (Dalziel and Marsden, 1984; McGeoch et al., 1985). This data, together with earlier findings that a similar sized polypeptide was attached to the genomic termini of HSV-1 (Wu et al., 1979; Hyman, 1980), indicated that Vmw21 and 22 might be involved in the packaging of viral DNA (Dalziel and Marsden, 1984). However, recent work has shown that the U_L11 gene products are not essential for virus growth in tissue culture (Umene, 1986; Brown and Harland, 1987; Longnecker and Roizman, 1986) and that they are localised within the nucleolus (Maclean et al., 1987). These results raise doubts as to whether U_L11 gene products are involved in nucleocapsid assembly.

13. HSV ENVELOPMENT AND EXIT FROM THE CELL

HSV nucleocapsids generally become enveloped by "budding" through the inner nuclear membrane of the cell (Darlington and Moss, 1968; Nii et al., 1968; Roizman and Furlong, 1974) at positions where the membrane is enriched for viral glycoproteins and lacks the normal complement of cellular proteins (Asher et al., 1969; Spear et al., 1970, Spear and Roizman, 1972). Envelopment is thought to be a rapid process since "budding" viruses are rarely seen in thin sections of virus-infected cells under the electron microscope, however enveloped particles are frequently observed between the nuclear membranes.

Although herpesvirus envelopment by "budding" has also been detected at cytoplasmic vacuoles (Nii, 1971; Seigneurin et al., 1977) and the Golgi complex membranes (Smith and de Harven, 1973), these are not considered to be the normal sites of envelopment. In addition, the occasional presence of enveloped capsids within the nuclei of cells infected with WT HSV-2 and some ts mutants of HSV-1 has prompted the suggestion that de novo synthesis of an HSV envelope can occur (Atkinson et al., 1978; Dargan and Subak-Sharpe, 1983). This idea, however, is not widely accepted.

From the analysis of proteins present in capsids and de-enveloped virions, Gibson and Roizman (1972) proposed that ICP35 plays a role in HSV envelopment. In subsequent work on CMV, Gibson (1981) observed that tegument proteins surrounding the capsid of this virus had exposed hydrophobic groups, and suggested that these structural proteins promoted nucleocapsid envelopment by interacting with hydrophobic elements, possibly viral glycoproteins, located on the cellular nuclear lamella. To date, no evidence has been obtained directly implicating either HSV tegument polypeptides or glycoproteins in this process. Nevertheless, such an interaction is an attractive explanation for the process of HSV envelopment. Following envelopment, the virion is transported to the cell membrane where it is released into the

extracellular space. Alternatively, virus is spread from cell to cell by fusion of adjacent cellular membranes.

There are two major theories for the transport and exit of HSV from the cell, of which the favoured mechanism is a modified version of the "reverse phagocytosis" hypothesis (Morgan et al., 1959). In this model envelopment occurs at the inner nuclear membrane and the virus particles are moved by transport vesicles from the rough endoplasmic reticulum to the Golgi complex, and from there to the cell surface (Johnson and Spear, 1982). This theory is supported by work with monensin, an ionophore which catalyses the exchange of sodium and potassium ions across biological membranes (Pressman, 1976), disrupting the ion gradients between membranes in the Golgi complex and blocking the secretion of soluble proteins (Tartakoff and Vassali, 1978; Uchida et al., 1979). The addition of monensin to virus-infected cells inhibits the transport of membrane proteins between the Golgi complex and the plasma membrane (Johnson and Schlesinger, 1980) and also prevents the transport of enveloped virions to the cell surface. As a consequence, infectious virions accumulate within large cytoplasmic vacuoles in the cell (Johnson and Spear, 1982), strongly suggesting that movement of virus to the cell surface occurs via the Golgi apparatus.

An alternative mechanism has been proposed by Schwartz and Roizman (1969) on the basis of electron- and light-microscope studies. They suggested that in virus-infected cells, a network of highly branched tubules is formed, connecting the perinuclear region with the extracellular space. These tubules are thought to facilitate the exit of enveloped virus particles from the cell.

Three virus glycoproteins, gD, gH (Gompels and Minson, 1986) and gE (Tognon et al., 1981) have been implicated in the process of virus egress; many antibodies to gD and gH are strongly neutralising in the absence of complement (Para et al., 1985; Buckmaster et al., 1984; Gompels and Minson, 1986), and also inhibit the fusion of cells infected by syncytial virus strains (Gompels and Minson, 1986). In addition, a monoclonal antibody to gH has been produced which inhibits plaque formation when added to the overlay of virus-infected cells (Buckmaster et al., 1984). Evidence that gH has a role in virus exit is supported by work on an HSV-1 mutant tsQ26, which has a ts lesion in gH (Weller et al., 1983). This mutant grows normally at the NPT in terms of the yield of infectious virus from cells, but fails to form plaques, that is, virus exit from the infected cell does not occur.

14. HSV ENCODED POLYPEPTIDES

14.1 Number and Genomic Location of HSV-induced Polypeptides

The HSV-1 genome encodes 71 unique genes (D.McGeoch

personal communication). At least 50 HSV-1 and HSV-2 induced polypeptides have been detected by 1-dimensional SDS-polyacrylamide gel electrophoresis (Honess and Roizman, 1973; Powell and Courtney, 1975). More recently, knowledge of the HSV-1 DNA sequence (McGeoch et al., 1985, 1986) has enabled gene products to be identified by means of antiserum raised against an oligopeptide specific for a small portion of the amino acid sequence of a gene (Palfreyman et al., 1983; Frame et al., 1986a, 1986b; Marsden et al., 1987).

14.2 Regulation of Synthesis of HSV-induced Polypeptides

Although the synthesis of HSV polypeptides is largely regulated at the level of mRNA transcription (Section 1.7), there have been claims that control also occurs at the level of translation. Work by Inglis and Newton (1981) indicated that HSV-1 mRNAs are no more efficiently translated than cellular transcripts in a cell free translation system, suggesting that any virally-induced inhibition of cellular polypeptide synthesis must take place at the post-transcriptional level. Also, work by Johnson and Spear (1984) showed that gD transcription increased until late times, even when little of the gene product was translated, suggesting that control was at the translational rather than transcriptional stage. However, this work conflicts with results obtained by Johnson et al., (1986) who found that whilst synthesis of gD increased steadily until late times in infection, as detected by immunoprecipitation of the polypeptide, transcription

of gD decreased after 5h pi.

15. POST-TRANSLATIONAL MODIFICATIONS OF VIRAL POLYPEPTIDES

A variety of post-translational modifications of HSV polypeptides occur, the most common of which are phosphorylation, cleavage, glycosylation and sulphation.

15.1 Phosphorylation

16 HSV-1 and 18 HSV-2 phosphorylated polypeptides including all of the IE polypeptides with the exception of Vmw12 (Hay and Hay, 1980) have been detected in virus-infected cells (Pereira et al., 1977; Marsden et al., 1978). Wilcox et al. (1980) demonstrated that the phosphate groups cycled on and off some HSV induced proteins, for example Vmw175, and that the DNA binding properties of certain proteins was altered after they were phosphorylated. These workers also concluded that more than one pathway existed for protein phosphorylation since Vmw175 was phosphorylated during pulse labeling, whilst Vmw63 and 68 were phosphorylated during the chase period (Wilcox et al., 1980).
In addition, a large number of structural polypeptides are phosphorylated, for example the tegument protein Vmw65 (H. Moss, personal communication).

15.2 Proteolytic Cleavage

An examination of gD in virus-infected cells showed that 25 amino acids were absent from the amino terminus of the polypeptide in comparison with the predicted

sequence (Eisenberg et al., 1984). This amino acid sequence is highly hydrophobic and probably represents the signal peptide located at the amino-terminus of many transmembrane glycoproteins (Kreil, 1981; Eisenberg et al., 1984) which is required for transfer of glycoprotein through the lipid membrane, and is cleaved during maturation. Similarly, HSV-2 gG was shown to be processed from a 120,000 MW precursor to a 108,000 MW polypeptide (Balachandran and Hutt-Fletcher, 1985). In addition, it is speculated that the non-glycosylated polypeptide, ICP35, is cleaved (Braun et al., 1983, 1984b), although no definite evidence for this has yet been obtained.

At late times in infection a number of polypeptides including Vmw175 and the large subunit of the ribonucleotide reductase (Vmw136) undergo cleavage (McDonald, 1980). This process did not occur when virus-infected cells were treated with the protease inhibitor tosylphenylchloromethyl ketone (TPCK), suggesting that protein degradation was due to specific proteolytic cleavage. The significance of this remains unknown.

15.3 Glycosylation

The map location of the HSV-1 and HSV-2 genes encoding glycosylated polypeptides, and the function of the gene products is given in Section 1.4.3.

Glycosylation of viral polypeptides is rapid, and can be detected 12-15min after the production of the precursor protein (Spear and Roizman, 1970). All HSV glycoproteins examined so far contain N-linked oligosaccharides, that is, linkages between N-acetyl glucosamine and asparagine residues (Eisenberg et al., 1979; Pizer et al., 1980). O-linked oligosaccharides between N-acetylglucosamine and serine or threonine have also been found in some HSV glycoproteins, for example, gB, gC, gD (Olofsson et al., 1981, 1983; Wenske and Courtney, 1981; Johnson and Spear, 1983).

15.4 Sulphation

Inorganic sulphate is added to all the major glycoproteins of HSV-1 and HSV-2, and it is thought that in the majority of cases the inorganic sulphate is attached to the N-linked oligosaccharides (Hope et al., 1982; Hope and Marsden, 1983).

In addition to these modifications poly ADP-ribosylation of Vmw175 (Preston and Notarianni, 1983) and fatty acid acylation of gE (Johnson and Spear, 1983) have also been reported.

16. GENETICS OF HSV

16.1 Mutations in Essential Genes

16.1.1 Ts Mutants

Most of the conditional lethal mutants which have been isolated from HSV are ts, and these have been extremely

useful in identifying the function of genes essential for virus growth in tissue culture. Since very few of the mutants characterised are from HSV-2, this section will concentrate largely on the analysis of HSV-1 mutants.

Ts mutants with lesions in essential genes grow normally at low temperatures (31-34 ^{o}) but not at higher temperatures (usually within the range of 38-39.5 ^{o}). Although these lesions can arise spontaneously, most ts mutations have been induced by treatment of virus-infected cells with 5'-bromo-2'-deoxyuridine (BUdR), by mutagenesis of virus with UV light, or by treatment of whole virus DNA or viral DNA fragments with nitrous acid, UV light or hydroxylamine (Timbury, 1971; Brown et al., 1973; Schaffer et al., 1973; Esparza et al., 1974; Manservigi, 1974; Chu et al., 1979; Sandri-Goldin et al., 1981). Complementation analyses of HSV-1 ts-mutants have identified at least 35 different cistrons (Schaffer et al., 1978; V. Preston, personal communication) suggesting that at least half the genes encoded by HSV are essential for virus growth in tissue culture.

In HSV-2, the study of ts mutants has been limited and only 20 different complementation groups have been identified (Timbury et al., 1976; Schaffer et al., 1978).

16.1.2 Host Range Mutants

An alternative approach to the one used above for selecting mutations in essential genes has been to make host range mutants. This has been achieved by transforming tissue culture cells with cloned restriction endonuclease fragments from WT virus. Mutant viruses containing an inactive essential gene will only grow on cell lines expressing the WT gene. Host range mutants with defects in Vmw175 (Davidson and Stow, 1985; DeLuca et al., 1985), gB (Cai et al., 1987) and the MDBP (Minson et al., 1978; Oberg and Schaffer, 1987) have been isolated and characterised.

16.2 Mutations in Nonessential Genes

Nonessential genes have been inactivated by insertions into the DNA; for example, the dUTPase gene (Preston and Fisher, 1984), or deletion of sequences, for example Vmw110 (Stow and Stow, 1986).

A novel method to produce specific deletions in a virus genome and thus identify nonessential genes has been described by Post and Roizman (1981). This method is composed of two steps: The selectable TK gene marker is inserted into the target DNA sequence, and this fragment is cotransfected into cells with TK^- viral DNA. Recombinant virus, where the TK is inserted into the target region, can be identified as TK^+ progeny. Cotransfection of this TK^+ virus DNA with a plasmid

containing a deletion in the target sequence will result in TK$^-$ virus progeny which carry the deletion. The isolation of such TK$^-$ virus indicates that the target gene is nonessential in tissue culture (Hubenthal-Voss and Roizman, 1985; Longnecker and Roizman, 1986). Specific nonessential genes have also been identified by a selection process, for example gC$^-$ (Holland et al., 1983), where gC$^-$ mutants were isolated from a mutagenised virus stock by means of resistance to anti-gC antibodies, and TK (Kit and Dubbs, 1963; Dubbs and Kit, 1964) where treatment of virus-infected cells with BUdR enabled the isolation of TK$^-$/BUdR resistant viruses.

From studies on dispensable genes it has become apparent that many genes are not required for growth in tissue culture. Evidence is emerging from studies of mutants with deletions in U_S that all of these genes, with the possible exception of gD, are nonessential for virus growth in vitro (Longnecker and Roizman, 1986, 1987; Mavromara-Nazos et al., 1986a; Umene, 1986; Brown and Harland, 1987; Weber et al., 1987).

16.3 Drug Resistance Mutants

The virus TK will phosphorylate a variety of nucleoside analogues, converting them from inactive to active agents which normally interfere with viral DNA replication by becoming incorporated into viral DNA and causing chain termination. Viruses exposed to these

drugs acquire resistance by mutation in the TK or virus polymerase genes. Many drug resistant mutants have an inactive TK gene. These TK^- viruses obtain dTMP by means of the cellular de novo pathway, and are, therefore, able to grow in tissue culture (Elion et al., 1977; Field et al., 1980). TK^+, acycloguanosine resistant mutants, which encode an altered viral DNA polymerase that fails to recognise the phosphorylated nucleoside analogue, have also been detected (Crumpacker et al., 1980; Darby et al., 1981). Drug resistant mutants have been very useful in determining the possible effects of antiviral drugs used in vivo. Interestingly, as is the case in vitro, most acycloguanosine drug resistant mutants isolated in vivo have mutations in the TK gene and not the DNA polymerase.

16.4 Syncytial (syn) Mutants

At least four loci which can give rise to syncytial plaque morphology have been identified in HSV-1 (Figure 3). Viruses with a syncytial phenotype are known as syn mutants. Little and Schaffer (1981) mapped the plaque morphology defect (syn 4) in the HSV-1 KOS syn mutant, 804, to 0.040-0.064 mu on the genome, and noted a reduction in the size of the gB dimer in mutant virus-infected cells. It was not determined, however, whether the syn mutation was responsible for this alteration of gB. Later work on the HSV-1 mutant,

tsB5, identified a syn locus (syn 3) at the carboxyl terminus of gB, separate from the gB ts mutation in this mutant (DeLuca et al., 1982). A syn locus (syn 5) was also detected to the right of the TK gene (Sanders et al., 1982). Ruyechan et al. (1979) claimed that two further loci were present at 0.70-0.83 mu (syn 1, syn 2). However, further analysis of this region identified only a single syn gene (syn 1) which lies within 0.735-0.740 mu. A mutation affecting virus plaque size (Bond and Person, 1984; Pogue-Geile et al., 1984) was also identified in this region, and this lesion maps within a gene predicted to encode a trans-membrane protein (Debroy et al., 1985).

In addition to these loci, gC can also affect plaque morphology. There appears to be an as yet undefined relationship between gC and the formation of syncytia, since many syn mutants are gC^-, but not all gC^- mutants have the syn plaque morphology.

16.5 Genetic Interactions of HSV

Recombination in HSV was first reported by Wildy (1955) who used lesion morphology in chick-chorioallantoic membranes and virulence in mice as markers of recombination. The ability of HSV to recombine was confirmed by Subak-Sharpe (1969) who demonstrated the presence of ts^+ recombinants amongst the progeny virus from cells mixedly infected with two different ts

mutants. Subsequent analyses of recombination frequencies from crosses with ts mutants led to the construction of linear linkage maps (Brown et al., 1973; Schaffer et al., 1974; Timbury and Calder, 1976). In a later study, the segregation of a variety of unselected markers amongst selected recombinant progeny was measured to determine the degree of linkage (Honess et al., 1980). In contrast to earlier findings, the results obtained were consistent with a circular genetic map, and it was proposed that recombination normally occurred between circular or concatemeric DNA molecules. This model was supported by a study on PRV recombination in which the appearance of both recombinant genomes and virus was monitored (Ben Porat et al., 1982). Evidence from this work suggested that recombination occurred mainly between parental genomes rather than progeny genomes and that it took place prior to DNA replication. These findings contrast with earlier data of Ritchie et al. (1977), who found that the frequency of recombination increased with time, indicating that both parental and progeny genomes participate in the process of recombination. The discrepancy between the two sets of data has yet to be resolved.

Recombination also occurs between HSV-1 and HSV-2 (Timbury and Subak-Sharpe, 1973; Esparza et al., 1976), and crossover sites have been detected in all regions

of the genome (Preston et al., 1978; Ruyechan et al., 1979). Intertypic recombinants and ts mutations have been exploited to map viral polypeptides on the virus genome (Marsden et al., 1978; Morse et al., 1978; Preston et al., 1978; Ruyechan et al., 1979) and this approach, together with the technique of marker rescue (Stow et al., 1978; Stow and Wilkie, 1978; Chartrand et al., 1979, 1981), has greatly facilitated the location of genes on the virus genome, and proved to be much more reliable than mapping by recombination analysis. As well as participating in generalised recombination between genomes, HSV also undergoes intramolecular recombination between IR_S and TR_S or IR_L and TR_L which results in the inversion of U_S relative to U_L. It is thought that inversion occurs by site-specific recombination between inverted copies of the a sequence (Mocarski et al., 1980; Mocarski and Roizman, 1981; Chou and Roizman, 1985), and that this process is mediated by as yet unidentified, trans-acting, viral gene products (Mocarski and Roizman, 1982b). Although the a sequence is particularly active in the inversion of U_S and U_L, recent work (Longnecker and Roizman, 1986) has shown that a second, less active site of inversion is present within the b sequence of the L repeat.

CHAPTER 2

MATERIALS

Viruses

Seed stocks of wild type HSV-1 strain 17 syn$^+$ (Brown *et al.*, 1973) and wild type HSV-2 strain HG52 (Timbury, 1971) were provided by Mrs M Murphy. The HG52 mutant, *ts*2203, was isolated by Dr V G Preston and contained one of the *ts* lesions present in the HG52 multiple mutant *ts*13. A cloned BglII *i* fragment from *ts*13 was recombined into wild type HG52 by marker rescue and the mutant *ts*2203 was isolated from this transfection experiment. Five independently isolated revertants of *ts*2203, which were *ts*$^+$ for growth at the NPT, were supplied by Dr V G Preston. These viruses were *ts*2203 rev-1, rev-2, rev-3, rev-4 and rev-5.

Wild type HSV-2 strain HVD25766 was kindly provided by Dr A C Minson, Cambridge. The *ts*13 mutation in BglII *i* fragment was transferred to this strain by marker rescue and the resulting *ts* virus isolate was referred to as *ts*2204. A *ts*$^+$ virus, *ts* 2204 (MR-1) was obtained by recombining cloned *ts*$^+$ HG52 BglII *i* into *ts*2204. Since *ts*2204 was subsequently shown to have a secondary *ts* mutation, a second *ts* mutant, *ts*2205, and *ts*$^+$ virus *ts* 2205(MR-2), were constructed in the same way as *ts*2204 and *ts* 2204(MR-1).

The HSV-1 strain 17 mutant *ts*1201 (Preston *et al.*, 1983) was supplied by Dr V G Preston.

TISSUE CULTURE CELLS AND MEDIUM

BHK C13 cells (Macpherson and Stoker, 1962) were used throughout this study.

These cells were grown in 1x Glasgow modified Eagle's medium (Busby et al., 1964), supplied as a 10x concentrate by Gibco Ltd. This medium was supplemented with 100 units/ml penicillin, 100ug/ml streptomycin and 0.2ug/ml amphotericin.

The following modified tissue culture media were also used:

ECn	Eagle's medium containing n% calf serum
EHUn	Eagle's medium containing n% human serum
Agar overlay	Eagle's medium containing 5% calf serum and 0.6% Noble 's agar

PLASMIDS

The plasmids pGZ73 and pGZ74 were constructed by Dr M Dalrymple and obtained from Dr V G Preston.

The construct containing HSV-2 HVD25766 BamHI a was kindly supplied by Dr A C Minson, Cambridge. pGZ1 containing HSV-2 HG52 BamHI g was obtained from Dr V G Preston.

The remaining plasmids, pTKN2 (Cordingley et al., 1983), pGX158, pMC1 and pMC4 (Campbell et al., 1984) were all obtained from Dr C M Preston.

Information about the composition of the above plasmids is given in Table 2 and a detailed description of the construction of these recombinant clones is given in the Results, Sections 3 and 4.

Table 2

Host bacteria and composition of recombinant plasmids used in this study

References:

1. Vieira and Messing (1982).

2. Hanahan (1983).

3. Bolivar *et al* (1977).

4. Brenner (1979).

5. Twigg and Sherratt (1980).

	Insert	Vector	Host bacteria
pGZ73	HSV-2 HG52 Bgl II i	pUC9[1]	E.coli k12 DH1
pGZ74	HSV-2 ts13 Bgl II i	pUC9	E.coli k12 DH1
	HSV-2 HVD 25766 BamHI a	pBR322[3]	E.coli k12 MRC1[4]
pGZ1	HSV-2 HG52 BamHI g	pBR322	E.coli K12 MRC1
pTKN2	HSV IE gene 3 promoter linked to HSV-1 TK coding sequences	pAT153[5]	E.coli K12 DH1
pGX158	HSV-1 BamHI f	pAT153	E.coli K12 DH1
pMC1	A 2.7 Kbp subclone of HSV-1 BamHI f	pAT153	E.coli k12 DH1
pMC4	HSV-1 BamHI f flanking one PvuII site	pAT153	E.coli k12 DH1

RADIOCHEMICALS

Radiolabelled compounds were obtained from Amersham International plc.

CHEMICALS

Analytical grade reagents were used wherever possible. Chemicals were obtained from the following suppliers: BDH Chemicals, Poole, England; BioRad Laboratories, California, USA; Koch-Light Laboratories, Suffolk, England; Pharmacia Fine Chemicals, Uppsala, Sweden; Sigma (London) Ltd.

Reagents for electron microscopy were obtained from Agar Aids, Stanstead, Essex, and Taab Laboratories, Emmer Green, Reading.

ENZYMES

Restriction endonucleases were obtained from Bethesda Research Laboratories (UK) Ltd, Cambridge, England.

OLIGONUCLEOTIDES

Phosphorylated XbaI oligonucleotide linkers were obtained from Pharmacia Fine Chemicals.

IMMUNOLOGICAL REAGENTS

Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG and rabbit anti-mouse IgG were purchased from Miles-Yeda Laboratories, Israel. Non-immune (pre-immune) rabbit serum was obtained from the Scottish Antibody Production Unit, Glasgow, Scotland. Protein-A sepharose was supplied by

Pharmacia Fine Chemicals, Sweden.

The monoclonal antibody LPI, specific for Vmw65 (McLean et al., 1982) was obtained from Dr A C Minson.

MISCELLANEOUS

Nitrocellulose membranes were obtained from Schleicher and Schull, Dassel, West Germany. Photographic film was supplied by Kodak Ltd, London. Plastic 850cm^2 roller bottles used for tissue culture were obtained from Becton Dickinson, Oxford. Plastic Petri dishes were supplied by Nunclon Ltd.

STANDARD SOLUTIONS

Phosphate buffered Saline (PBS)	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 pH7.2 (Dulbecco and Vogt, 1954).
PBS-calf	PBS, 5% calf serum (v/v).
Tris-Saline	140mM NaCl, 30mM KCl, 280mM Na_2HPO_4 1mg/ml glucose, 0.0015% (w/v) phenol red, 25mM Tris-HCl pH7.4, 100 units/ml penicillin, 100ug/ml streptomycin.
Versene	0.6mM EDTA in PBS containing 0.002% (w/v) phenol red.
Trypsin	0.25% (w/v) trypsin in tris-saline.
Giemsa stain	1.5% (w/v) giemsa in glycerol heated at 56°C for 120min and diluted with an equal volume of methanol.
Reticulocyte soluble 1.5mM buffer (RSB)	10mM Tris-HCl pH7.5, 10mM KCl, $MgCl_2$.
TSE	10mM Tris-HCl pH8.0, 1mM EDTA, 0.25% (w/v) SDS.
NTE	10mM Tris-HCl pH7.5, 100mM NaCl, 1mM EDTA.
Hepes buffered saline (HEBS)	130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4 5.5mM D-glucose, 21mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid) pH7.05.

Plating medium	50% (conditioned) EC10, 44% (fresh) EC10, 10% HEBS, 0.4mM $CaCl_2$.
Cell lysis buffer	0.6% SDS, 10mM EDTA, 10mM Tris-HCl pH 7.4.
Gel Soak I (1x)	200mM NaOH, 600mM NaCl.
Gel Soak II (1x)	1mM Tris-HCl pH8.0, 0.59M NaCl.
Nick translation buffer	50mM Tris-HCl pH7.5, 5mM $MgCl_2$, 50ug/ml BSA, 1mM DTT.
T4 DNA polymerase buffer	330 mM tris acetate pH7.9, 600mM potassium acetate, 100mM magnesium acetate, 5mM dithiothreitol, 1mg/ml BSA.
Denhardt's buffer (x5)	0.1% (w/v) Ficoll, 0.1% (w/v) polyvinyl pyrollidone, 0.1% (w/v) BSA.
Hybridization buffer	5 x Denhardt's buffer, 0.1% (w/v) SDS, 6 x SSC, 30ug/ml denatured calf thymus carrier DNA.
SSC buffer (20x)	3M NaCl, 0.3m tri-sodium citrate.
TE buffer (1x)	10mM Tris-HCl pH7.4, 1mM EDTA.
Resolving gel buffer	1.5M Tris-HCl pH8.9, 0.4% (w/v) SDS.
Stacking gel buffer	0.49m Tris-HCl pH6.7, 0.4% (w/v) SDS.
SDS-polyacrylamide	53mM tris, 53mM glycine, 0.1% (w/v) gel tank buffer SDS.

Dye Ficoll	15% (w/v) Ficoll, 0.2% (w/v) bromophenol blue, 100mM EDTA (pH8.0).
TBE buffer (10x)	8.9mM Tris-HCl pH8.3, 8.9mM boric acid, 0.3mM EDTA containing 0.5mg/ml ethidium bromide.
Electro-elution	0.05M sodium acetate, 0.01M EDTA, buffer (x10) 0.4M Tris-HCl pH7.5.
Zweig's buffer	0.1M Tris-HCl pH8, 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate, 0.2mM phenylmethylsulphonyl fluoride (PMSF).
Elution buffer	2% SDS, 20% glycerol, 5% (v/v) β-mercapthoethanol, 0.125M Tris-HCl pH6.8, 0.004% (w/v%) bromophenol blue.
Washing buffer	600mM LiCl, 100mM Tris-HCl pH7.4, 1% (v/v)β-mercaptoethanol.
TK Lysis buffer	20mM Tris-HCl pH7.5, 2mM $MgCl_2$, 10mM NaCl, 0.5% (v/v) NP40, 6.5mM β-mercaptoethanol (added just prior to use).
TK reaction buffer	100mM sodium phosphate buffer (pH6.0), 50um, dTTP, 5mM ATP, 10mM $MgCl_2$, 100uCi [^{3}H]-thymidine/ ml.

STET buffer	8% (w/v) sucrose, 5% (v/v) NP40, 50mM EDTA, 50mM Tris-HCl, pH 8.0.
Ligation buffer	20mM Tris-HCl pH7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP.
L-broth	170mM NaCl, 10g/l Difco bactotryptone, 5g/l yeast extract.
Permeabilization buffer	10% (w/v) sucrose, 1% calf serum, 0.5% buffer (v/v) NP40, (in PBS).
Fix solution	2% (v/v) formaldehyde, 2% (w/v) sucrose (in PBS).

METHODS

1. Cell Culture

BHK C13 cells were grown at 37°C in rotating 850cm^2 culture bottles containing 200ml ETC10 in an atmosphere of 95% air, 5% CO_2. A confluent monolayer of approximately 3×10^8 cells from one bottle was used to seed five further 850cm^2 bottles.

Cells were harvested by washing the monolayers first with 20ml versene, then with 40ml trypsin:versene (1:1). The cells were shaken off the glass and resuspended in culture medium.

A density of 2×10^6 cells per 50mm petri dish and 1×10^6 cells per 30mm dish was sufficient to obtain subconfluent monolayers after an overnight incubation at 37°C.

2. Virus Culture

2.1 Production of Virus Stocks

Confluent BHK cell monolayers in 850cm^2 glass roller bottles were infected with a moi of 0.003 pfu virus per cell in 50ml EC5. After incubation at 31°C for 3-4 days when extensive CPE had developed, virus-infected cells were shaken into the medium and pelleted at 1,500rpm for 15min at 4°C.

Cell-released virus in the supernatant was concentrated by centrifugation at 12,000rpm for 2h at 4°. The virus pellet was resuspended in virus-infected cell medium by sonication, and stored at -70°.

The virus-infected cell pellet was sonicated in a small volume of EC5 to release cell-associated virus, and the cell debris was removed by centrifugation at 3,000rpm for 15min at 4°. The sonication step was repeated if necessary and the cell-associated virus stocks were pooled and stored in aliquots at -70°.

2.2 Sterility Check

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

2.3 Titration of Virus Stocks

Serial tenfold dilutions of virus stock were made in PBS-calf. The medium was removed from 75-90% confluent cell monolayers, and 100ul of diluted virus was added to each dish. Following virus absorption for 45-60min 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

4ml EHU5 to prevent the spread of virus plaques. After incubation at 38.5°C for 2 days or 31°C for 3 days, the medium was removed from the dishes and the cells stained with Giemsa for 10min at RT. The monolayers were washed with water to remove the stain, and virus plaques were counted under a dissecting microscope.

2.4 Virus Plaque Purification from Cells Overlaid with Agar Medium

A single plaque together with the overlying agar medium were scraped up into a Pasteur pipette, transferred to 500ul PBS-calf, sonicated and stored at -70°C prior to virus titration.

2.5 Ts Mutant Infections

Virus infections were carried out at the PT(31°C) or NPT (38.5°C). Cell monolayers were incubated at the required temperature for a minimum of 15min prior to the addition of virus, and the overlay medium was pre-warmed to 42°C before use. All manipulations at the NPT were carried out as rapidly as possible to prevent any drop in temperature.

2.6 Isolation of Spontaneous ts$^+$ Revertants of ts2203

Single plaques were isolated from a low passage stock of ts2203 titrated on BHK cells at 31°C. BHK cell monolayers from five 50mm petri dishes were infected with virus from a single plaque and a small seed stock

grown up at 31°. The seed stocks were titrated at the NPT and any cell monolayers showing CPE after incubation for 2 days were harvested. Progeny virus from each cell monolayer were tested for the ability to form plaques at the NPT. Those which grew at the NPT were plaque-purified once at the NPT and twice at the PT.

Seed stocks from five $\underline{ts}^{+}$ revertants, all derived from different plaques of $\underline{ts}$2203, were made and each used to grow up a larger virus stock.

2.7 Single-step Growth of Virus

Subconfluent monolayers of BHK cells in 30mm dishes were infected at a moi of 5pfu virus per cell at the PT or NPT. After incubation for 1h, the cells were washed 3x with PBS-calf to remove unabsorbed virus and overlaid with 1.5ml EC10. At various times post absorption, the cells were scraped into medium, sonicated and the virus stored at -70° prior to titration at the PT.

2.8 Virion Thermostability Assay

Virus stocks at a concentration of 1×10^5 pfu per ml PBS containing 0.05% calf serum, in plastic 15ml Falcon tubes, were incubated in a water bath at 40°. At various times 100ul aliquots were removed and added to 900ul PBS-calf serum. Samples of virus were stored at -70° prior to titration on BHK cells at 31°.

2.9 Recombination Assay

BHK cells in 30mm dishes were infected at a moi of 10pfu virus per cell (5pfu of each virus in mixed infections). After incubation for 1hr at 31°, monolayers were washed twice with PBS-calf to remove any unabsorbed virus and incubated in 1.5ml EC10 for 24h at the PT.

Virus-infected cells were scraped into the growth medium, sonicated and stored at -70° before virus titration at the PT and NPT. Recombination could then be assessed by growth at the NPT.

3. Preparation of Viral DNA

3.1 Large-Scale Preparation of Virion DNA

Virus-infected cells showing extensive cpe (normally from 5-10 850cm^2 roller bottles), were harvested into the medium, pelleted by low speed centrifugation and resuspended in 20ml RSB containing 0.5% NP40. After incubation on ice for 10min, the sample was centrifuged at 2,500rpm for 10min at 4° to separate the nuclei from the cytoplasmic fraction. The pellet of nuclei and cell debris was resuspended in 20ml RSB-NP40 buffer, left on ice for 10min and centrifuged again. The cytoplasmic supernatant fractions were then combined with the virus-infected cell medium and the virus concentrated by centrifugation for 2-3h at 4°.
The virus pellet was sonicated in 5ml NTE and lysed by

the addition of SDS to a final concentration of 2% (w/v). The virus DNA sample was sequentially extracted three times with phenol saturated with TE, once with chloroform, incubated overnight at RT with RNase I (10 ug/ml), and finally treated with proteinase K at 31°C for 1-2h. The solution was then deproteinised with an equal volume of phenol:chloroform (1:1), followed by extraction with chloroform. Sodium acetate was added to a concentration of 0.3M and the viral DNA was precipitated by the addition of 2 volumes of ethanol. The DNA was pelleted by low speed centrifugation, lyophilised and resuspended in 2-3ml TE.

3.2 Small-scale Preparation of Virus-infected Cell DNA

BHK cell monolayers (4×10^6 cells per 50mm dish) were infected with a moi of 5pfu virus per cell at the required temperature. After incubation for 18h, the cells were harvested and either total virus-infected cell DNA or encapsidated DNA prepared from the samples.

3.3 Total Virus-infected Cell DNA

The medium was removed from the cells and cell-released virus pelleted by centrifugation at 18,000rpm for 1h at 4°C in an SS34 rotor. The virus pellet and cells were incubated in 2ml lysis buffer, containing 500ug/ml pronase for 4h at 37°C. NaCl was added to a final concentration of 200mM, and the DNA sequentially extracted once with phenol:chloroform (1:1), once with

chloroform, and precipitated in ethanol. The DNA pellet was lyophilised and resuspended in 400ul H O containing 10ug/ml RNase I.

3.4 Encapsidated (DNase resistant) DNA

The medium was removed from the dishes and cell released virus was concentrated as described for the total virus DNA. The virus pellet and cells were incubated in 0.5ml RSB containing 0.5% (v/v) NP40, 100ug/ml DNase I for 2h at 37°. SDS, EDTA and pronase were then added to concentrations of 0.6%, 10mM and 500ug/ml respectively, and the sample was incubated for a further 2h at 37°.

Virus DNA was purified as described previously.

4. Analysis of Viral DNA

4.1 Restriction Endonuclease Digestion of DNA

DNA was digested with restriction endonuclease using conditions recommended by the suppliers.

4.2 Separation of Restriction Endonuclease DNA Fragments

4.2.1 Agarose Gel Electrophoresis

Large DNA fragments were separated on horizontal 0.8-1.0% (w/v) agarose gels. DNA samples in 10% (v/v) dye Ficoll were loaded into wells formed with plastic combs, and the gel was electrophoresed at 2-10v/cm in 1x TBE buffer containing 0.5ug/ml EtBr at RT for 16-24h. The separated fragments were visualised under UV light.

4.2.2 Polyacrylamide Gel Electrophoresis (PAGE)

DNA fragments in a size range of 70-1,000 bp were separated by PAGE. Gels were prepared from 80ml 10-16% polyacrylamide (using a stock solution of acrylamide/N-N'-methylene bisacrylamide in a ratio of 29:1 (w/w) in 0.55xTBE. Ammonium persulphate and N,N,N'-N'-tetramethylethylene diamine (TEMED) were added to a final concentration of 0.006% (w/v) and 0.004% (w/v) respectively to polymerise the gel. DNA fragments in 10% (v/v) dye Ficoll were loaded on to the gel and separated by electrophoresis at 3v/cm^2 for 16-24h in 0.55x TBE.

DNA bands were visualized under UV light after the gel had been stained in 0.55xTBE containing 0.5ug/ml EtBr for 30min.

4.3 Southern Blot Analysis

4.3.1 Transfer of DNA Fragments to Nitrocellulose

The experimental procedure used was essentially the same as the method described by Southern (1975). DNA restriction endonuclease fragments were separated by agarose gel electrophoresis, and the gel piece containing the DNA was shaken gently in 0.2M HCl at RT to depurinate the DNA. After 30min, the gel was washed in deionised water and treated with GSI for 60min to denature the DNA. This solution was neutralized by soaking the gel in GSII for 60min.

The gel was transferred onto Whatman 3mm filter paper, the ends of which were in contact with a reservoir of 10xSSC buffer. A sheet of moistened nitrocellulose was placed on top of the gel and any air bubbles were smoothed out. Four sheets of 3mm filter paper, slightly smaller than the gel, were placed over the nitrocellulose sheet and a weighted stack of paper towels laid on top acted as a wick to draw the 10xSSC from the reservoir.

The next day the nitrocellulose paper was removed and washed in 2xSSC, air dried and baked in a vacuum oven at 72°C for 2hrs. To ensure that efficient transfer of DNA fragments from agarose to nitrocellulose had occurred, blotted gels were soaked in 1xTBE containing 1mg/ml EtBr for 30min and visualised under shortwave UV light.

4.3.2 In Vitro [^{32}P]-Labelling of DNA by Nick Translation (Rigby et al., 1977)

The reaction mixture (50ul), containing plasmid DNA (0.2-0.6ug), 1x10^{-6} mg/ml DNase I, 1xNT buffer, 20uM dATP and 20uM dTTP, was placed in a 37°C water bath for 2-3min prior to the addition of 10uCi each of [α-^{32}P]-dCTP and [α-^{32}P]-dGTP together with 1 unit of E.coli DNA polymerase I (pol I). After incubation at 16°C for 2hrs, the reaction was terminated by the addition of 20mM EDTA, and the sample extracted once with phenol:chloroform (1:1).

Unincorporated [α-^{32}P]-dNTPs were separated from the [^{32}P]-labelled DNA by fractionation through a Sephadex column. The DNA was collected in a 100ul volume and denatured in 0.2M NaOH at RT for 10min. The solution was neutralised by the addition of 0.2M HCl. A specific activity of $1x10^{7}$ -$1x10^{8}$ cpm per ug plasmid DNA was routinely obtained.

4.3.3 Separation of Deoxyribonucleotide Triphosphates from [^{32}P]-labelled DNA: The Spun Column Technique (Maniatis et al., 1982)

A small plug of sterile siliconised glass wool was inserted into a 1ml disposable syringe and the syringe was then placed in a 1.5ml reaction vial. A solution of fine Sephadex G50 in TE was spun through the syringe at 3,000rpm for 2min at 4^{o}. This procedure was repeated until the volume of Sephadex G50 retained in the syringe was 0.8-1.0ml. A 100ul volume of TE was spun through the column, followed by 100ul of nick-translated DNA in TE which was collected into a fresh reaction vial.

4.3.4 DNA Blot Hybridisation

High stringency conditions of hybridisation based on those described by Southern (1975) and Denhardt (1966) were used.

Vacuum-dried nitrocellulose filters were incubated in 20ml of hybridisation buffer in sealed plastic bags

submerged in a shaking water bath at 72°. After 2-3h, this solution was replaced with fresh hybridisation buffer containing $2x10^7$ cpm denatured [^{32}P]-labelled DNA probe, and incubation was continued at 72° for a further 16-18hrs. Following hybridisation, the nitrocellulose sheets were washed four times in 2xSSC, once in 1xSSC and once in 0.5xSSC.

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 4-24h.

5. Insertion of Oligonucleotide Linkers into Plasmid DNA

5.1 Production of Linear Partial DNA

Plasmid DNA (2ug) was digested for various times with 2 units of a multi-cutting restriction endonuclease in the presence of decreasing concentrations of EtBr to determine the optimal conditions for the production of linear partials of the plasmid DNA.

Digested DNA was analysed on a 0.8-1.0% agarose gel and a bulk restriction was carried out under optimal conditions. The linear DNA was then eluted from agarose gel slices.

5.2 Purification of DNA from Agarose Gels

DNA was eluted from agarose gel by high-voltage

electrophoresis in elution buffer, and purified by sequential extraction in saturated butan-2-ol, phenol:chloroform (1:1) and finally chloroform. Sodium acetate (pH6.6) was added to a final concentration of 0.3M and the DNA precipitated with an equal volume of isopropanol. After 2h at RT, the DNA was pelleted at 10,000rpm for 10 min, lyophilised and resuspended in TE.

5.3 Ligation of Linear Partial Plasmid DNA with a 6bp Oligonucleotide Linker Fragment

Purified linear partial DNA was treated with 5 units of calf intestinal phosphatase in 50mM Tris-HCl, 0.1mM EDTA (pH8.0) for 60min at 37°. The DNA was extracted once with phenol:chloroform (1:1), once with chloroform, and precipitated with isopropanol in the presence of 0.3M sodium acetate (pH6.6).

Plasmid DNA (10-50ng) was incubated overnight at 4° with a 10-100 x molar excess of Pharmacia XbaI (5'TCTAGA 3') phosphorylated oligonucleotide linker in a total volume of 10ul containing 1-2 units of T4 DNA ligase in 1x ligation buffer.

5.4 The Production of Competent Bacteria and DNA Transfections

The method used was based on that described by Cohen et al., (1972). E.coli K12 strain DH-1 was grown in L-broth to an optical density (OD 590nm) of approximately 0.2. The cells were pelleted at 3,000

rpm for 10 min at 4°, and resuspended in 1/4 volume ice-cold 100mM $CaCl_2$. After incubation on ice for 60min, the cells were concentrated by centrifugation and resuspended in 1/10 volume of ice-cold 100mM $CaCl_2$.

A sample of cells were tested for competence using uncleaved vector DNA. Plasmid DNA (10ng) was added to 100ul of the cells and the sample was left on ice for 60min before heat-shock treatment for 90-120sec at 42-45°. The bacteria were then added to 1.5ml L-broth and shaken at 37°. After 60min, 100ul samples were plated onto L-broth agar containing 100ug/ml ampicillin, and plates were incubated overnight at 37°. Routinely, more than 1×10^6 colonies per ug plasmid vector were obtained, and since the competence of DH-1 bacteria increases 5-fold after an overnight incubation at 4°, competent bacteria were added to the ligation reactions 16h after their initial preparation.

5.5 Small-scale Preparation of Plasmid DNA (STET Preparations)

(A Modification of the method described by Maniatis et al., [1982])

Bacteria from a single colony were grown in 1.5ml L-broth containing 100ug/ml ampicillin in an orbital shaker at 37° for 16-18h. After being pelleted at 10,000rpm for 90sec, the cells were resuspended in 75ul STET buffer, treated with 6ul 10mg/ml lysozyme (Holmes

and Quigley 1981) and boiled for 1-2min before centrifugation at 10,000rpm for 10min. The supernatant was retained and extracted sequentially once with phenol:chloroform (1:1) and once with chloroform. Plasmid DNA in a solution containing 0.3M sodium acetate was precipitated with an equal volume of isopropanol, pelleted at 10,000rpm for 10min, lyophilised and resuspended in 40-50ul 10mM Tris-HCl (pH7.4).

5.6 Bulk Preparation of Plasmid DNA

This is based on the procedure described by Godson and Vapnek (1973). A flask containing 1l L-broth supplemented with 100ug/ml ampicillin (for growth of *E.coli* K12 MRC1, 50ug/ml diaminopimelic acid and 200ug/ml N-acetyl glucosamine were also added) was inoculated in 10ml of an overnight bacterial culture, and incubated at 37°C in an orbital shaker for 6-7h. Chloramphenicol was added to a final concentration of 25ug/ml, and incubation was continued for a further 16h. The bacteria were pelleted by centrifugation at 8,000rpm for 5min at 4°C, and resuspended in 25ml 25% sucrose on 50mM Tris-HCl pH7.9. The mixture was incubated on ice for 30min at which time EDTA (pH7.9) was added to a final concentration of 50mM. Five minutes later, NaCl and SDS were added to final concentrations of 750mM and 2.5% (w/v) respectively. After incubation at 4°C for 2-3h, the sample was

centrifuged at 20,000rpm for 60min at 0°C to remove debris and high molecular weight bacterial DNA. The resulting supernatant was extracted once with phenol:chloroform (1:1), once with chloroform, and the DNA was precipitated with 2 volumes of ethanol in the presence of 0.3M sodium acetate. The pelleted DNA was washed with 70% ethanol, lyophilised and incubated overnight in 2-5ml 10mM Tris-HCl pH7.5 containing 10ug/ml RNase I. The DNA was then further purified by equilibrium centrifugation on caesium chloride-ethidium bromide gradients.

5.7 Caesium-chloride-ethidium bromide Gradients (CsCl-EtBr)

To each ml of plasmid DNA preparation, 1g caesium chloride was added to give a solution with a final density of 1.55g/ml. This solution, plus ethidium bromide (500ug/ml), was centrifuged at 45,000rpm for 18h at 15°C in a Beckman TV865 rotor. The tubes were examined under long wave UV light, and the lower band of DNA, representing supercoiled plasmid DNA, was withdrawn through a syringe inserted in the side of the tube.

The ethidium bromide was removed by sequential extraction of the solution with isopropanol saturated with caesium chloride. After overnight dialysis against TE buffer to remove the caesium chloride, the DNA was concentrated by ethanol precipitation, and pelleted by centrifugation.

5.8 Storage of Bacterial Stocks

Concentrated bacteria were resuspended in 40% glycerol, 1% bactopeptone broth, and stored at -20°.

6. Expression of TK in Transfected Cells

6.1 Transfection of Plasmid DNA into BHK Cells

Calcium chloride was added to a solution containing a total of 3ug DNA (a mixture of test plasmid and carrier DNA) to a final concentration of 136mM in 1x HEBS. The solution was briefly mixed and left for 30min at RT to allow the formation of a fine precipitate. The suspension was added dropwise to drained, 50% confluent cell monolayers in 30mm dishes and the cells were incubated at 37°. After 45min the cells were overlaid with 2ml plating medium and incubation continued at 37° for 3-4h at which time cells were treated with 25% DMSO in 1x HEBS for 4 mins. After extensive washing to remove DMSO, cells were incubated for 16h at 37° in EC10.

6.2 Preparation of Cell Extracts

Cells were washed once with ice-cold PBS, scraped into 1ml of PBS and pelleted by centrifugation at 10,000rpm for 2min. The cell pellet was resuspended in 150ul ice-cold lysis buffer and samples incubated on ice for 5min. The vials were then briefly vortexed and placed on ice for a further 5min. After a final vortex, the samples were centrifuged at 10,000rpm for 2min, and the supernatant was retained for immediate use, or frozen in dry ice and stored at -70°.

6.3 Thymidine Kinase Assay

Routinely, 50ul of reaction mixture containing 5ul of cell extract, 100mM sodium phosphate buffer (pH6.0), 10mM $MgCl_2$, 50um dTTP, 5mM ATP and 100uCi [^{3}H]-thymidine/ml were incubated at 30^{o} for 60min. The reaction was terminated by the addition of 10ul of 2mM thymidine.

The tubes were heated for 3min at 100^{o} in a heating block, incubated on ice for 5min and centrifuged at 10,000rpm for 2min. A sample (40ul) of the supernatant was spotted onto DE81 discs, and when dry, the discs were washed three times in a solution of 4mM ammonium formate, 10mM thymidine for 10min at 37^{o}, followed by a brief wash in 100% ethanol. Discs were dried under a heat lamp, placed in scintillation vials with 5ml scintillation fluid (Ecoscint) and counted in a scintillation counter for 1min.

7. Marker Rescue Technique

Experiments were performed as described by Stow et al., (1978) with the modifications of Preston (1981).

Calcium chloride was added to a final concentration of 130mM in a 500ul solution containing 0.5ug intact mutant virion DNA, a 10-fold molar excess of an HSV DNA fragment and 10ug/ml calf thymus carrier DNA in HEBS. Samples were then left undisturbed for 5min at RT to

allow the development of a fine suspension which was added to drained BHK cell monolayers in 50mm dishes. After incubation at 37°C for 45min, the monolayers were overlaid with EC5 and incubation was continued at 37°C.

At about 4h from the time of addition of DNA to cells, the transfected cells were washed once with EC5 and boosted with 1ml 25% (v/v) DMSO in hepes for 4min at RT. The monolayers were washed three times in EC5 to remove the DMSO, and cells were incubated in 4mls EC10 at 31°C for 3-4 days until extensive CPE had developed. Cells were scraped into the growth medium, disrupted by sonication and virus yield at both the PT and the NPT determined.

8. Analysis of Virus-induced Polypeptides

8.1 Preparation of Radiolabelled Cell Extracts

Subconfluent monolayers of BHK cells in 30mm dishes were infected at a moi of 20 pfu virus per cell. After incubation for 45min at either 31°C or 38.5°C, cells were washed once with EC5 to remove any unabsorbed virus, overlaid with EC10, and incubated at either the PT or NPT until the designated labelling time.

8.2 Labelling Polypeptides with [^{35}S]-methionine

Virus-infected cells were washed once with pre-warmed PBS and incubated in pre-warmed PBS containing 100uCi/ml [^{35}S]-methionine for 1-2h. The monolayers

were washed three times with PBS before harvesting.

8.3 Harvesting Radiolabelled Infected Cells for Analysis by SDS-PAGE

Virus-infected cells were harvested in 300ul disruption buffer and stored at -70°.

8.4 Immunoprecipitation

Radiolabelled virus-infected cells were harvested in 500ul Zweig's buffer, transferred to glass vials and sonicated. After incubation on ice for 60min, samples were centrifuged at 10,000rpm for 5min. The supernatant was retained and stored at -70° until required.

Routinely, 25ul antigen (equivalent to approximately 1 x 10^6 cpm) was incubated on ice with 10ul rabbit anti-mouse immunoglobulin and 20ml of a 1/40 dilution of monoclonal antibody LPI. After 3h, 70ul Protein A Sepharose (33% w/v suspension) was added and samples rotated at 4° for 60min.

Immunoprecipitates bound to Protein A Sepharose were washed 6 times by centrifugation at 10,000rpm for 20sec followed each time by resuspension in 50ul lithium chloride washing buffer. The samples were finally resuspended in 50ul disruption buffer, heated at 100° for 5min, then centrifuged at 10,000rpm for 5 min. The supernatant was analysed by SDS-PAGE.

8.5 SDS-PAGE

Polypeptides were separated on 8% polyacrylamide gels (acrylamide was crosslinked with 1 part in 40 (w/w) N,N'-methylenebisacrylamide) in resolving gel buffer. Ammonium persulphate (0.006% w/v) and TEMED (0.004% v/v) were added to the solution to polymerise the gel. After the gel had been poured, it was overlaid with 1/4-strength resolving gel buffer to ensure a smooth gel surface on polymerisation.

A stacking gel, containing 5% polyacrylamide (with the same ratio of acrylamide to N,N'-methylenebisacrylamide as above) in stacking gel buffer was poured on top of the resolving gel and a Teflon comb inserted to form the wells. Samples were boiled for 5min and loaded into the wells, and the gels electrophoresed at 0.45 A/cm^2 for 3-4h in fresh tank buffer.

8.6 Fluorography

Gels were fixed in a solution of methanol:water:acetic acid (50:50:1) for 1h, soaked for 18h in a solution of methanol:water:acetic acid (5:88:17), treated with En^{3}hance (New England Nuclear, Boston, USA) for 1h at RT, and rehydrated in water for 30min at RT. Gels were then dried onto a sheet of Whatman (3mm) filter paper at 80^{o} under vacuum, placed in contact with flashed X-Omat XS-1 film at -70^{o}, and the film exposed for a minimum of 18h.

8.7 Immunofluorescence Assay

The procedure used was essentially that of Randall and Dinwoodie (1986). Cells were grown to 25% confluence on 13mm diameter coverslips, infected with virus at a moi of 5 pfu per cell, or mock infected (mi), and incubated at the PT or the NPT. At 8h pi, the growth medium was removed and the cells were fixed in a solution of 5% formaldehyde. After 10min at RT, the coverslips were washed four times in PBS containing 1% calf serum (wash buffer), immersed in permeabilisation buffer for 5min at RT, and then washed three more times with wash buffer.

LPI monoclonal antibody (25ul of a 1/40 dilution in wash buffer) was added to each coverslip, and the samples were left for 60min at RT. Unbound antibody was removed by washing the coverslips in wash buffer. Cells were incubated at RT for a further 60min in 25ul isothiocyanate-conjugate rabbit anti-mouse immunoglobulin diluted 1:40 in wash buffer. Coverslips were washed 1x in permeabilisation buffer, 4x in wash buffer and 1x in water, then mounted in Gelvatol on glass slides. Fluorescence was visualised under a Leitz UV microscope with an appropriate filter for maximum absorption of fluorescein isothiocyanate conjugate.

9. Electron Microscopy

9.1 Preparation of Samples for Thin Sectioning

Monolayers of BHK cells in 30mm dishes were infected with a moi of 5 pfu virus per cell, and incubated at 31°C or 38.5°C. At various times after infection, samples were harvested; the growth medium was removed and the cells were washed three times with ice-cold PBS before being fixed in 1-2ml 2.5% (v/v) glutaraldehyde in PBS. After at least 1h at 4°C, the cells were scraped into the glutaraldehyde-PBS solution and 500ul of cell suspension transferred to a soft Beem capsule. The cells were centrifuged at 5,000rpm for 10min and the pellet washed three times with ice-cold PBS. Cells were subsequently treated with 1% (w/v) osmium tetroxide for 60min, washed three times with PBS and sequentially dehydrated in solutions of 30%, 50%, 70%, 90% and 100% (v/v) ethanol in PBS for a minimum of 90min in each solution.

Cells were left overnight in fresh ethanol (100%) and embedded, first in 50% (v/v) epon resin in ethanol, then in 100% epon resin. Finally, samples were incubated in fresh 100% epon resin at 65°C for 2 days to polymerise the resin.

9.2 Thin Sectioning

Thin sections of virus-infected cells embedded in resin were cut with a Rawleyer diamond knife or a glass knife on a Reichart-Jung Ultramicrotome and collected on

parlodium-coated copper grids. The samples were

stained for 60min with saturated uranyl acetate in 50% (v/v) ethanol, rinsed in deionized water, counter stained with lead citrate for 10min and examined at 60-100Kv in a Jeol 100s electron microscope.

9.3 Virus Particle Counts

Virus stock was mixed with an equal volume of a latex bead suspension of known concentration and the same volume of phosphotungstic acid (1% (v/v) pH6.2). A small sample was spotted onto a parlodium coated copper grid, air dried, and examined in the electron microscope.

The numbers of latex beads and virus particles in a minimum of 10 fields of the grid were determined, and the concentration of particles in the virus stock calculated.

CHAPTER 3

3. CHARACTERISATION OF THE HSV-2 MUTANT, TS2203, WHICH HAS A TS DEFECT IN A GENE ENCODING A STRUCTURAL POLYPEPTIDE

Introduction

The mutant, ts2203, was derived from the HG52 multiple mutant ts13, one of thirty-three ts mutants isolated from HG52 virus mutagenised with BUdr (Timbury, 1971). Thirteen of these mutants (ts1...ts13) were classified into different complementation groups (Timbury, 1971; Halliburton and Timbury, 1976) and analysed biochemically (Halliburton and Timbury, 1973, 1976). Ts13, which produced limited amounts of viral DNA at the NPT, was the only virus unable to induce virus-specific alkaline exonuclease activity at the NPT (Francke et al., 1978). In addition to this defect, ts13 was shown to have a thermolabile virus particle (Halliburton and Timbury, 1976), and this phenotype was thought to result from a second ts lesion in the virus, within a gene encoding a structural polypeptide (Moss et al., 1979).

This was later confirmed by the isolation of ts^{+} revertants of ts13 which were thermostable at 39^{o} but which retained the apparently non-lethal alkaline exonuclease defect (Moss et al., 1979). The particle defect was subsequently mapped to a different portion of the genome from the exonuclease lesion. The conditional lethal ts mutation was located within the region common to EcoRI l and XbaI d (mu 0.638 to 0.700) by intra- and

intertypic marker rescue (Chartrand et al., 1981), whilst the defect in the alkaline exonuclease gene was mapped within 0.145-0.185mu using a modified marker rescue assay (Moss et al., 1979; Moss, 1986). Further work suggested that the structural polypeptide affected by the ts lesion in ts13 was Vmw65, a major component of the tegument; the monoclonal antibody, MA1044, specific for Vmw65, failed to react with the ts13 polypeptide, but did recognise Vmw65 induced by a ts$^+$ revertant of ts13 (H Moss and J Palfreyman, personal communication).

More recently, Campbell et al. (1984) demonstrated that the HSV-1 Vmw65 virion polypeptide was also the trans-inducing factor (TIF) of IE transcription, and this aspect is dealt with in Section 4. Vmw65, therefore, appears to have a dual role in the growth of the virus; it is important at very early times of the lytic cycle for accelerating IE transcription and at late times it is present as a major component of the virion. To study the structural role of Vmw65, the ts mutation within mu 0.622 to 0.720 in ts13 was cloned from the virus, recombined into WT HG52, and the mutant ts2203 isolated.

3.1 Growth Properties of ts2203

As well as the ts phenotype at 38.5^{o}, ts2203 grew

less well than WT HG52 at 31°, as demonstrated by a one-step growth experiment (Figure 13), and ts2203 virus stocks had titres generally 5-10 fold lower than those of WT HG52. This property at 31° correlated with a high number of non-infectious virus particles in ts2203 stocks, where the particle: pfu ratio was approximately 17 times higher than that of HG52 (Table 3).

3.2 Ts2203 has a ts Lesion in the Vmw65 Gene

Evidence that ts2203 has a ts lesion within the Vmw65 gene was obtained using a variety of experimental approaches.

3.2.1 Intratypic Marker Rescue

The marker rescue technique has been extremely useful in mapping mutations to very small regions on the HSV genome. Using this approach, it has been possible to rescue a mutant with a DNA fragment as small as 320bp (Preston, 1981), although clearly, marker rescue with a fragment of this size is at the limits of the technique.

Initially, pGZ74, containing ts13 BglII i, was analysed for the ability to marker rescue ts2203 (Table 4a). pGZ74 failed to do this, suggesting that the ts13

Figure 13

Growth of ts2203 and HG52

Subconfluent monolayers were infected at a moi of 5pfu virus per cell with ts2203 or wild type (WT) HG52 and incubated at 31°C for 2 to 31h pi. Cells were harvested and virus titrated at 31°C.

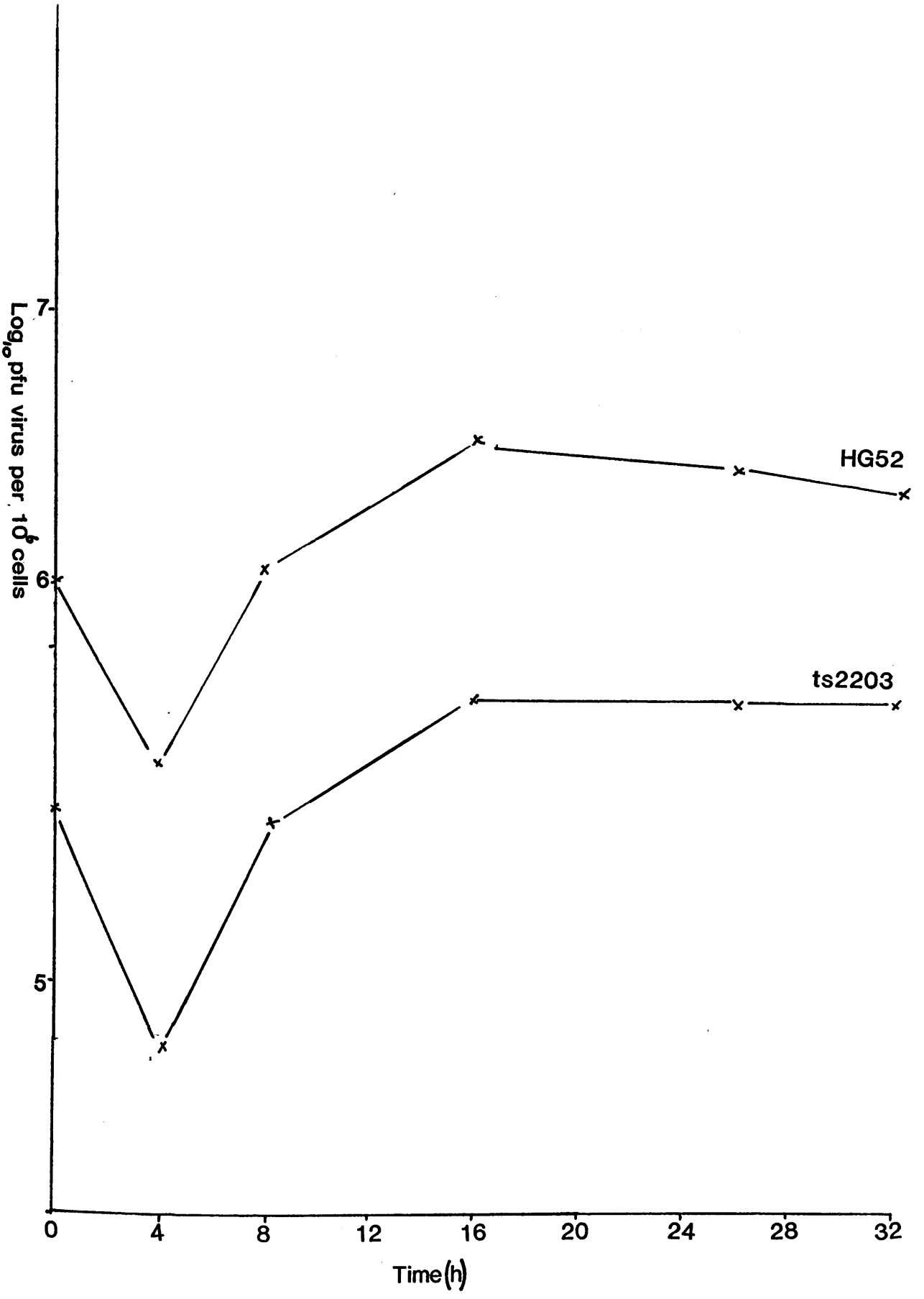

Log10 pfu virus per 10^6 cells
7
6
5
HG52
ts2203
0
4
8
12
16
20
24
28
32
Time (h)

Table 3

Particle: pfu ratios of HSV-2 HG52 and ts2203 virus stocks

Table 3

Particle: pfu ratio

HG52	3×10^{2}
*ts*2203	5×10^{3}

Table 4

Summary of marker rescue results for ts2203

a. Control samples, BglII i digested pGZ73, and BglII i digested pGZ74,

b. Unseparated KpnI plus BglII, digested pGZ73 DNA,

c. Separated KpnI plus BglII; KpnI, BglII plus XbaI, digested pGZ73 DNA,

d. Unseparated XhoI-, SstI- and BamHI- digested pGZ73 DNA,

were used in marker rescue experiments. The relative efficiency of plating (eop NPT/PT) was calculated from the yield of progeny virus from the transfected cells.

Table 4

HSV-2 DNA Fragment	eop $\frac{NPT}{PT}$ x 100	Rescue
a. pGZ73 (HG52 BglII i)	47.368	+
pGZ74 (ts13 BglII i)	0.002	-
ts2203 virus	0.0001	-
b. Unseparated KpnI digested pGZ73	9.000	+
c. Separated KpnI fragments of pGZ73:		
KpnI c	8.700	+
KpnI i	0.152	-
KpnI s	0.700	-
KpnI c1	9.600	+
KpnI c2	0.0042	-
d. pGZ73 (HG52 BglII i)	140.000	+
pGZ74 (ts13 BglII i)	0.001	-
ts2203 virus	0.0002	-
Unseparated BamHI digested pGZ73	0.00006	-
pGZ73 (HG52 BglII i)	66.670	+
pGZ74 (ts13 BglII i)	0.083	-
ts2203 virus	0.067	-
Unseparated XhoI digested pGZ73	0.001	-
Unseparated SstI digested pGZ73	0.003	-

lesion had indeed been transferred to the HG52 virus in ts2203.

To fine map the ts mutation within ts2203 BglII i, pGZ73 (HG52 BglII i) was digested with KpnI and BglII (Figure 14), and marker rescue performed with the unseparated fragments (Table 4b). As the result was positive, the fragments were separated by agarose gel electrophoresis, and used individually in marker rescue (Figure 15, Table 4c). These results suggested that the mutation was within the KpnI c fragment, and the map location was further refined using KpnI, BglII and XbaI digested fragments of pGZ73 in the marker rescue experiment. The addition of XbaI to the digestion cleaved KpnI c, producing two smaller fragments, termed here KpnI c1, and KpnI c2 (Table 4c).

Unseparated fragments of BamHI-, XhoI- or SstI-digested pGZ73 were also assessed for marker rescue of ts2203 (Table 4d). However, no rescue was observed, and it was concluded that the restriction sites for these enzymes lie close to the position of the ts lesion, rendering the fragments incapable of rescue.

Figure 14

Map of restriction endonuclease sites within HG52 BglII i fragment (0.622-0.720mu)

KpnI and KpnI plus XbaI fragments used in marker rescue experiments are given, together with the fragments generated by BamHI digestion of BglII i.

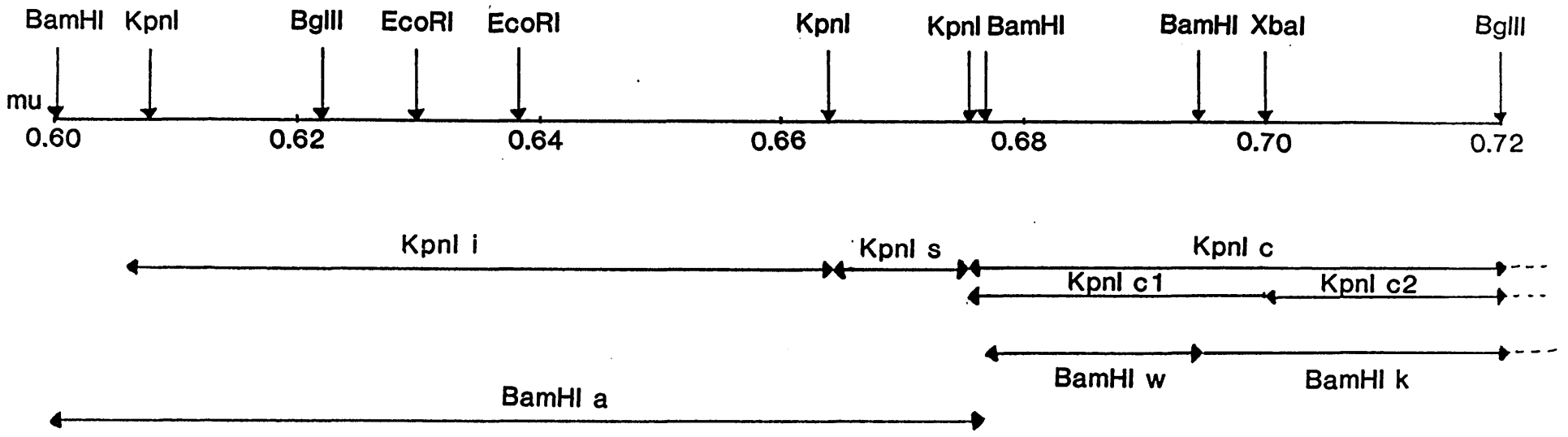

BamHI
KpnI
BglII
EcoRI
EcoRI
KpnI
KpnI BamHI
BamHI XbaI
BglII
mu
0.60
0.62
0.64
0.66
0.68
0.70
0.72
KpnI i
KpnI s
KpnI c
KpnI c1
KpnI c2
BamHI w
BamHI k
BamHI a

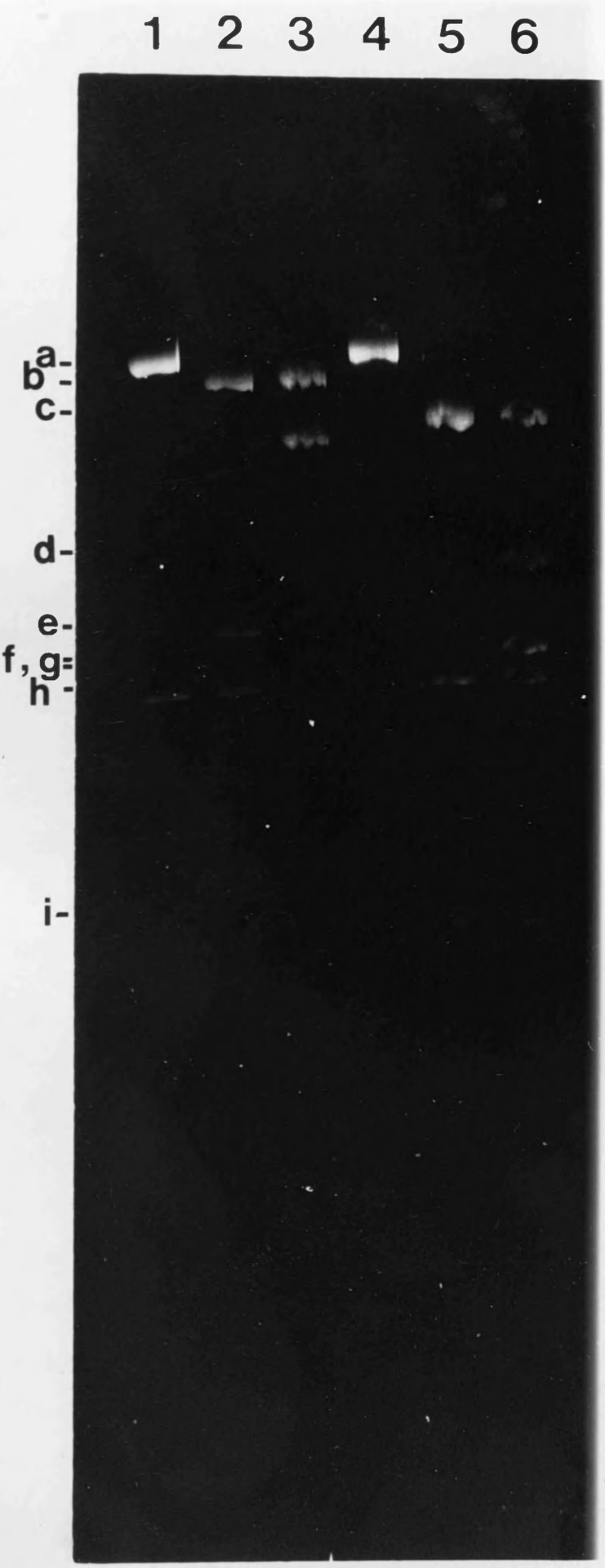
Figure 15

Restriction fragments of HSV-2 BglII i (pGZ73)

The restriction endonuclease fragments indicated in Figure 14 were generated by digestion of pGZ73 with BglII (track 1); BamHI plus BglII (track 2); BamHI (track 3); KpnI (track 4); KpnI plus BglII (track 5); KpnI, BglII plus XbaI (track 6).

Fragments are: (a) BglII i; (b) BamHI a; (c) KpnI i and KpnI c (track 5), KpnI i (track 6); (d) KpnI c1; (e) BamHI k; (f) BamHI w (tracks 2,3); (g) KpnI c2 (track 6); (h) pUC9 vector; (i) KpnI s (tracks 4,5,6).

HSV fragments carrying vector sequences in tracks 3 and 4 are not labelled.


1 2 3 4 5 6
a
b
c
d
e
f, g
h
i

Therefore, the failure of BamHI unseparated fragments, and the ability of the KpnI c fragment, to rescue the ts2203 lesion, suggests that it lies close to the BamHI site at 0.678 or 0.695mu within KpnI c (0.676 to 0.790 mu). This information is summarised in Table 4, and the map locations of the fragments are given in Figure 14.

3.2.2. Intertypic Marker Rescue

The HSV-1 cloned DNA BamHI f fragment (in pGX158) and plasmid pMC1, which encode the HSV-1 Vmw65 gene, were analysed for the ability to marker rescue ts2203. The results (Table 5) suggested that the HSV-1 Vmw65 gene had rescued ts2203, indicating that the ts lesion in ts2203 is located within the HSV-2 gene homologous to HSV-1 Vmw65.

3.2.3. Ts^+ Revertants of ts2203

Stocks of five independently isolated ts^+ revertants of ts2203, (ts^+ rev1...rev5), were grown up from seed stocks supplied by Dr V G Preston, and the eop $\frac{NPT}{PT}$ of each virus determined (Table 6). The fact that the eop $\frac{NPT}{PT}$ of these viruses is similar to WT virus confirmed MR data suggesting that ts2203 contains a single ts mutation.

Table 5

Marker rescue of ts2203 with HSV-1 and HSV-2 DNA fragments

pGX158 (containing HSV-1 BamHI f), pMC1 (containing HSV-1 Vmw65), pGZ73 (containing HG52 BglII i) and pGZ74 (containing ts13 BglII i) DNA, digested with BamHI, EcoRI plus EcoRV, BglII and BglII, respectively, were tested for the ability to marker rescue ts2203.

The results from 2 independent experiments are shown.

Table 5

HSV DNA Fragment	eop NPT (x100) PT	(x100)	Rescue
	a	b	
ts2203 alone	0.001	0.002	-
pGZ73 (HG52 BglII *i*)	80.000	35.900	+
pGZ74 (*ts*13 BglII *i*)	0.010	0.250	-
pGX158 (HSV-1 BamHI *f*)	9.000	2.700	+
pMC1 (HSV-1 Vmw65)	4.800	1.000	+

Table 6

Eop NPT/PT of ts+ revertants of ts2203

Table 6

Eop NPT of ts+ revertants of ts2203

PT

Virus	eop NPT PT
HG52	2.0
ts+ rev-1	1.6
ts+ rev-2	1.8
ts+ rev-3	0.9
ts+ rev-4	1.2
ts+ rev-5	2.0

3.3 The Location of Vmw65 Gene within HSV-2 BglII i

The results of the marker rescue experiments showing that pMC1, which contains HSV-1 Vmw65, rescues ts2203, together with earlier work on ts13 using a monoclonal antibody specific for Vmw65 (Moss and Palfreyman, pc), strongly suggested that the ts2203 lesion mapped within the Vmw65 gene. In order to determine the location of Vmw65 within HSV-2 BglII i, pMC1 was cross-hybridised to subfragments of pGZ73, which contains HG52 BglII i.

3.3.1 Cross-hybridisation of HSV-1 and HSV-2 Vmw65

In order to remove vector sequences, pGZ73 was digested with BglII, and the fragments separated by agarose gel electrophoresis. HSV-2 BglII i DNA was then recovered from the larger fragment band by elution of DNA from the agarose gel slices (Section 2.5.2). Digestion of pMC1 with EcoRI and EcoRV similarly removed the pUC9 (2.7kb) vector together with 5' noncoding sequences adjacent to the Vmw65 coding region (Figure 16).

BglII i DNA was digested with the restriction endonucleases BamHI, KpnI and KpnI plus XbaI. The separated fragments (Figure 17) were transferred from an agarose gel onto nitrocellulose membrane, and the DNA was then hybridised with [^{32}P]-labelled HSV-1 Vmw65 DNA under low stringency hybridisation conditions to promote annealing between HSV-1 and HSV-2 DNA.

Figure 16

Structure of pMC1

pMC1 contains approximately 2.7kb HSV-1 sequences, including Vmw65, ligated into pUC9.

Details of the structure of pMC1 are given in Section 4 and Figure 49.

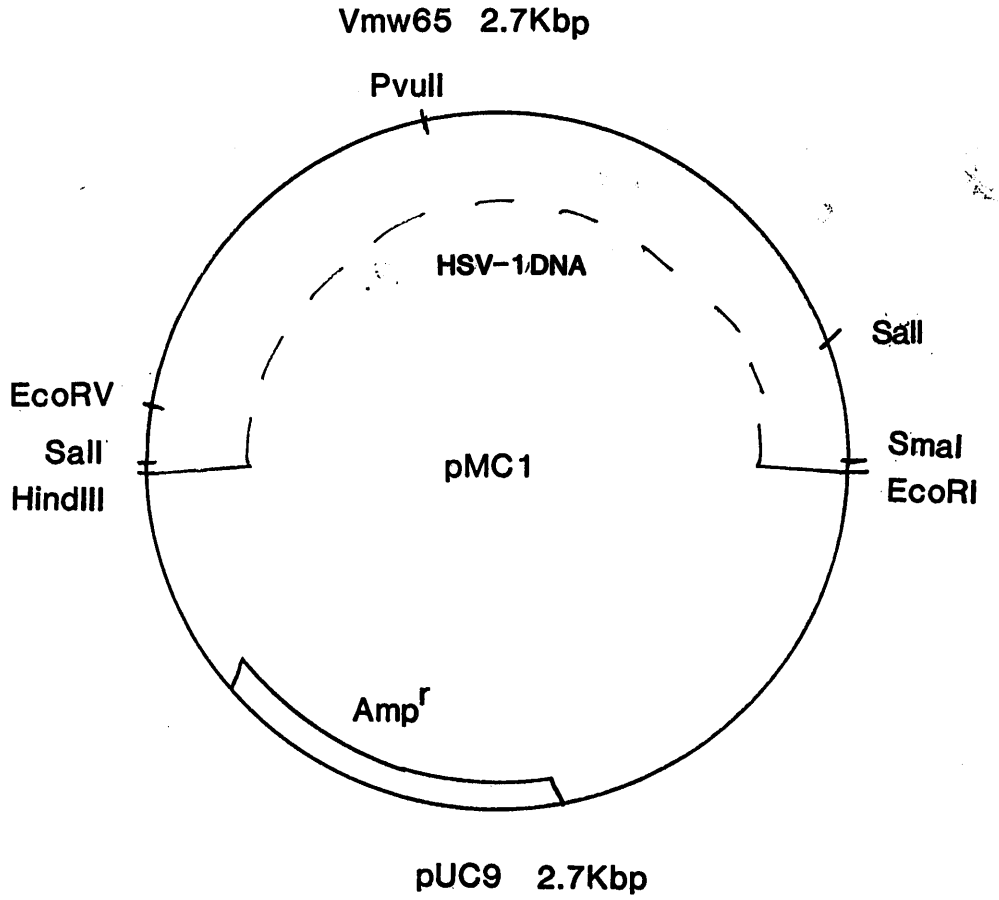
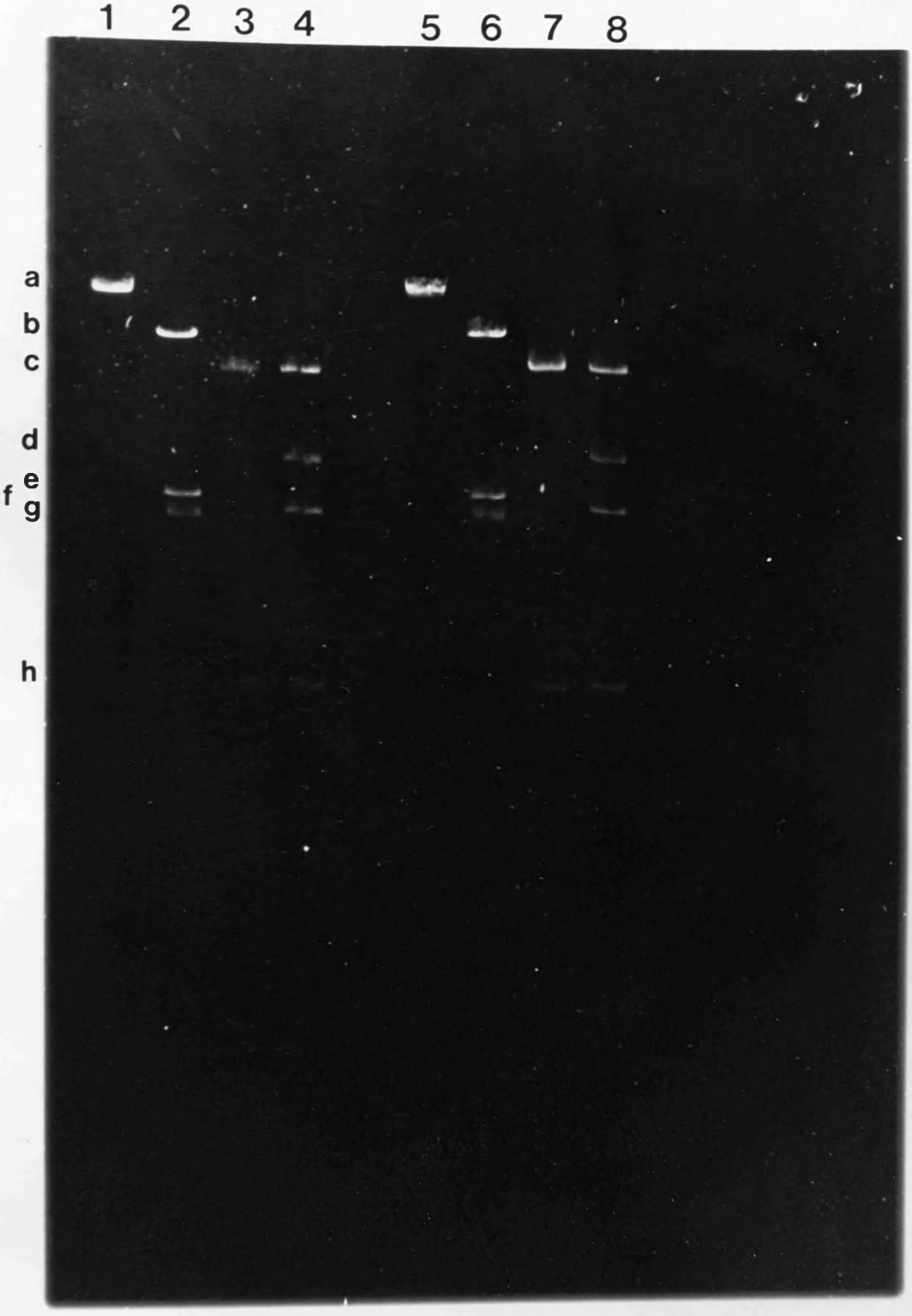

Vmw65 2.7Kbp
PvuII
HSV-1 DNA
SalI
EcoRV
SalI
HindIII
pMC1
SmaI
EcoRI
Amp^r
pUC9 2.7Kbp

Figure 17

Separation of HSV-2 BgllI i DNA fragments

[^{32}P]-labelled (tracks 1-4) and unlabelled (tracks 5-8) HSV-2 BglII i DNA was digested with BglII (tracks 1,5); BamHI (tracks 2,6); KpnI (tracks 3,7) and KpnI plus XbaI (tracks 4,8), and fragments separated electrophoretically on a 1% agarose gel.

Fragments are: (a) BglII i; (b) BamHI a (c) KpnI i, KpnI c; (d) KpnI c1; (e) BamHI k; (f) KpnI c2 (tracks 4,8); (g) BamHI w (tracks 2,6) and (h) KpnI s.


1
2
3
4
5
6
7
8
a
b
c
d
e
f
g
h

Where BglII i DNA was digested with BamHI, the fragments BamHI a, BamHI k and BamHI w were produced. Digestion with KpnI resulted in three fragments, KpnI i and KpnI c, which migrate together, and KpnI s, a much smaller fragment. The addition of XbaI to a KpnI digestion resulted in the separation of KpnI i, KpnI c1 and KpnI c2 (Figures 17 and 18a).

The results of hybridising unlabelled, digested HSV-2 BglII i DNA with [^{32}P]-labelled HSV-1 Vmw65 are shown in Figure 18b). The HSV-2 BglII i control fragment hybridised with HSV-1 Vmw65 (track 1), as did the BamHI a fragment (track 2), but not BamHI k or BamHI w. Similarly, KpnI s, which is contained within the BamHI a fragment, hybridised with HSV-1 Vmw65, but the addition of XbaI to the KpnI digest of HSV-2 BglII i showed that of the large KpnI fragments, only KpnI c1 hybridised with the HSV-1 sequences.

These results, together with the marker rescue results, are summarised in Figure 19. The results suggest that in HSV-2 BglII i the sequences colinear to HSV-1 Vmw65 extend through KpnI s into KpnI c, but are not present in BamHI w, or are limited to such a small region that they cannot be detected by hybridisation.

Figure 18

Cross-hybridisation of HSV-1 and HSV-2 Vmw65 gene sequences

a. [^{32}P]-labelled HSV-2 BglII *i* DNA digested with (1) BglII, (2) BamHI, (3) KpnI, (4) KpnI plus XbaI.

b. Unlabelled HSV-2 BglII *i* DNA digested as in a. and hybridised with [^{32}P]-labelled HSV-1 Vmw65 DNA.

HSV-2 fragments hybridising with HSV-1 Vmw65 are indicated.

a. is a composite of two exposures of the sample, and c. represents the exposure for tracks 3 and 4, plus overexposure of tracks 1 and 2.

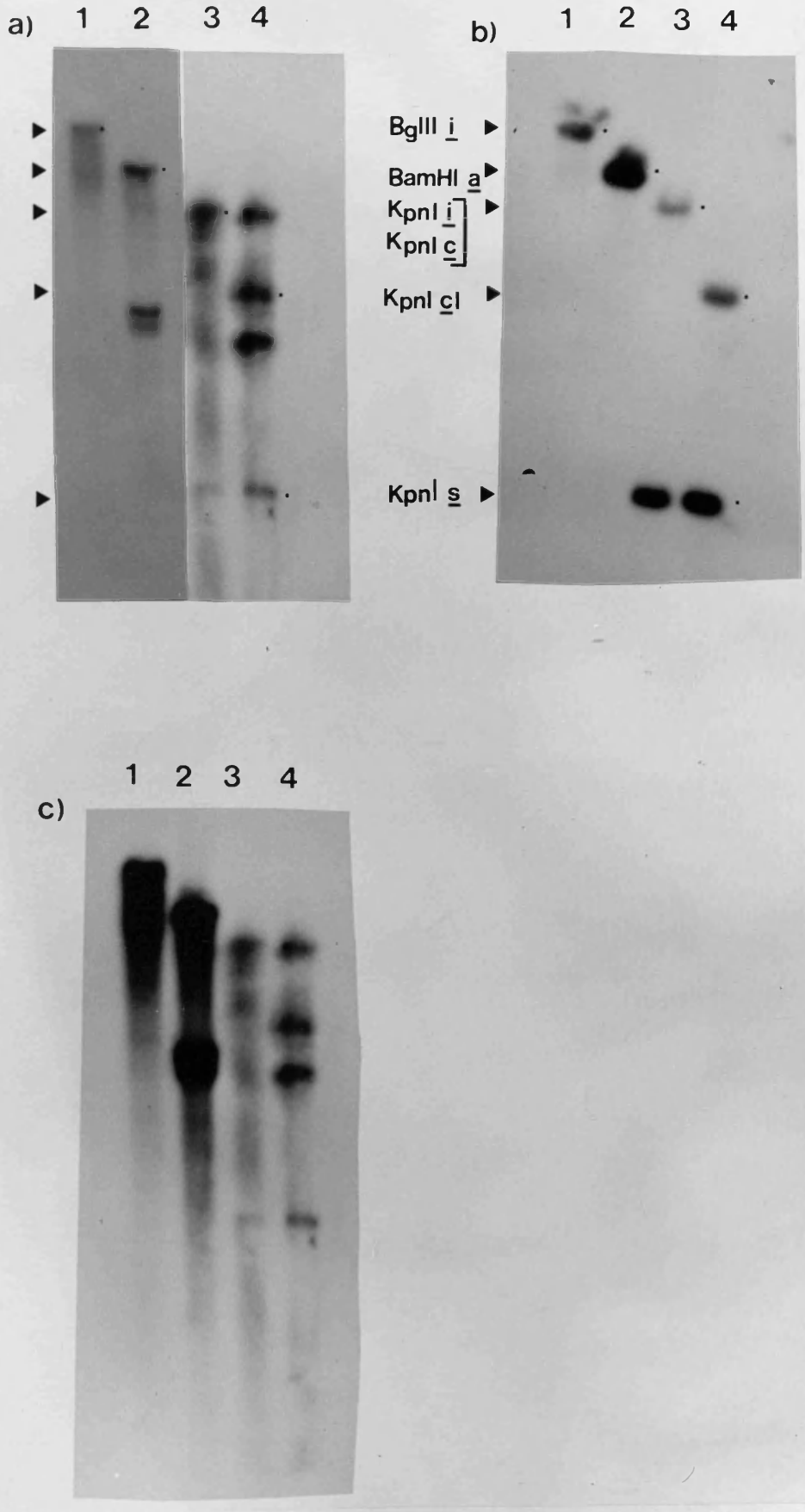
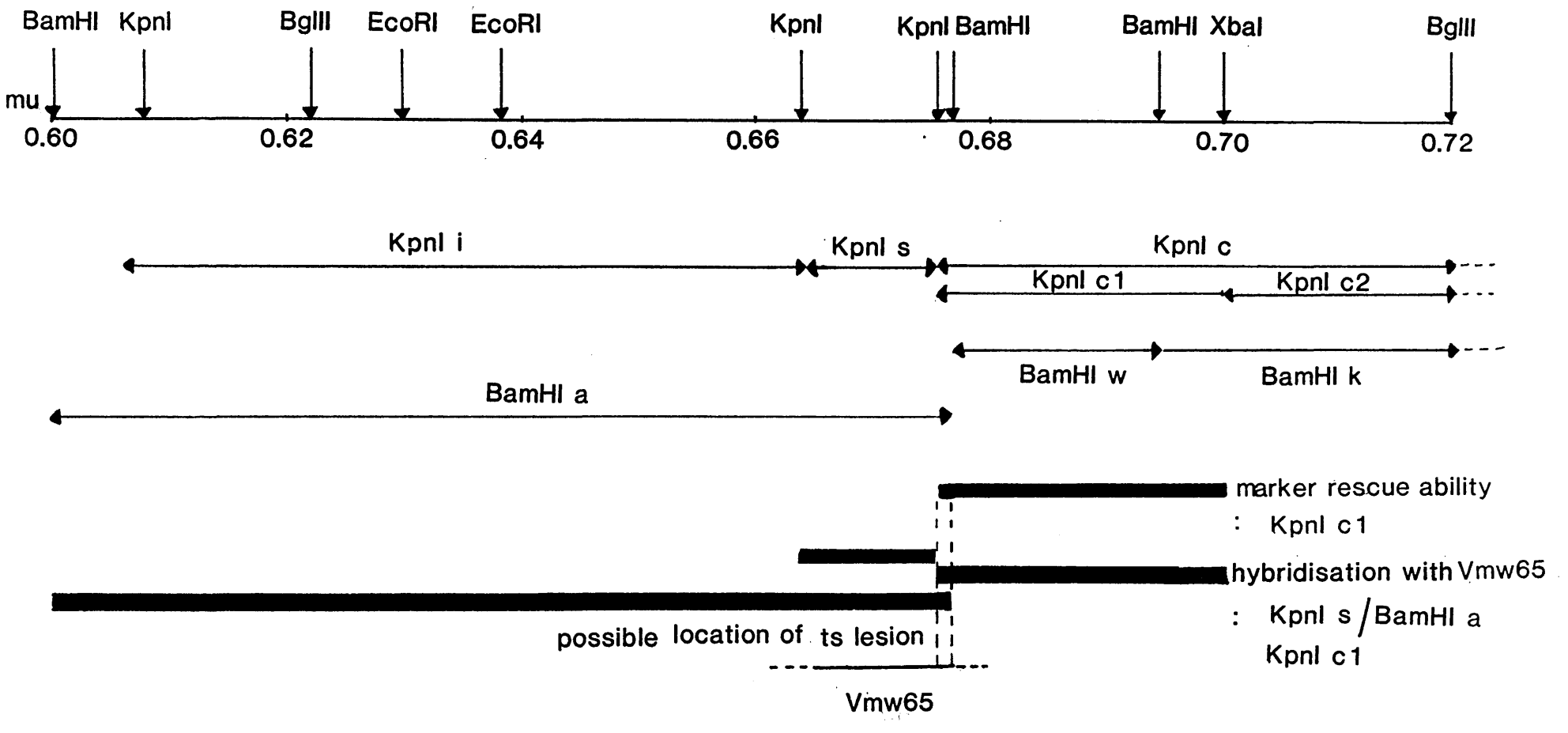

a)
1
2
3
4
b)
1
2
3
4
BglII i
BamHI a
KpnI i
KpnI c
KpnI cI
KpnI s
c)
1
2
3
4

Figure 19

Summary of marker rescue and cross-hybridisation data

The map locations of HSV-2 BglII i restriction endonuclease fragments which rescued ts2203, and those which hybridised with HSV-1 Vmw65 are given, together with the location of Vmw65 on the HSV-2 genome as determined by this data.

The proposed location of the ts lesion in ts2203, bounded by KpnI and BamHI at (0.676 and 0.678mu) restriction endonuclease sites is also given.


BamHI
KpnI
BglII
EcoRI
EcoRI
KpnI
KpnI BamHI
BamHI XbaI
BglII
mu
0.60
0.62
0.64
0.66
0.68
0.70
0.72
KpnI i
KpnI s
KpnI c
KpnI c1
KpnI c2
BamHI w
BamHI k
BamHI a
marker rescue ability
: KpnI c1
hybridisation with Vmw65
: KpnI s / BamHI a
KpnI c1
possible location of ts lesion
Vmw65

From the mapping data, the region between the KpnI site at 0.676mu and BamHI site at 0.678mu is approximately 300bp. HSV-2 BglII i, pGZ73, was digested with KpnI, BamHI or KpnI plus BamHI. On separation by agarose gel electrophoresis, a unique 600bp fragment was produced, indicating that available HSV-2 HG52 restriction maps are inaccurate in this region (Figure 20).

The results therefore indicate that the bulk of HSV-2 Vmw65 lies between the KpnI site at 0.664mu and BamHI site at 0.678mu, approximately 2.17kbp, and that the ts lesion is located towards the 5' terminus of the gene in a 600bp subfragment of KpnI c1. In addition, there may be some 5' regulatory sequences of Vmw65 located within BamHI w, and 3' sequences within BamHI a.

3.4 Thermolability of ts2203 Virus Particle

Early work on ts13 (Halliburton and Timbury, 1976) suggested that one of the ts lesions in this virus was in a structural polypeptide, since ts13 virions were unstable at 39°. Ts2203 was similarly analysed to determine if the virion instability was due to a lesion in the Vmw65 gene, rather than a secondary defect present in ts13.

Samples of HSV-2 HG52, ts2203 or ts13 virus stocks were incubated in plastic tubes at 40° for 0-8h, and

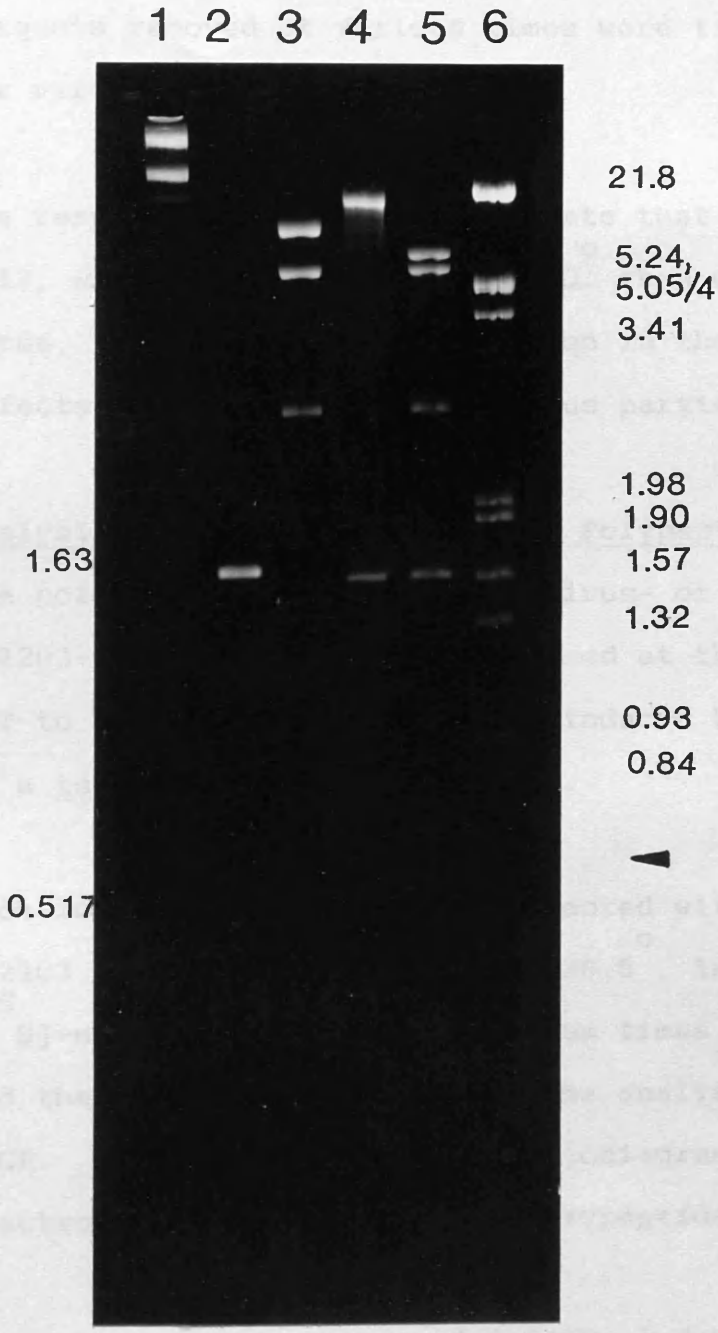
Figure 20

Restriction endonuclease digestions of pGZ73 (HG52 BglII i)

Samples were digested with BamHI (track 3), KpnI (track 4) and BamHI plus KpnI (track 5), and separated on a 1% agarose gel. A unique fragment of approximate size, 600bp, was generated by the BamHI/KpnI digest. pAT DNA digested with HinfI (track 2) and λ DNA, digested with EcoRI plus HindIII (track 6), were used as size markers.

Track 1 = undigested pGZ73.

Fragments were also separated on a 2% agarose gel, but no further fragments were detected.


1 2 3 4 5 6
21.8
5.24,
5.05/4.21
3.41
1.98
1.90
1.63
1.57
1.32
0.93
0.84
0.517

aliquots removed at various times were titrated at 31°C for virus infectivity.

The results (Figure 21) demonstrate that ts2203, like ts13, was more thermolabile at 40°C than wild type HG52 virus, indicating that a ts lesion in the Vmw65 gene affects the stability of the virus particle.

3.5 Analysis of ts2203-infected Cell Polypeptides

The polypeptide profiles of WT virus- or ts2203-infected cells were analysed at the PT and the NPT to identify any differences induced by the presence of a ts lesion in the Vmw65 gene.

Mock-infected cells or cells infected with HG52 or ts2203 were incubated at 31°C or 38.5°C, labelled with [^{35}S]-methionine for 1h at various times pi, harvested, and the radiolabelled polypeptides analysed by SDS PAGE. Figures 22 and 23 show autodiagrams of the electrophoretically separated polypeptides.

The polypeptide profiles of ts2203- and WT HG52 virus-infected cells at the PT and NPT were very similar, consistent with the suggestion that at the NPT the block to ts2203 infection occurs late in the virus growth cycle, after DNA replication. One noticeable difference, however, was that the Vmw65 induced by

Figure 21

Thermolability of the HSV virion

Virus samples containing 1×10^4 pfu per ml of HG52, ts2203 or ts13 were incubated at 40°. Aliquots were removed at various times, diluted, and virus titrated on BHK cells at 31°. Results are given as percentage (%) of virus infectivity at Oh.

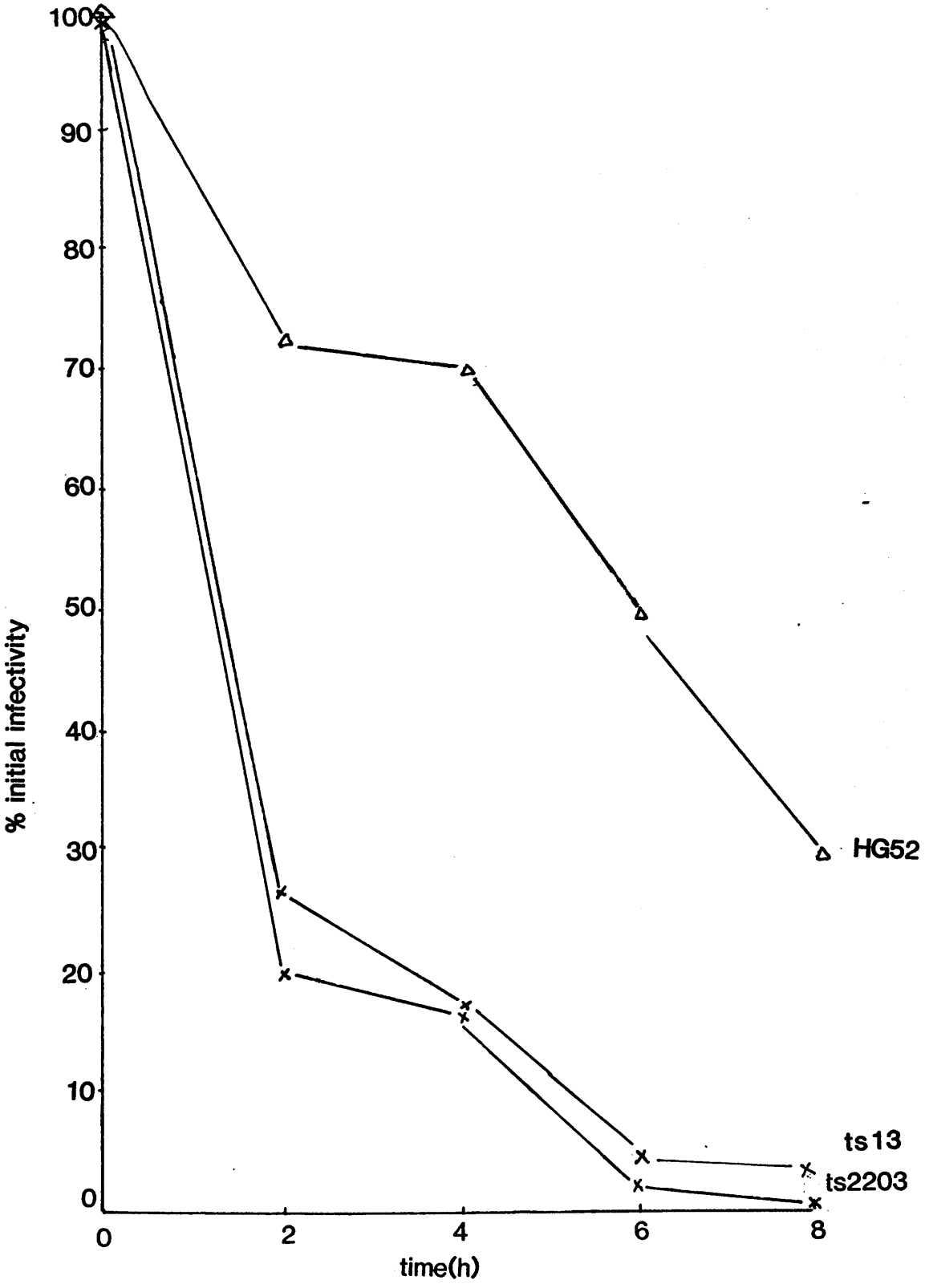

100
90
80
70
60
50
40
30
20
10
0
% initial infectivity
0
2
4
6
8
time(h)
HG52
ts13
ts2203

Figure 22

Autoradiogram of electrophoretically separated polypeptides from mock-and virus-infected cells incubated at the NPT

Cells were labelled with [^{35}S]-methionine from 2-3h (tracks 1-3) and 6-7h pi (tracks 4-6) and harvested. Protein samples were analysed on a 9% single concentration SDS-polyacrylamide gel.

mock-infected (MI) cell polypeptides (tracks 1, 4);
HG52-infected cell polypeptides (tracks 2, 6);
ts2203-infected cell polypeptides (tracks 3, 5).

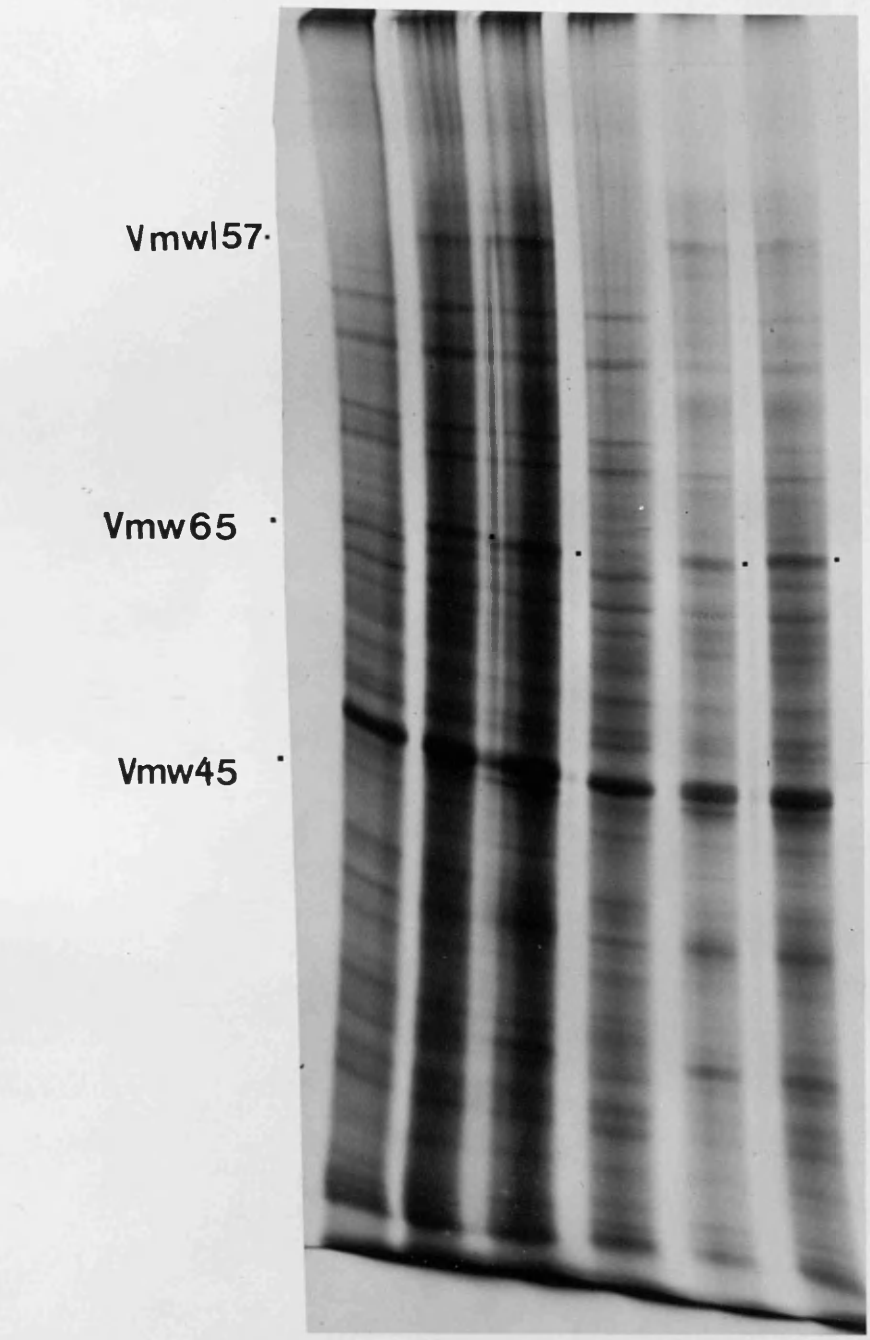
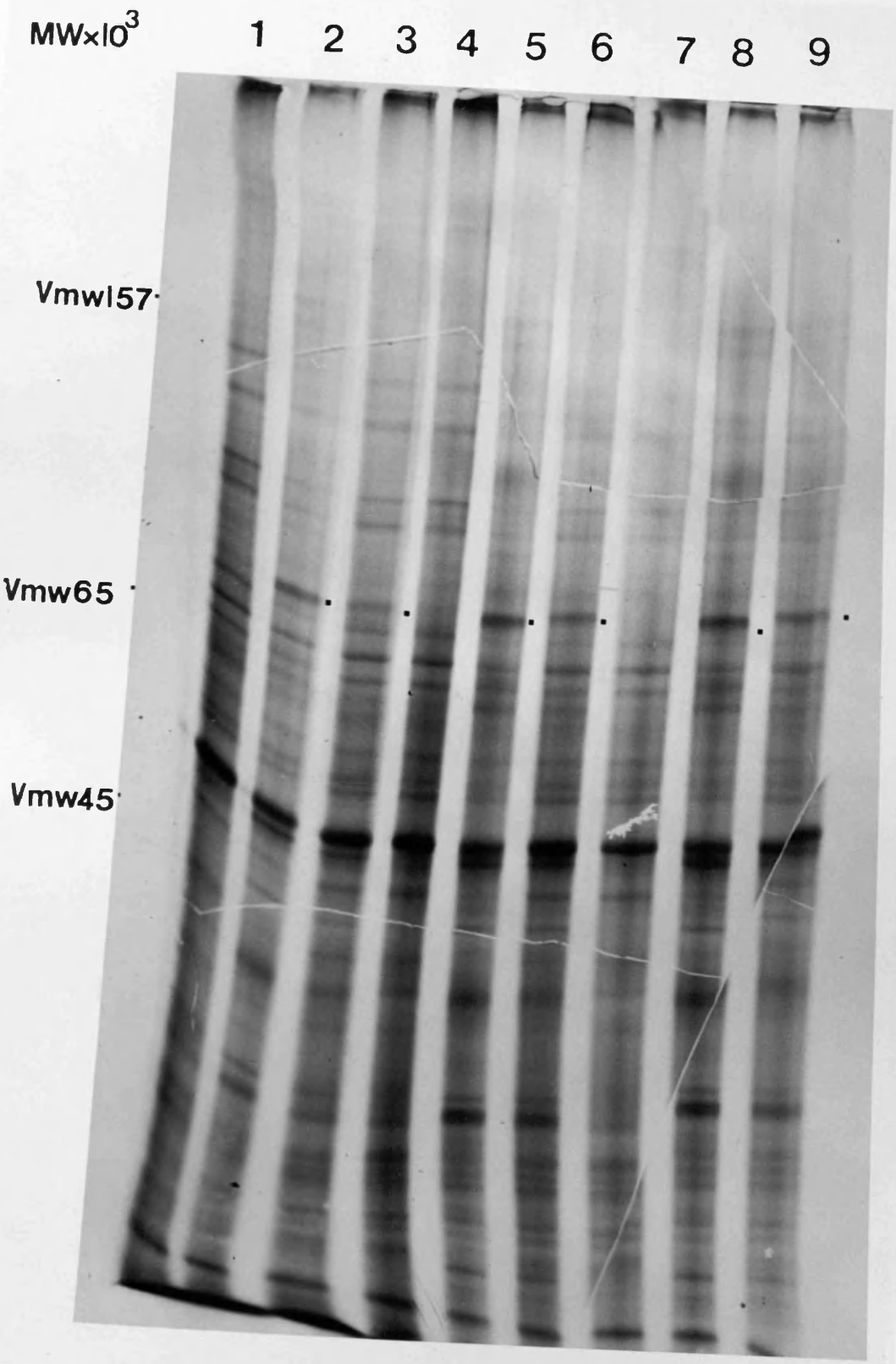

MW×10^3
1
2
3
4
5
6
Vmw157
Vmw65
Vmw45

Figure 23

Autoradiogram of electrophoretically separated polypeptides from mock-and virus-infected cells incubated at the PT

Cells were labelled with [^{35}S]-methionine from 4-5 (tracks 1-3), 6-7 (tracks 4-6) and 7-8 (tracks 7-9)h pi and harvested. Protein samples were analysed on a 9% single concentration SDS-polyacrylamide gel (SDS Page).

mock-infected (MI$^{\bullet}$) cell polypeptides (tracks 1, 4, 7);
ts2203-infected cell polypeptides (tracks 2, 5, 8);
HG52-infected cell polypeptides (tracks 3, 6, 9).


MW×10³
1
2
3
4
5
6
7
8
9
Vmw157
Vmw65
Vmw45

ts2203 at both 31° and 38.5° migrated slightly faster than the WT species. This finding is in agreement with earlier results of Moss and Palfreyman, who also found that ts13 Vmw65 had a lower apparent MW than HG52 WT Vmw65.

3.6 Analysis of Vmw65 Synthesised by ts2203

Ts2203 Vmw65 is recognised by the monoclonal antibody, LPI, specific for Vmw65.

As mentioned earlier, previous work by Moss and Palfreyman (personal communication) suggested that the structural defect in ts13 was located within the Vmw65 gene. This conclusion was based on the finding that MA1044, the type-common monoclonal antibody to Vmw65 immunoprecipitated WT HG52 and ts13 ts+ revertant Vmw65, but not the Vmw65 synthesised by ts13 at the PT or the NPT. In addition, these workers noted that the ts13 Vmw65 synthesised at both these temperatures had an aberrant mobility on SDS PAGE.

Analysis of polypeptides induced by ts2203 (Figures 22 and 23) demonstrated that the Vmw65 synthesised at both the PT and NPT by this virus had an altered mobility on SDS PAGE, in agreement with the findings of Moss and Palfreyman, suggesting that the production of an

aberrant polypeptide was due to the presence of a lesion in Vmw65 gene rather than an alternative location. To further examine this, $\underline{ts}^{+}$ revertants of $\underline{ts}$2203 were analysed for the synthesis of an altered Vmw65.

Immune precipitation experiments using a second monoclonal antibody to Vmw65, LPI, were carried out on extracts prepared from cells infected with WT HG52, $\underline{ts}$2203 and each of five independently isolated $\underline{ts}^{+}$ revertants of $\underline{ts}$2203, and incubated at the PT or NPT. SDS PAGE of the immune precipitations revealed that LPI recognises Vmw65 synthesised by $\underline{ts}$2203 as well as that synthesised by each of the revertant viruses (Figures 24 and 25).

$\underline{Ts}^{+}$ rev-1, rev-2 and rev-4 Vmw65 had the same electrophoretic mobility as the WT polypeptide indicating that the $\underline{ts}$ mutation itself is responsible for the alteration in the apparent MW of Vmw65. However, $\underline{ts}^{+}$ rev-3 and rev-5 Vmw65 retained the aberrant mobility of the $\underline{ts}$2203 species, suggesting that these viruses retained the original mutation affecting Vmw65 mobility, and have a second mutation converting the virus to the $\underline{ts}^{+}$ phenotype.

Figure 24

Analysis of Vmw65 induced by HG52, ts2203 or ts$^+$ revertants of ts2203

Cells infected at 38.5^o with HSV-2 HG52, ts2203 or ts$^+$ revertants of ts2203, (ts$^+$ rev-1...rev-5), were labelled with [^{35}S]-methionine from 5-6h pi, and harvested into Zweig's buffer. Immunoprecipitation was carried out using monoclonal antibody LPI which is specific for Vmw65. Immunoprecipitated polypeptides were analysed on a 9% SDS polyacrylamide gel.

Tracks 1-8 = whole cell extracts of mock infected (MI) (track 1), HG52 (track 2), ts2203 (track 3), ts$^+$ rev-1...rev-5 (tracks 4-8 respectively) infected cells.

Tracks 9-16 = immunoprecipitations of MI (track 9), HG52 (track 10), ts2203 (track 11), ts$^+$ rev-1...rev-5 (tracks 12-16 respectively), virus infected cell polypeptides.

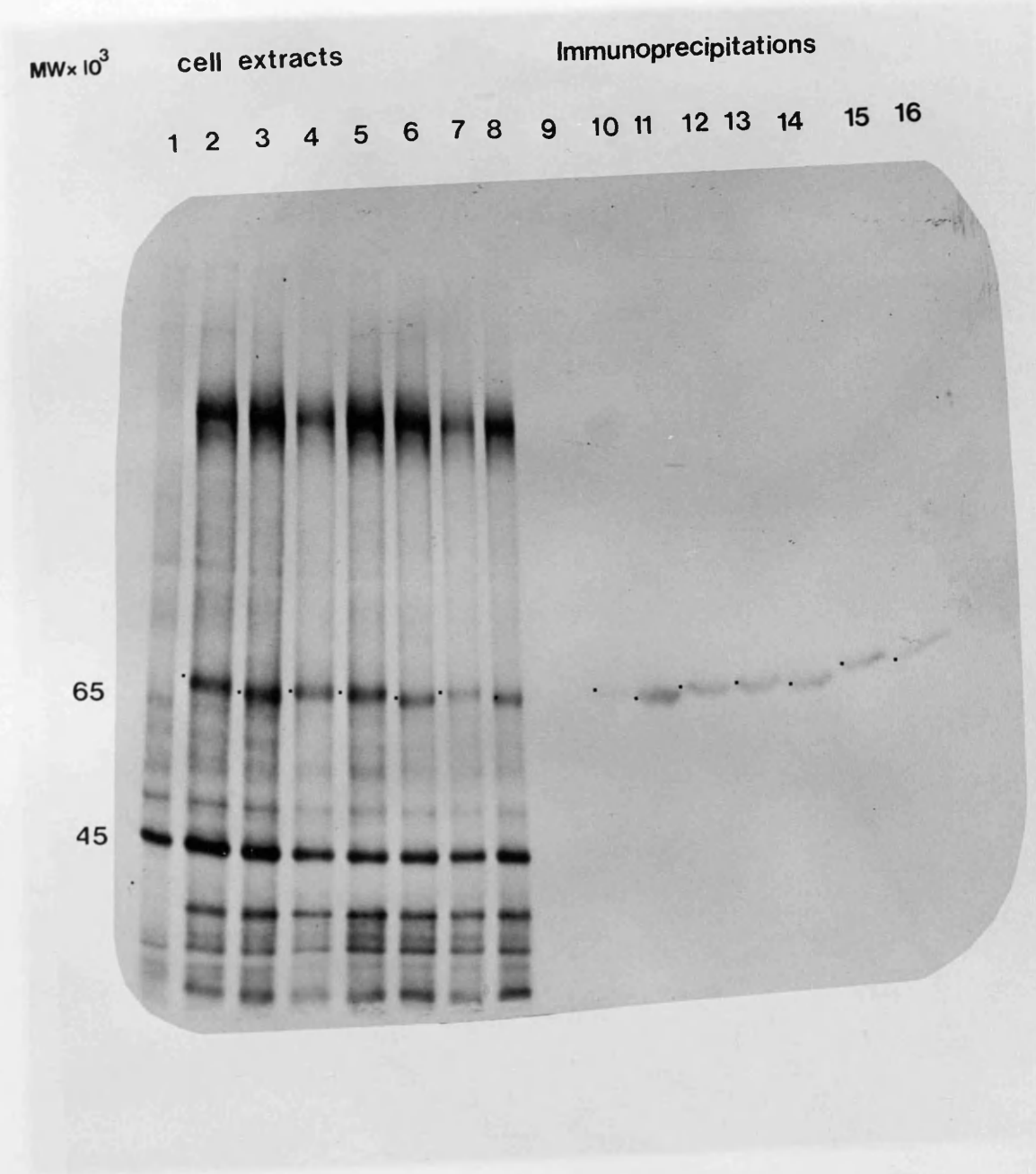

MW× 10³
cell extracts
Immunoprecipitations
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
65
45

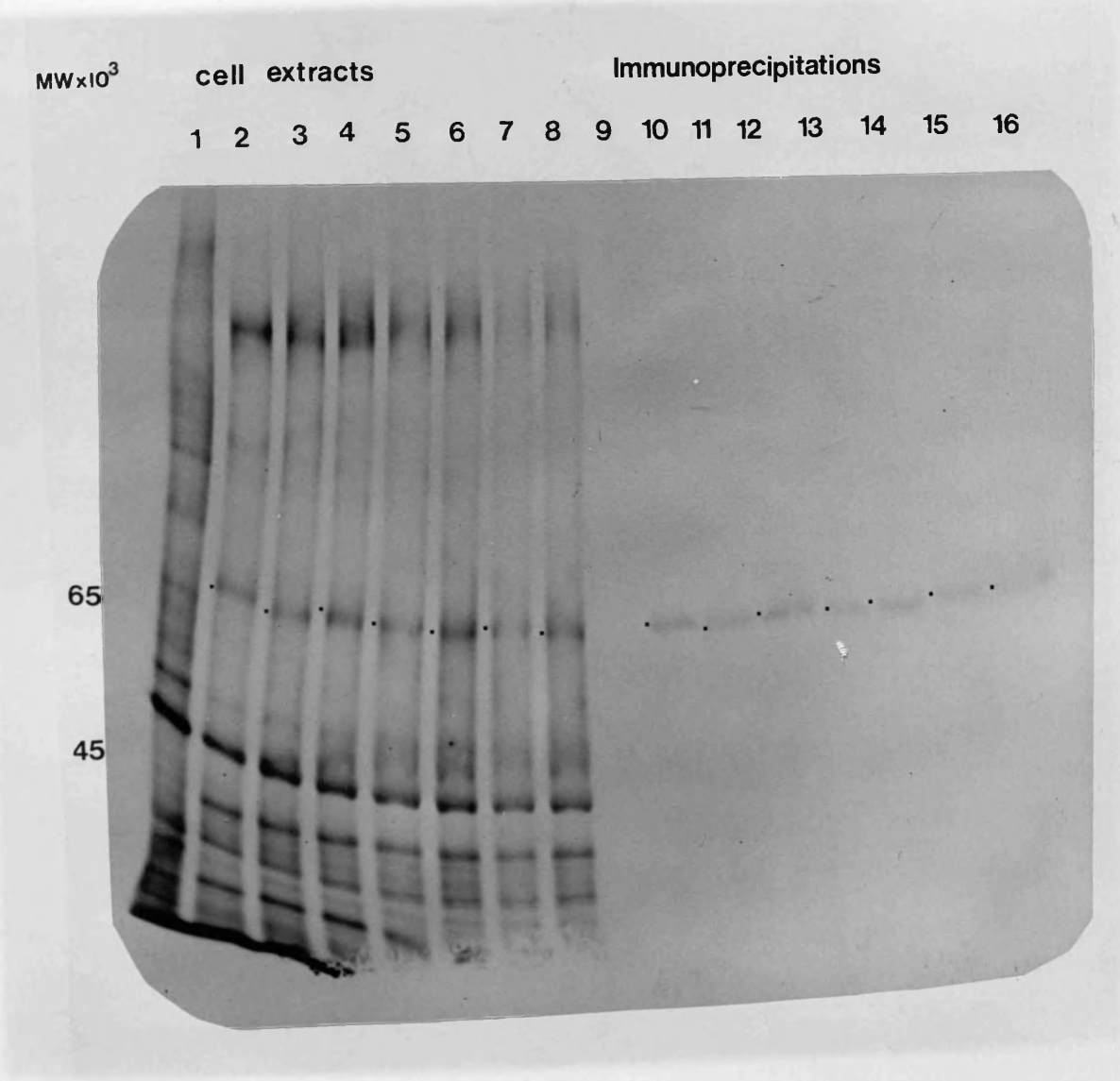
Figure 25

Analysis of Vmw65 induced by HG52, ts2203 or ts^{+} revertants of ts2203

Cells infected at 31^{o} with HSV-2 HG52, ts2203 or ts^{+} revertants of ts2203, (ts^{+} rev-1...rev-5), were labelled with [^{35}S]-methionine from 5-6h pi, and harvested into Zweig's buffer. Immunoprecipitation was carried out using monoclonal antibody LPI which is specific for Vmw65. Immunoprecipitated polypeptides were analysed on a 9% SDS polyacrylamide gel.

Tracks 1-8 = whole cell extracts of mock infected (MI) (track 1), HG52 (track 2), ts2203 (track 3), ts^{+} rev-1...rev-5 (tracks 4-8 respectively) infected cells.

Tracks 9-16 = immunoprecipitations of MI (track 9), HG52 (track 10), ts2203 (track 11), ts^{+} rev-1...rev-5 (tracks 12-16 respectively), virus infected cell polypeptides.


MW x10³
cell extracts
Immunoprecipitations
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
65
45

It can be concluded, therefore, since LPI recognises ts2203 Vmw65, as well as the WT and ts+ revertant Vmw65, that the epitope recognised by this antibody is not affected by the ts mutation.

3.7 Cellular Location of Vmw65

Immunofluorescence assays were performed to determine the intracellular location of Vmw65 synthesised by HG52, ts2203 and ts+ revertants of ts2203.

Hfl cells were infected at the PT or NPT for 6-8h, then fixed and permeabilised, and incubated with monoclonal antibody LPI and fluorescein, or control serum. Cells were examined for fluorescence under UV light (Figure 26).

In WT-, ts2203- and ts+ 2203 revertant-infected cell samples, grown at either the PT or NPT, Vmw65 was distributed throughout the cell cytoplasm and nucleus, although more intense immunofluorescent staining was observed in the nucleus. Since Vmw65 is present in the nucleus and is a component of the capsids prior to envelopment, it is not surprising that it is predominantly nuclear in location. Moreover, the results suggest that the localisation of ts2203 Vmw65 within the cell at the NPT is unaffected by the

Figure 26

Localisation of Vmw65 in ts2203, HG52 and ts^+ revertant virus-infected cells

Immunofluoresence at 38.5°C Monolayers of Hfl cells were mock infected (MI) or infected with 5pfu HSV-2 HG52, ts2203 or ts^+ rev-1, rev-2, rev-3, rev-4 or rev-5. Cells were harvested at 10h pi and prepared for immunofluorescence assay with

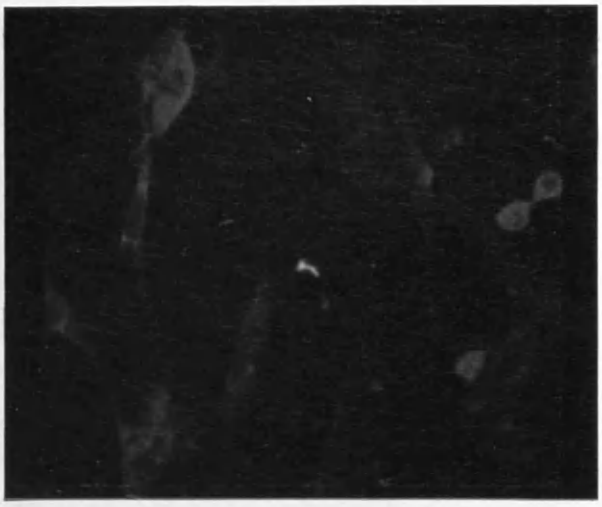
a. LPI, a type common monoclonal antibody specific for Vmw65 or
b. rabbit control serum (RCS).

ts^+ revertant viruses were indistinguishable in their reactions to the antibodies, and ts^+ rev-1 only is shown here.

MI Cells + LPI (1), + RCS (2); HG52-infected cells + LPI (3), + RCS (4); ts2203-infected cells + LPI (5), + RCS (6); ts^+ 2203 rev-1 infected cells + LPI (7), + RCS (8). Samples were identical at 38.5°C and 31°C, therefore 38.5°C samples alone are shown here.

1

2



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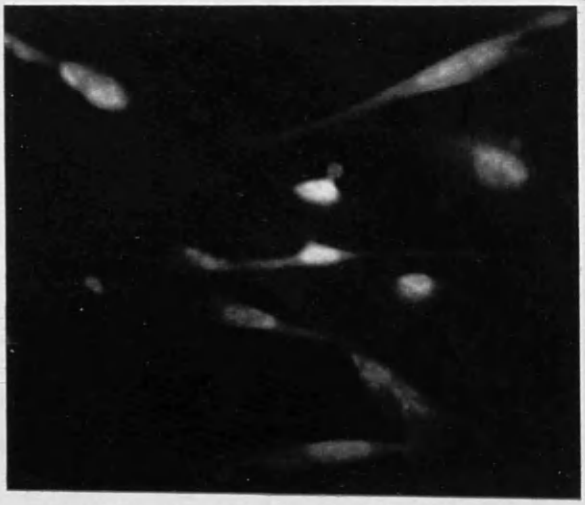


4

5

6

7

8

ts lesion. It is, therefore, likely that the lesion in the gene directly affects the ability of Vmw65 to participate in virion assembly.

3.8 Ts2203 BglII i Stimulates IE Gene Transcription at the PT and NPT

Previous experiments have shown that HSV-1 Vmw65, encoded within HSV-1 sequences present in pMC1 (Campbell et al., 1984) can trans-activate HSV IE gene transcription in vitro. From the marker rescue data, cross-hybridisation studies and immunological evidence, it appears that the ts lesion in ts2203 is located within the Vmw65 gene. It was, therefore, of interest to determine whether the ts2203 defect affected IE gene transcription at the NPT.

Preliminary experiments (M Dalrymple, 1986) suggested that the ts lesion in ts13 BglII i did not impair the ability of Vmw65 to stimulate TK expression from plasmid pTKN2, a chimaeric plasmid in which the HSV TK coding sequences were linked to the IE175 promoter sequences (Section 4.1, Figure 44). Using a short term cotransfection assay, plasmids pGZ73 (containing WT HSV-2 BglII i) and pGZ74 (containing ts13 BglII i) were compared with pMC1 for the ability to stimulate TK activity from pTKN2.

All three DNA clones stimulated TK expression at 31°C and 38.5°C (Table 7), confirming that the ts lesion in ts2203 does not affect the transcriptional function of Vmw65.

3.9 Electron Microscope Analysis of ts2203- and HG52-infected Cells Grown at the PT and NPT

Defects in capsid assembly or viral DNA encapsidation can easily be detected using the electron microscope, since empty, partially cored and dark-staining capsids containing DNA can be clearly differentiated under the electron microscope. Therefore, to determine whether the ts2203 lesion affected virus assembly, samples of virus-infected BHK cells grown at the PT or NPT were examined under the electron microscope and compared with WT virus-infected cell samples.

At 31°C, in both HG52 and ts2203-infected cells, large quantities of capsids, including numerous fully cored capsids, and enveloped virus particles, were visible. However, although both HG52 and ts2203 synthesised numerous capsids at the NPT, very few of the capsids had dense cores (Figure 27, Table 12). This suggested that both viruses packaged DNA inefficiently at the NPT, and in addition, very few enveloped virus particles were observed.

Table 7

Stimulation of IE-TK expression by pGZ73, pGZ74 and pMC1

Each of the above 3 plasmids was assayed for the ability to stimulate TK activity when co-transfected into cells with pTKN2, containing the IE175 promoter linked to TK coding sequences.

The results are presented as $\frac{\text{cpm plasmid}}{\text{cpm pTKN2}}$

Table 7

Cloned HSV DNA	Level of Stimulation	
	31°C	38.5°C
pMC1	8.65	5.50
pGZ73	8.78	15.83
pGZ74	7.81	10.73

Figure 27

Electron microscopic analysis of HG52 and ts2203-infected cells

Cells were infected at a moi of 5pfu virus per cell at the NPT and uncubated for 18h. They were then harvested and embedded in epon resin. Thin section preparations of virus-infected cells were examined under the electron microscope. The bar represents 0.5um.

1,2 = ts2203-infected cells

3,4 = HG52-infected cells

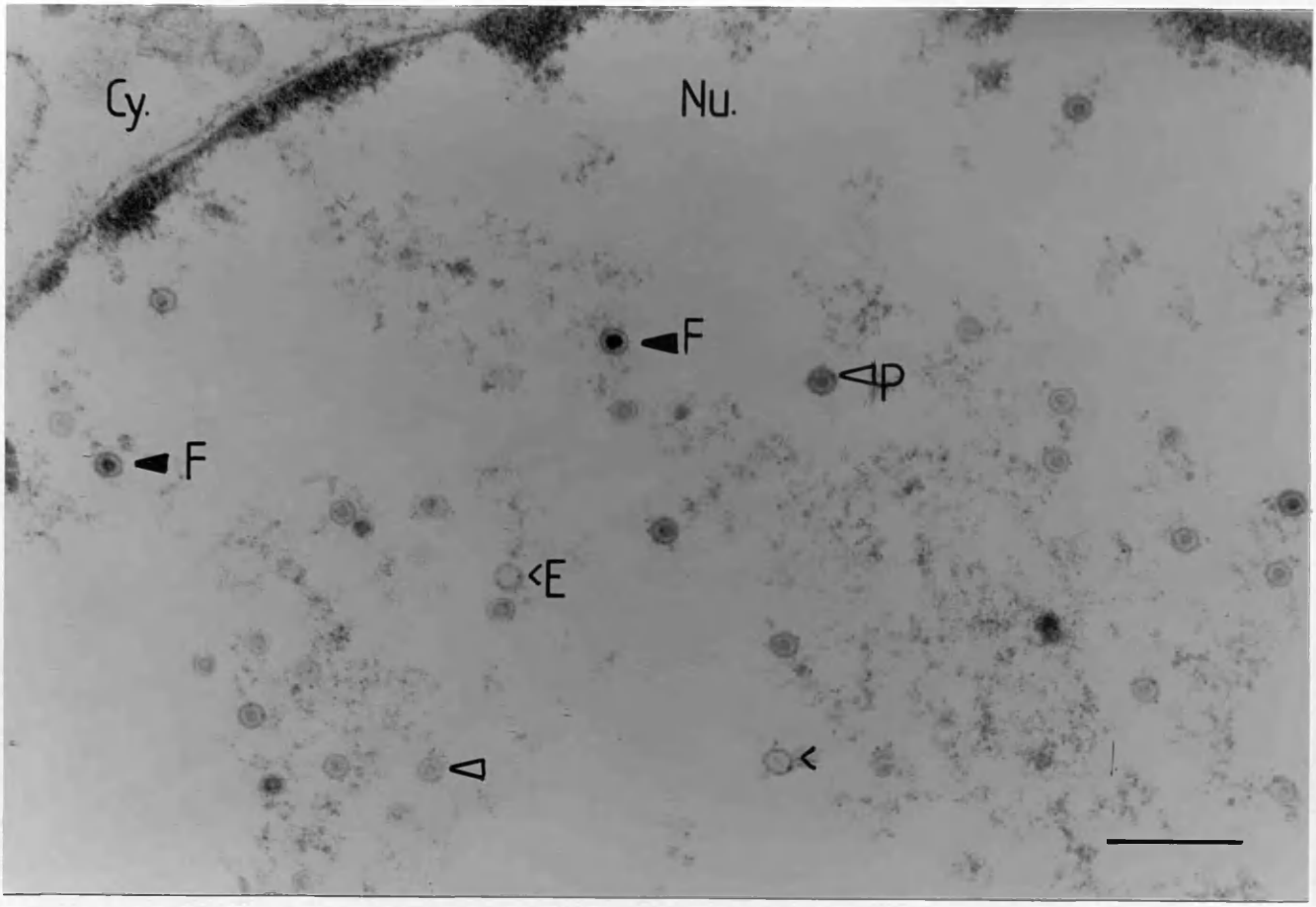
P = partially cored capsid ; ◁

F = dense-cored capsid ; ◀

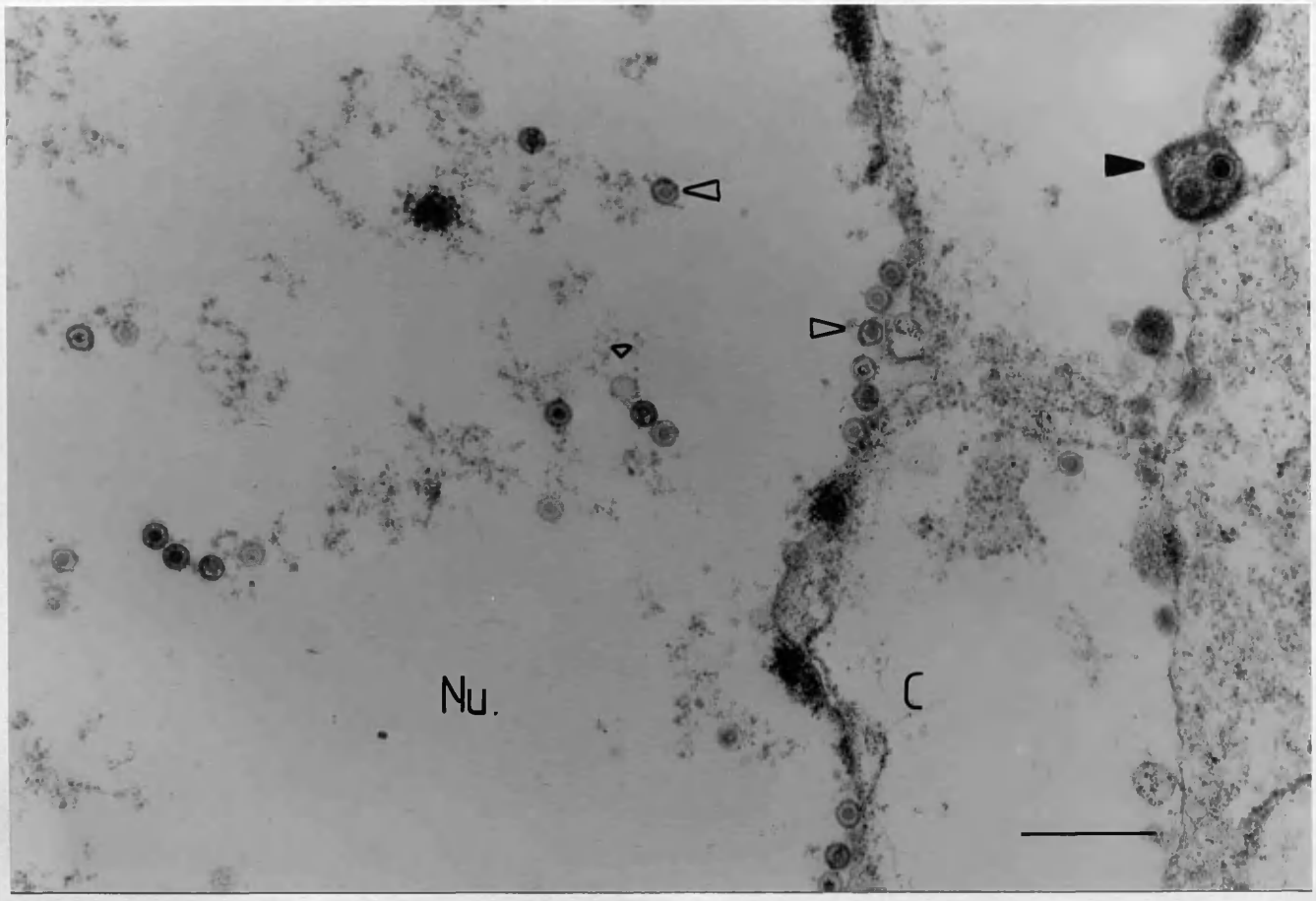
E = empty capsid ; ◁

Nu = nucleus; Cy = cytoplasm.

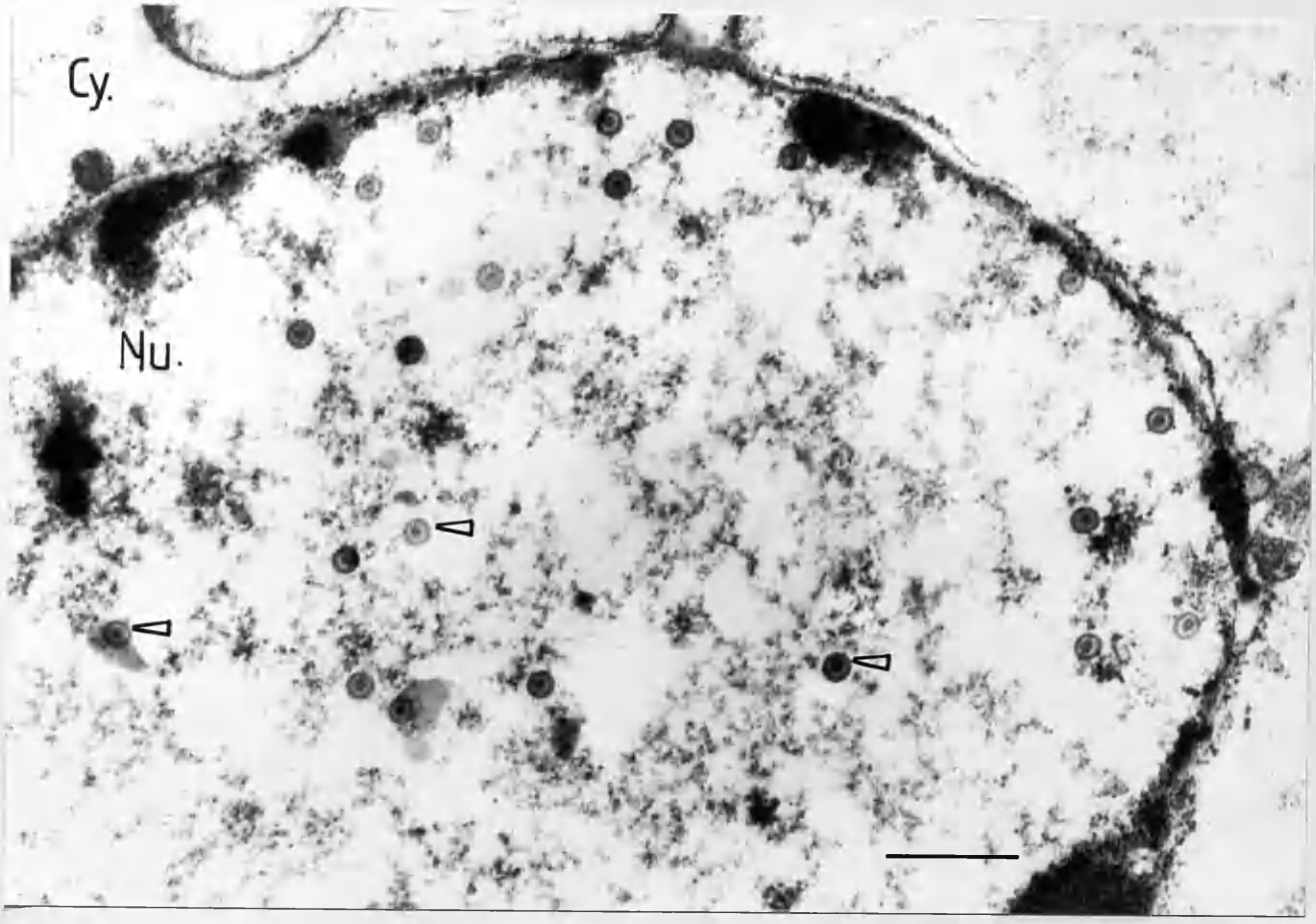
1



2



3



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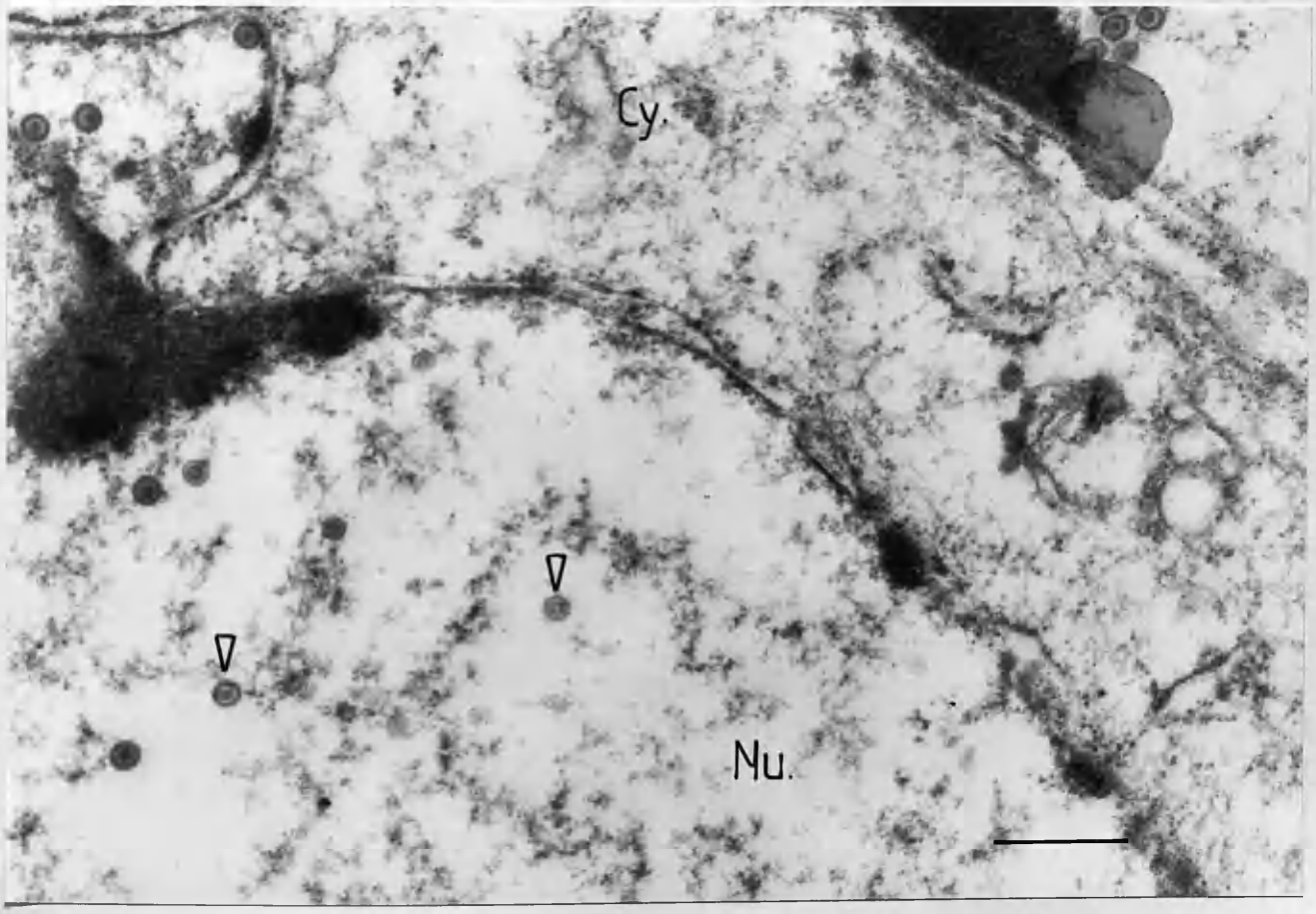


Therefore, although the results indicated that the lesion in ts2203 does not affect the synthesis of capsids, it was difficult to ascertain whether there were any differences between the mutant and wild type virus at the stage of viral DNA encapsidation or capsid envelopment.

3.10 Synthesis of DNA in HG52 and ts2203-infected Cells

The results from electron microscopic analysis suggested that both HG52 and ts2203 packaged DNA inefficiently at the NPT. One possible explanation for this finding is that the viruses synthesise reduced amounts of DNA at the NPT.

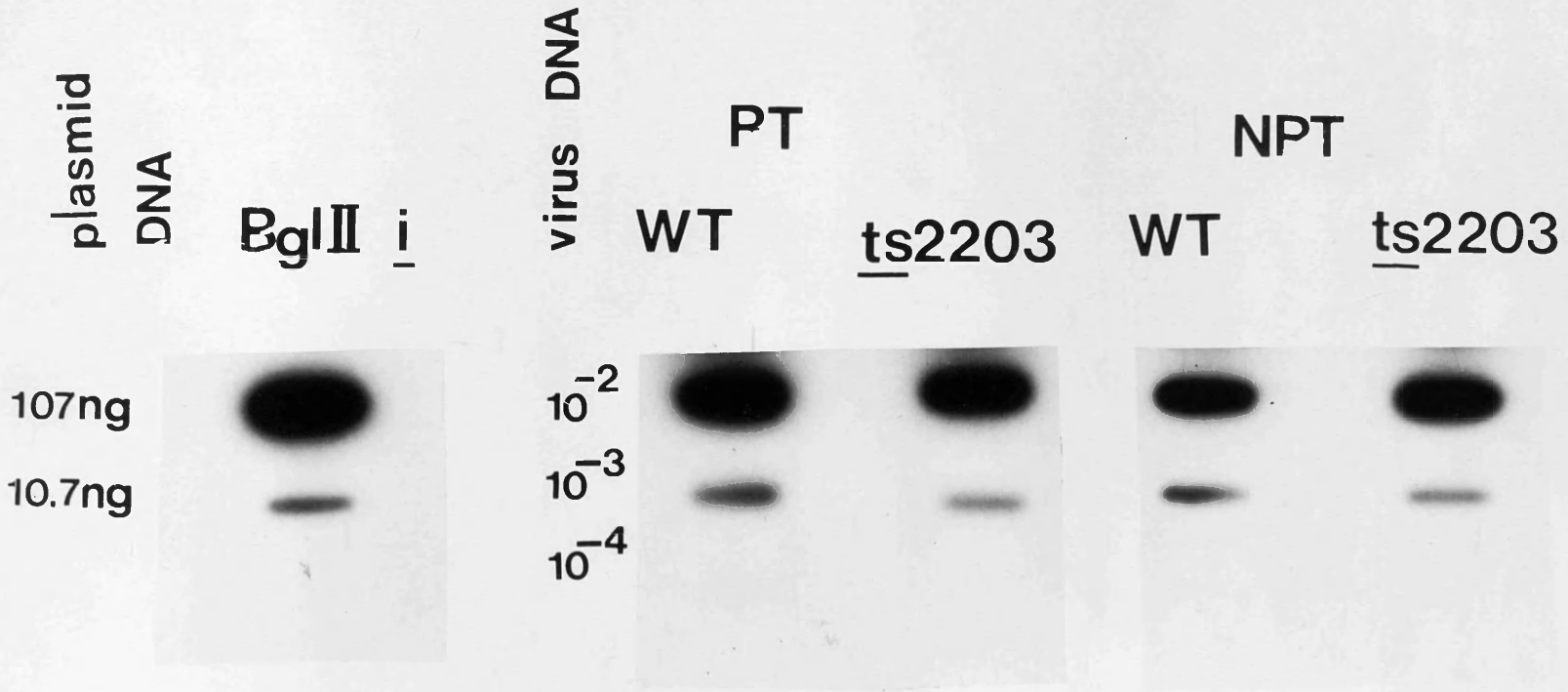
BHK cells were infected with ts2203 or HG52 at the PT or NPT and harvested at 18h pi. Serial dilutions of total virus-infected cell DNA were taken then loaded onto nitrocellulose membrane using a "slot-blot" apparatus. Known amounts of pGZ73 (HSV-2 BglII i) DNA were also loaded onto the membrane so that the numbers of genomes synthesised in WT and ts2203 infected cells could be determined. The DNA samples were then hybridised to [^{32}P]-labelled pGZ73 (Figure 28).

It was apparent from these results that HSV-2 and ts2203 synthesised similar quantities of DNA at both the

Figure 28

Autodiogram of DNA synthesized by HG52 and ts2203 at the PT and NPT

Dilutions (10^{-2}, 10^{-3}, 10^{-4}) of virus infected cell DNA and samples of pGZ73 (HG52 BglII i fragment) equivalent to 10^{2} and 10^{3} copies/cell ie. 10.70ng and 107.00ng pGZ73, were loaded onto the nitrocellulose membrane using a slot-blot apparatus. Samples were hybridised to [^{32}P]-labelled pGZ73.


plasmid DNA
BglII i
107ng
10.7ng
virus DNA
PT
WT
ts2203
NPT
WT
ts2203
10^-2
10^-3
10^-4

PT and NPT, equivalent to approximately 10^5 genome copies per cell as assessed by densitrometric analysis (Table 8). Ts2203 does not, therefore, have a defect in viral DNA synthesis unlike ts13, the virus from which it was derived (Moss et al, 1979; Moss, 1986).

3.11 Processing of Viral DNA in ts2203- and HG52-infected Cells

An analysis of the amount of viral DNA synthesised by ts2203 and HG52 showed that both viruses made similar amounts of DNA at the NPT and the PT. It was, therefore, likely that HG52 and ts2203 were ts for encapsidation of DNA, and experiments were performed to assess the ability of ts2203 and HG52 to cleave and package unit lengths of viral DNA, from concatemers, at 38.5^{o}.

Total virus-infected cell DNA and encapsidated viral DNA samples were prepared from ts2203- or HG52-infected cells incubated at the PT or NPT for 18h. The viral DNA was then analysed by the Southern bloting technique. DNA samples were digested with BamHI, which cleaves the long and short repeat sequences as well as the unique sequences. The fragments were separated on an agarose gel, transferred onto a nitrocellulose membrane and hybridised to [^{32}P]-labelled pGZ1. This

Table 8

Densitometric analysis of [^{32}P]-labelled virus-infected cell DNA

Densitometric analysis was made of an autoradiogram of DNA extracted from HG52- and ts2203-infected cells incubated at the PT and NPT (Figure 28).

The approximate number of genome copies per cell per 50mm dish was determined by a comparison with the equivalent of 10^3 copies/cell/50mm dish of pGZ73 (containing HSV-2 BglII i DNA fragment).

Table 8

Copy number: genomes/cell/50mm plate

	31°C	38.5°C
HSV-2 HG52	10^4	8×10^4
ts2203	10^4	8×10^4

contains the joint-spanning fragment of HG52, BamHI g, thus giving a measure of the proportion of BamHI g joint fragment to the terminal fragments BamHI v and u (which also hybridise to BamHI g) (A map of BamHI restriction sites in HSV-2 is given in Figure 30). An estimate can therefore be made of the amount of viral DNA cleavage and packaging.

Figure 29 shows an autoradiogram of a Southern blot of BamHI-digested total virus DNA and encapsidated DNA. As anticipated from the previous "slot-blot" hybridisation analysis of viral DNA present in ts2203- and HG52-infected cells, HG52 and ts2203 synthesised similar amounts of viral DNA at the PT and NPT. However, it was clear that at the NPT, both HG52 and ts2203 DNA samples contained low amounts of the terminal fragments BamHI v and u, suggesting that most of the viral DNA was "endless".

Analysis of encapsidated viral DNA confirmed that both HG52 and ts2203 packaged viral DNA inefficiently at the NPT. Although in three independent experiments ts2203 packaged less DNA than HG52 at the NPT, ts2203 encapsidated DNA was always detectable. It was, therefore, difficult to determine whether the ts2203 lesion within Vmw65 affected this stage of capsid maturation or a later stage such as envelopment. Alternatively, ts2203 Vmw65 may simply be "leaky" at 38.5°, and able to function to a limited extent.

Figure 29

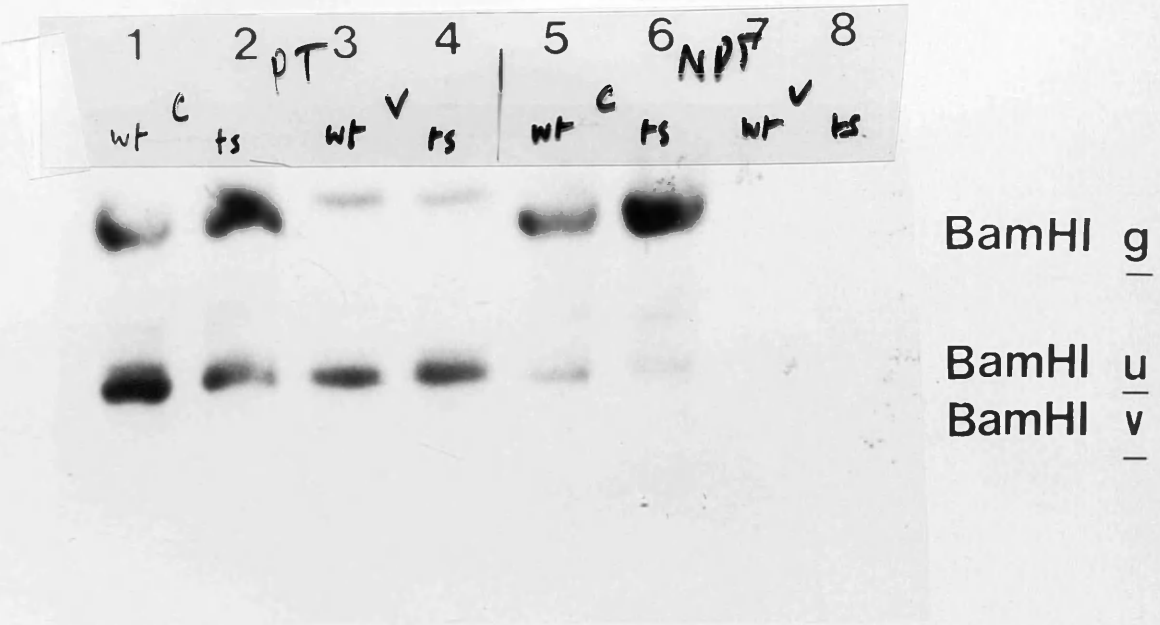
The processing of viral DNA in ts2203- and HG52-infected cells

Total virus-infected cell DNA and DNase-resistant (packaged) DNA were purified from virus-infected cells incubated at the PT or NPT for 18h.

1/20 total virus-infected cell DNA and 1/10 DNase-resistant DNA samples were digested with BamHI and the fragments separated by agarose gel electrophoresis. The fragments were transferred to nitrocellulose and hybridised to [^{32}P]-labelled pGZ1 which contains HG52 BamHI g. An autoradiogram was made of the Southern blot.

a. HG52- and ts2203-infected cell DNA (PT) [tracks 1,2 respectively]; HG52- and ts2203 encapsidated DNA (PT) [tracks 3,4]; HG52- and ts2203-infected cell DNA (NPT) [tracks 5,6]; HG52- and ts2203 encapsidated DNA (NPT) [tracks 7,8].

b. represents a longer exposure of the tracks shown in a.


1
2
pT
3
4
5
6
NPT
7
8
wt
c
ts
wt
v
ts
wt
c
ts
wt
v
ts
BamHI g
BamHI u
BamHI v

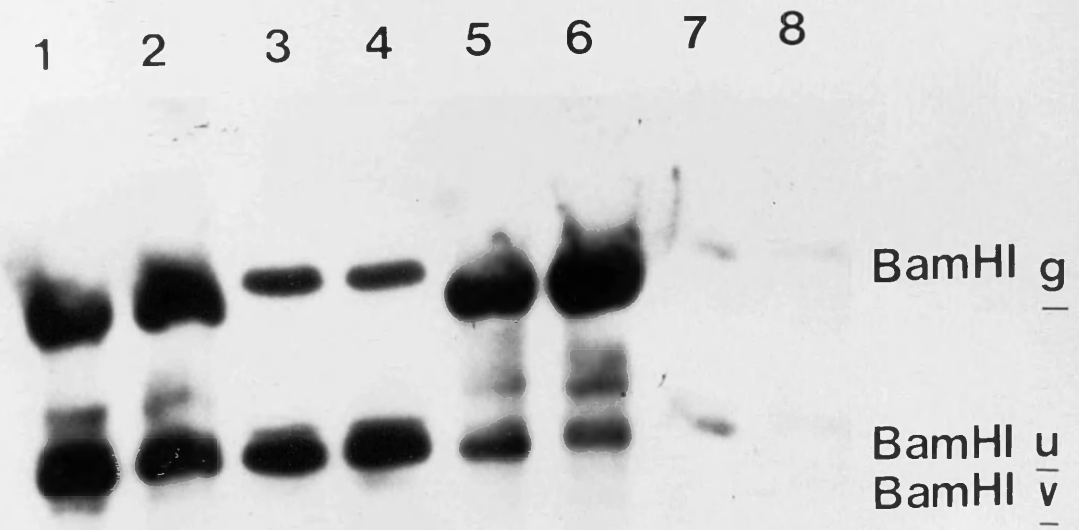

1
2
3
4
5
6
7
8
BamHI g
BamHI u
BamHI v

Figure 30

Physical map of HSV-2 for restriction endonuclease BamHI, and an autoradiogram of [^{32}P]-labelled HSV-2 DNA disgested with BamHI

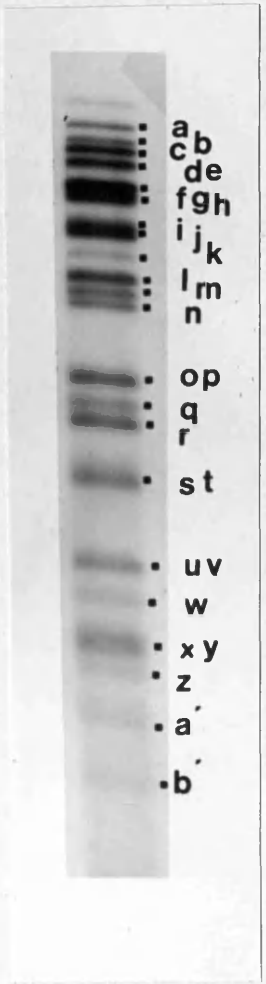
(a) [^{32}P]-labelled DNA samples were digested with BamHI and separated on a 1% agarose gel. The letters refer to specific HSV-2 DNA fragments, and the physical map locations of these are shown in (b). The autoradiogram was supplied by Dr V.G. Preston.

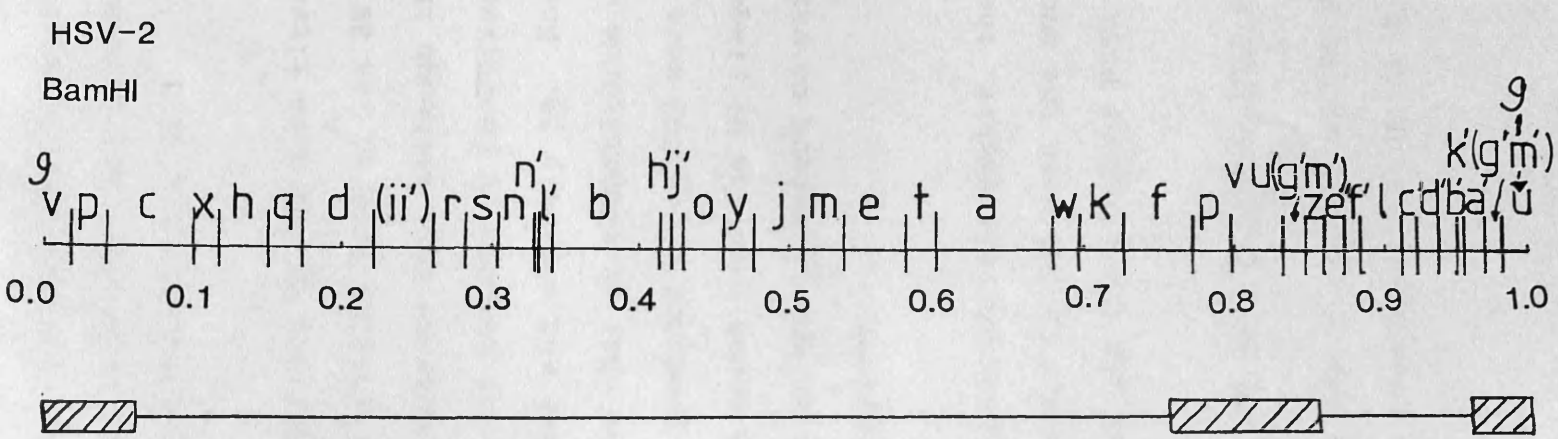
(b) Physical map of HSV-2 DNA for restriction endonuclease BamHI, taken from Davison (1981).

Composition of the joint spanning fragment g=v+u

a

b


a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x
y
z
a'
b'


HSV-2
BamHI
g
v p c x h q d (ii') r s n n' l' b h'j' o y j m e t a w k f p v u(g'm') z e f l c d' b' a' k'(g'm') u
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

3.12 Temperature Sensitivity of HSV-2 HG52 and ts2203

Since HG52 and ts2203 packaged DNA inefficiently at the NPT, it was important to determine whether the NPT could be lowered to a temperature at which ts2203 was still ts, but at which HG52 packaged DNA efficiently. Therefore, an assessment was made of virus growth at various temperatures below 38.5°.

Stocks of HG52 and ts2203 were titrated at various temperatures ranging from 31° to 38.5° (Table 9). The results demonstrate that although the titre of ts2203 below 38.5° was reduced in comparison with WT virus, the defect was not entirely ts. However, it should also be noted that at temperatures of 36.4° and above, the plaques produced by ts2203 were smaller and more difficult to count than the WT plaques, presumably due to an effect of the ts defect on virus encapsidation and/or envelopment.

As a consequence of this result, the NPT could not be reduced in order to increase the amount of DNA encapsidation and the yield of HG52 virus.

3.13 Transfer of the ts13 Mutation into HSV-2 Strain HVD25766

One possible way of overcoming the problem described above was to recombine ts13 BglII i into a different

Table 9

Plaque-formation of HG52 and ts2203 at various temperatures

Virus stocks were titrated at temperatures ranging from 31 °C (PT) to 38.5 °C (NPT).

Table 9

Incubation Temperature	HG52	ts2203
31°C	8.0×10^8	1.0×10^8
35.6°C	1.0×10^9	9.0×10^7
36.4°C	9.0×10^8	6.0×10^7
37.5°C	2.0×10^9	2.3×10^5
38.0°C	1.0×10^9	2.0×10^5
38.5°C	5.0×10^8	$<10^3$

HSV-2 strain. The strain HVD25766 was reported to grow well at 38.5°, and to produce large quantities of cell-released infectious virions. Preliminary results confirmed this: electron microscopic analysis of thin section preparations of HVD-infected cells, grown at 38.5° for 18h, showed that HVD synthesised large quantities of dense-cored capsids and enveloped particles. In contrast, samples of HG52-infected cells grown under the same conditions contained few dense-cored capsids, and very little enveloped virus (Figure 31, Table 12). Single step growth of HVD confirmed that the virus grew well at 38.5° (Figure 32). In addition, the HVD virus produced large amounts of cell released (CR) and cell associated (CA) virus at low moi at 31°, which had equivalent titres. The CA Virus had a titre 5 to 10 fold higher than an HG52 CA stock grown at 31°.

In view of these results the ts13 mutation in the BglII i DNA fragment was transferred into HVD25766 virus.

Intact HVD25766 WT virus DNA and pGZ74 (containing ts13 BglII i) cleaved with BglII, were cotransfected into BHK cells, and the resulting virus progeny titrated at 31° under agar. Well isolated plaques formed at 31° were picked, and the virus assayed at 31° and 38.5° for temperature-sensitivity. Potential ts virus isolates

Figure 31

Electron microscopic analysis of HG52 and HVD infected cells

BHK cells were infected at a moi of 5pfu virus per cell and incubated at the NPT for 18h. Cells were harvested, fixed and dehydrated, then embedded in epon resin. Thin sections of infected cells were stained and examined under the electron microscope. The bar represents 0.5um.

HG52-infected cells at 18h pi (1); HVD-infected cells at 18h pi (2).

Dense-cored capsids:

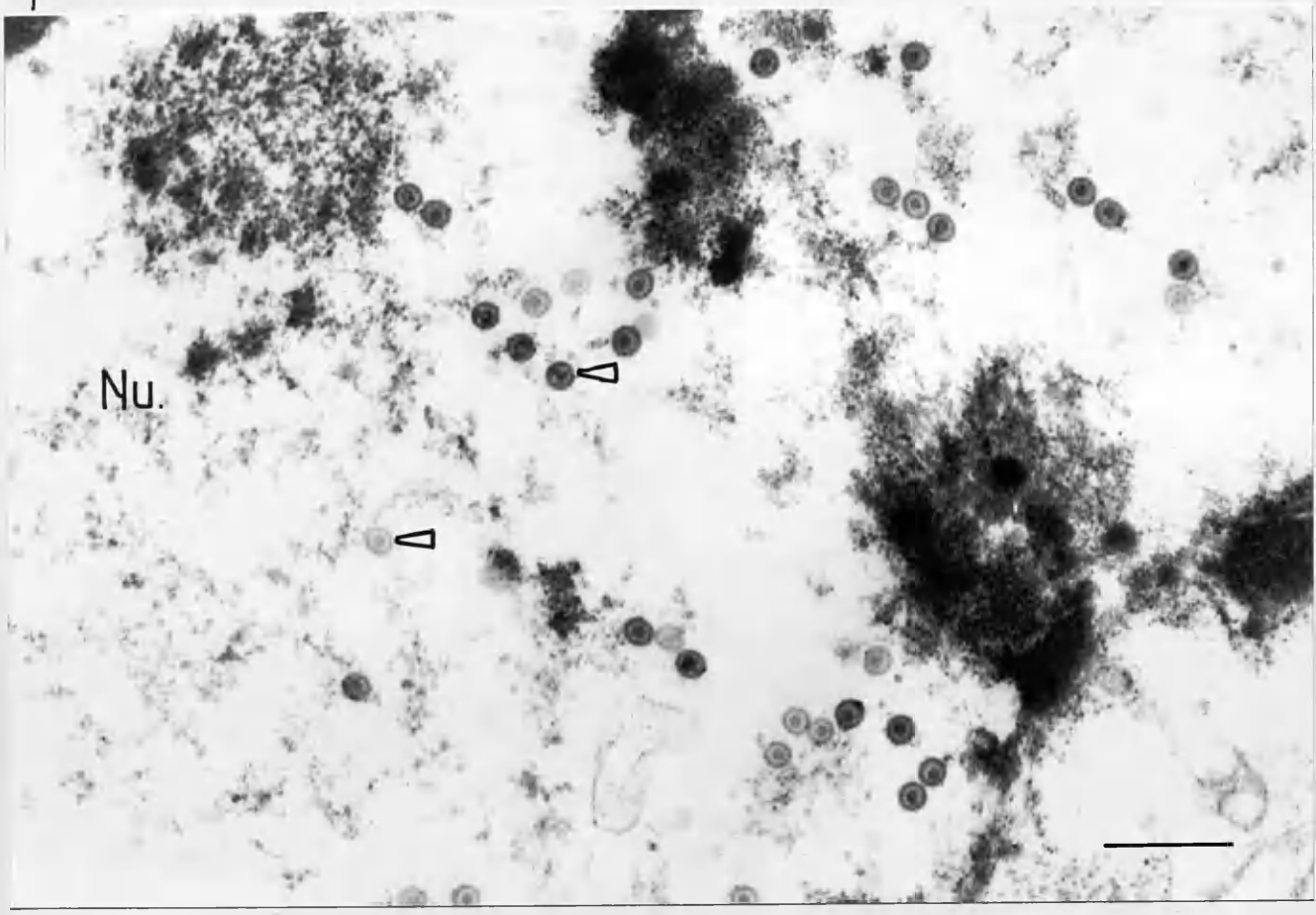
Partially-cored capsids:

Empty capsids:

Virions:

Nu; nucleus; Cy; cytoplasm

1



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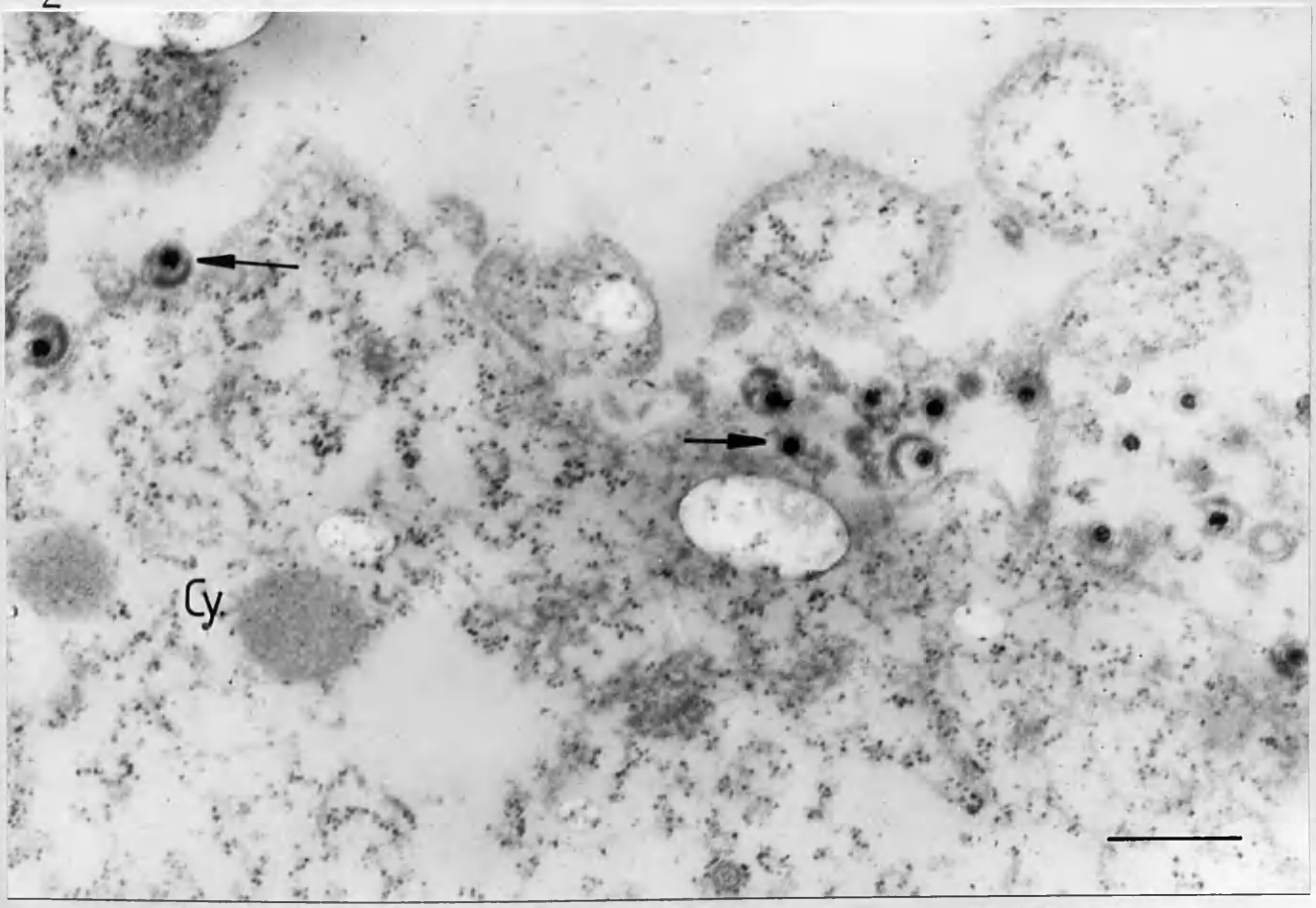
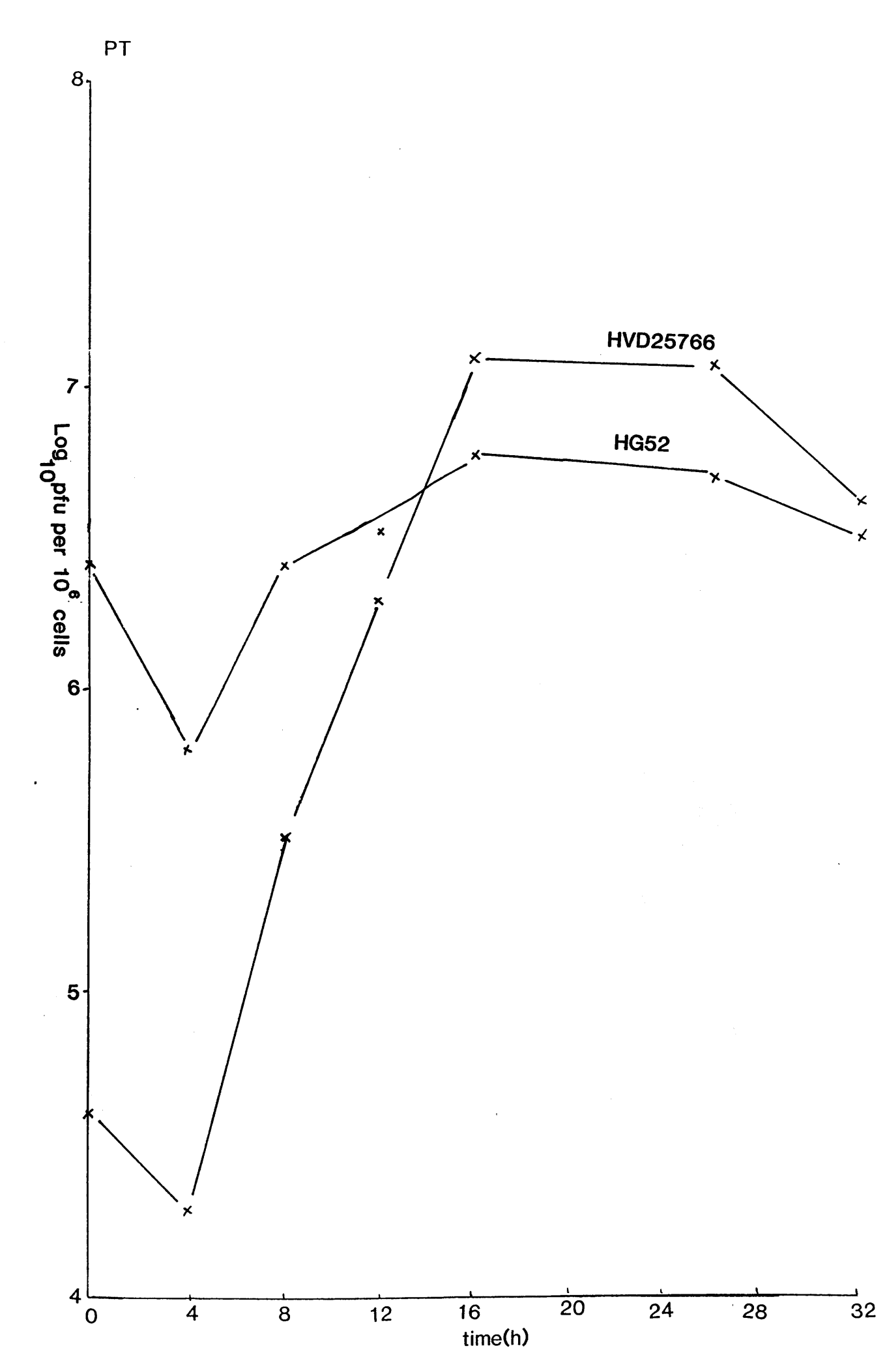
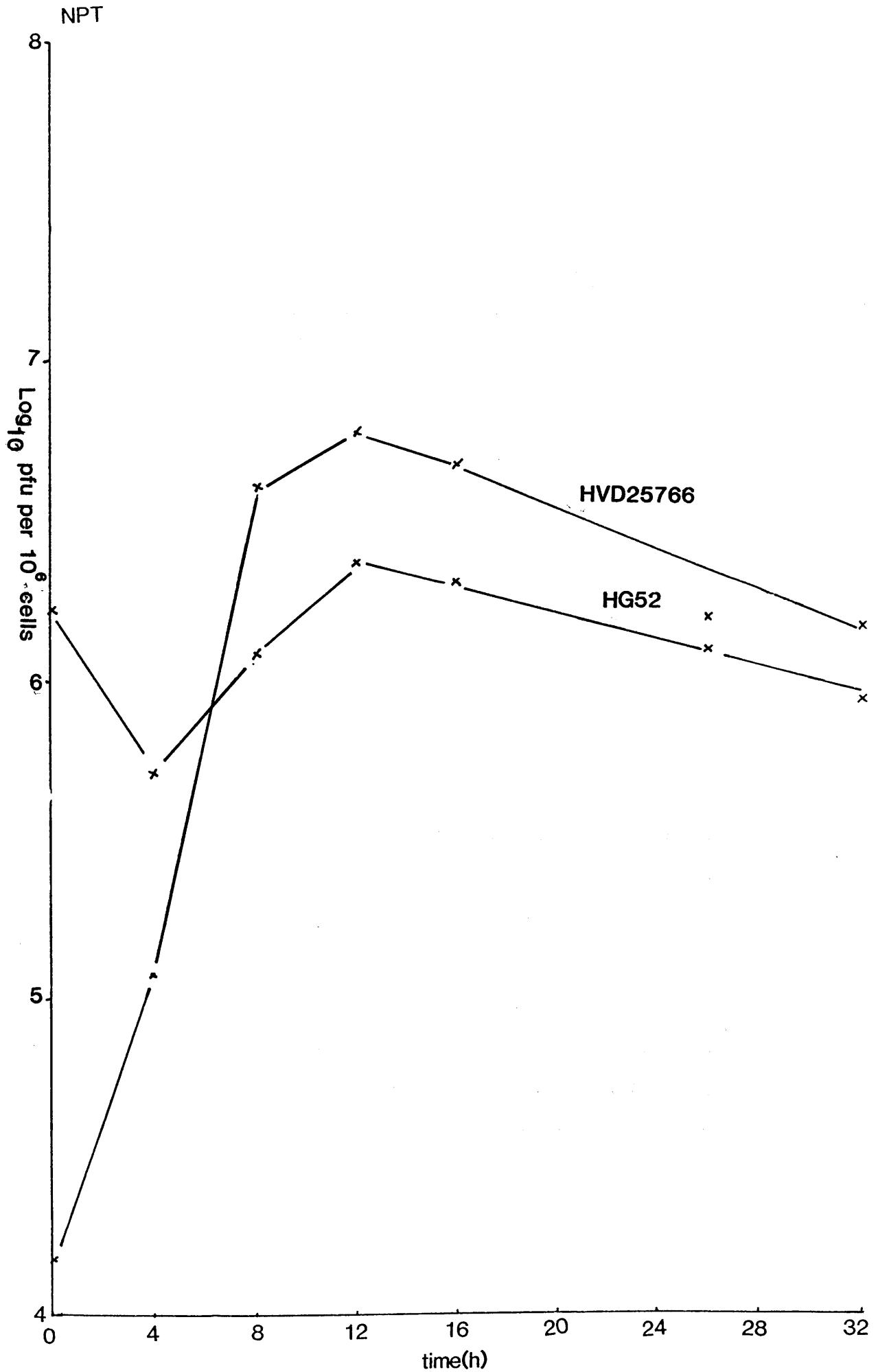


Figure 32

Single cycle growth of HG52 and HVD 25766 at the PT and NPT

Subconfluent monolayers of cells in 30mm dishes were infected at the PT and NPT with CA stocks of virus at a moi of 5pfu per cell. Cells were then harvested at various times pi and the virus yield was titrated on BHK cells at the PT (Section 2).


PT
8
7
6
5
4
HVD25766
HG52
Log10pfu per 10^6 cells
0
4
8
12
16
20
24
28
32
time(h)


NPT
8
7
6
5
4
Log10 pfu per 10^8 cells
HVD25766
HG52
0
4
8
12
16
20
24
28
32
time(h)

were plaque-purified 3 times and stocks screened for the ability to recombine with ts2203.

One of the 3 ts isolates which failed to recombine with ts2203, No.171, was renamed ts2204, and a high titre stock of this virus was prepared. To confirm that ts2204 contained the ts13 lesion, the virus was re-checked for recombination with ts2203. Ts2201, an HG52 ts mutant which recombined well with ts2203, was also included in the recombination assay (Table 10).

3.14 Processing of Viral DNA in ts2204-infected Cells

Ts2204 was compared with WT HVD25766 for the ability to cleave and package viral DNA at the PT and NPT. BamHI-digested total virus-infected cell DNA and packaged DNA samples were separated on agarose gels, transferred to a nitrocellulose filter, and hybridised to [^{32}P]-labelled pGZ1 (which contains HG52 BamHI g), and a plasmid containing HVD BamHI a.

Figure 33 shows an autoradiogram of the Southern blot obtained from this experiment. Both WT HVD and ts2204 synthesised large quantities of viral DNA at the PT and NPT, and at the PT packaged similar amounts of viral DNA. At the NPT, in contrast, terminal BamHI fragments were barely detectable in the ts2204-total virus DNA sample digested with BamHI, and almost no encapsidated

Table 10

Recombination analysis of ts2204

ts2204 was assayed for the ability to recombine with ts2203 or ts2201 at the PT.

The recombination frequency R(f) was calculated using the formula given in Brown et al., (1973).

Table 10

Virus	Titre of Virus (pfu per ml)		R(f)
	31^{o}	38.5^{o}	
<u>ts</u>2204(CA)	2.0×10^{5}	$<10^{2}$	/
<u>ts</u>2204(CR)	2.0×10^{5}	$<10^{2}$	/
<u>ts</u>2203	3.5×10^{4}	$<10^{2}$	/
<u>ts</u>2201	1.0×10^{5}	$<10^{3}$	/
<u>ts</u>2204(CA)/<u>ts</u>2203	1.0×10^{5}	$<10^{2}$	0
<u>ts</u>2204(CR)/<u>ts</u>2203	2.0×10^{5}	$<10^{2}$	0
<u>ts</u>2204(CA)/<u>ts</u>2201	2.0×10^{5}	9.5×10^{3}	8.45
<u>ts</u>2203 /<u>ts</u>2201	9.0×10^{4}	3×10^{3}	5.37

Figure 33

The processing of viral DNA in ts2204-and HVD-infected Cells

Total virus-infected cell DNA and DNase-resistant (packaged) DNA were purified from virus-infected cells incubated at the PT or NPT for 18h.

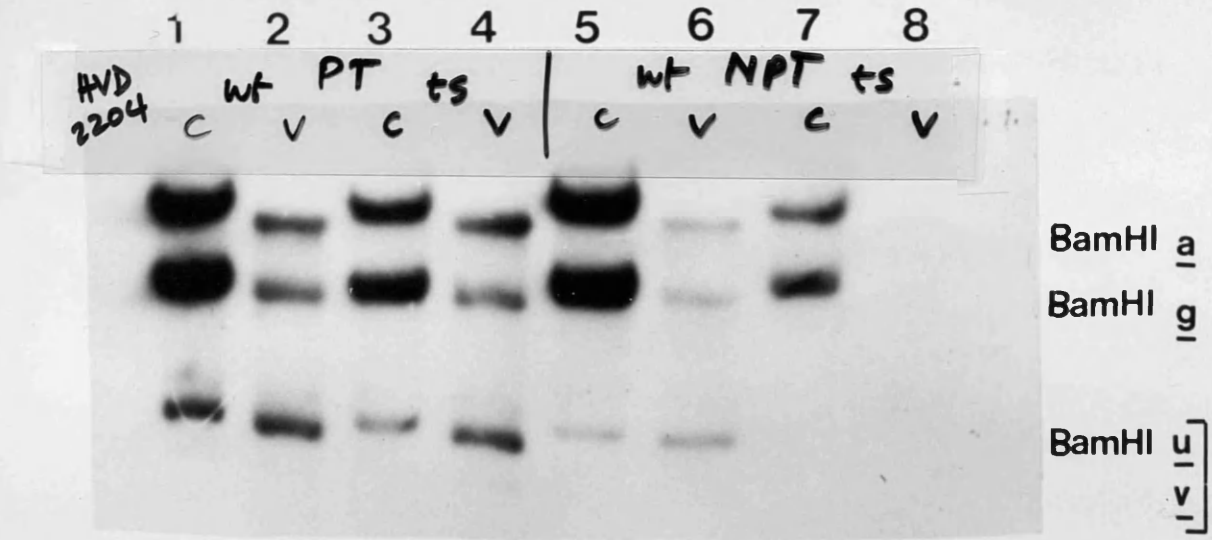
1/20 total virus infected cell DNA and 1/10 DNase-resistant DNA samples were digested with BamHI, and the fragments separated by agarose gel electrophoresis. The fragments were transferred to nitrocellulose and hybridised to [^{32}P]-labelled pGZ1 which contains HG52 BamHI g, and [^{32}P]-labelled HVD p(BamHI a).

Tracks 1-4: samples infected at the PT

HVD-infected cell DNA, (track 1); HVD encapsidated DNA (track 2); ts2204-infected cell DNA (track 3); ts2204 encapsidated DNA (track 4).

Tracks 5-8: samples infected at the NPT

HVD-infected cell DNA (track 5); HVD encapsidated DNA (track 6); ts2204-infected cell DNA (track 7); ts2204 encapsidated DNA (track 8).


1 2 3 4 5 6 7 8
HVD 2204
wt PT ts
wt NPT ts
C V C V C V C V
BamHI a
BamHI g
BamHI u
I
v
I

DNA at all could be seen in the ts2204 encapsidated DNA sample at 38.5°. Ts2204, therefore, appeared to have a defect in cleavage and packaging of viral DNA at the NPT.

3.15 Densitometric Analysis of Total Viral and Encapsidated DNA

Since the joint-spanning fragment, BamHI g, contains sequences from the long and short repeats, it will hybridise not only to itself but also to the terminal BamHI fragments, v and u. In virion DNA digested with BamHI, BamHI g, v and u are in equimolar amounts. However, in the Southern blot analysis described here, the probe pGZ1 containing BamHI g was treated with DNase I prior to *in vitro* labelling with [^{32}P]-dNTP by nick translation. This means that the size of the denatured probe should be small, and therefore the proportion annealing to BamHI g = BamHI v + u. That is, the amount of hybridisation depends on the size and molarity of the fragment.

At late times in infection, unpackaged, replicated DNA is present as concatemers, and as a consequence BamHI g increases relative to the terminal fragments u and v, so that the number of BamHI g fragments per concatemer ie. the molarity of BamHI g = 2n-1 (where n is the number of unit length genomes in the concatemer), and

the molarity of BamHI $\underline{v}$ = $\underline{u}$ = 1.

Thus the molar ratio of BamHI $\frac{\underline{g}}{\underline{v} + \underline{u}} = \frac{2n-1}{2}$.

This assumes that the ends of the concatemers are the same as in virion DNA. However, this may not be so since plasmids containing ori_S but not the $\underline{a}$ sequence, will form concatemers in presence of helper virus. It is, therefore, likely that in WT virus-infected cells, virus concatemers contain a mixture of ends, some terminating in the $\underline{a}$ sequence as in virion DNA, (if one or more cleavage steps have taken place from this end of the concatemer), and some terminating elsewhere in the genome, possibly near an origin of replication.

A better measurement of packaging can be made by comparing the amount of a unique fragment in total virus DNA with the amount present in encapsidated DNA. In these experiments BamHI $\underline{a}$, which occurs in the U_L, was used.

Thus, % packaging = $\frac{\text{BamHI } \underline{a} \text{ (encapsidated DNA)}}{2 \times \text{BamHI } \underline{a} \text{ (total virus-infected cell DNA)}}$

since twice the amount of BamHI-digested, encapsidated DNA samples was loaded onto agarose gels compared to the amount of BamHI-digested total virus-infected cell DNA used in the experiments.

3.16 Construction of Marker Rescued Virus, ts$^+$ 2204 (MR-1)

To confirm that the ts2204 lesion in Vmw65 was responsible for the packaging defect at the NPT, a recombinant virus was constructed by co-transfecting cells at 31^o with intact ts2204 DNA and pGZ73 (containing HG52 BglII i), cleaved with BglII.

Ts$^+$ virus amongst the progeny was plaque purified once at the NPT and twice at the PT, and one isolate was grown to a high titre virus stock. This virus, termed HVD ts$^+$ 2204 (MR-1) appeared to have a similar eop $\frac{NPT}{PT}$ to WT HVD.

3.17 Ts2204 has a Second ts Lesion

The synthesis and maturation of WT HVD, ts2204 and HVD ts$^+$ 2204 (MR-1) at the PT and NPT was analysed by the Southern blot technique as described in the previous section. The results (Figure 34) showed that at the NPT, in contrast to the WT HVD sample, both ts2204 and HVD ts$^+$ 2204 (MR-1) DNA samples contained a low proportion of terminal BamHI fragments, indicating that HVDts$^+$ 2204 (MR-1) had a defect in the processing of viral DNA at this temperature. This suggested that HVD ts$^+$ 2204 (MR-1) and possibly ts2204 had a ts mutation which mapped outside the Vmw65 gene.

Figure 34

Processing of viral DNA in ts2204, WT HVD and HVDts$^{+}_{-}$2204(MR-1) infected cells

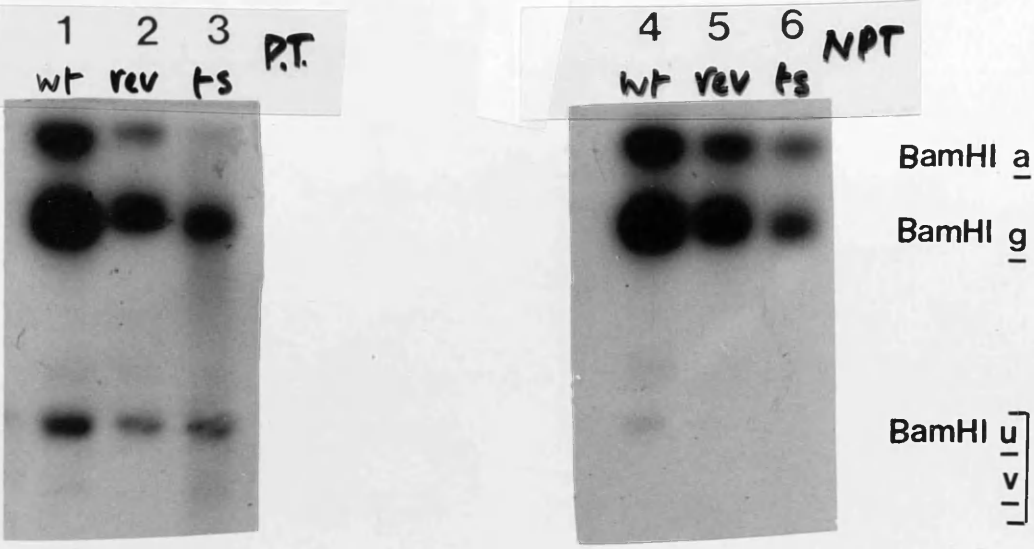
Total virus-infected cell DNA was purified from virus-infected cells incubated at the PT and NPT for 18h.

1/20 total virus-infected cell DNA samples were digested with BamHI, and the fragments separated by agarose gel electrophoresis. The fragments were transferred to nitrocellulose and hybridised to [^{32}P]-labelled pGZ1 containing HG52 BamHI g and [^{32}P]-labelled HVD p(BamHI a).

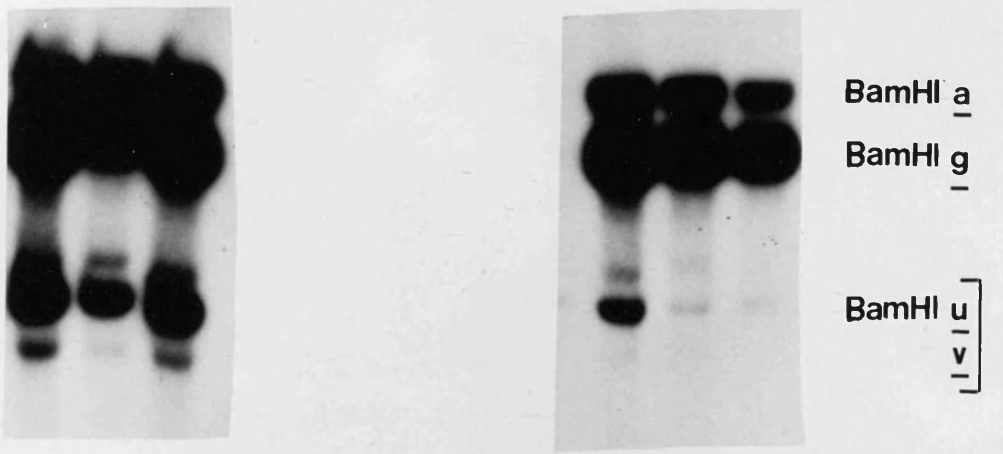
a. HVD WT, HVD ts^{+}2204 (MR-1) and ts2204 infected cell DNA at the PT (tracks 1-3 respectively) and NPT (tracks 4-6 respectively).

b. represents a longer exposure of the blots presented in a.

a)


1 2 3
wt rev ts
P.T.
4 5 6
wt rev ts
NPT
BamHI a
BamHI g
BamHI u
v

b)


1 2 3
4 5 6
BamHI a
BamHI g
BamHI u
v

Preliminary experiments revealed that the defect in HVD ts 2204 (MR-1) was also in ts2204, since at temperatures above 38.5°C ts progeny from cells cotransfected at 31°C with ts2204 and pGZ73 (containing WT HG52 BglII i), formed small plaques in comparison with those formed by WT HVD (V G Preston, personal communication). As a consequence of this finding, the process of recombination between HVD WT DNA and ts13 BglII i (pGZ74) was repeated in order to isolate a recombinant virus which had a single ts lesion.

3.18 Isolation of ts2205 and ts 2205(MR-2)

Cells were cotransfected at 31°C with intact WT HVD DNA and pGZ74 cleaved with BglII as described in Section 3.13. A ts virus which failed to recombine with ts2203 was isolated, and designated ts2205. The HG52 BglII i fragment was recombined into ts2205, and a ts virus, HVD ts 2205 (MR-2) which had a similar eop $\frac{NPT}{PT}$ to WT HVD, was isolated. Unlike HVD ts 2204 (MR-1), plaques formed by this virus at temperatures greater than 38.5°C were found to be similar in size to those of WT HVD.

3.19 Processing of Viral DNA

Figure 35 shows an autoradiogram of a Southern blot in which BamHI-digested DNA samples, prepared from BHK cells infected with ts2205, HVD or ts 2205 (MR-2), were

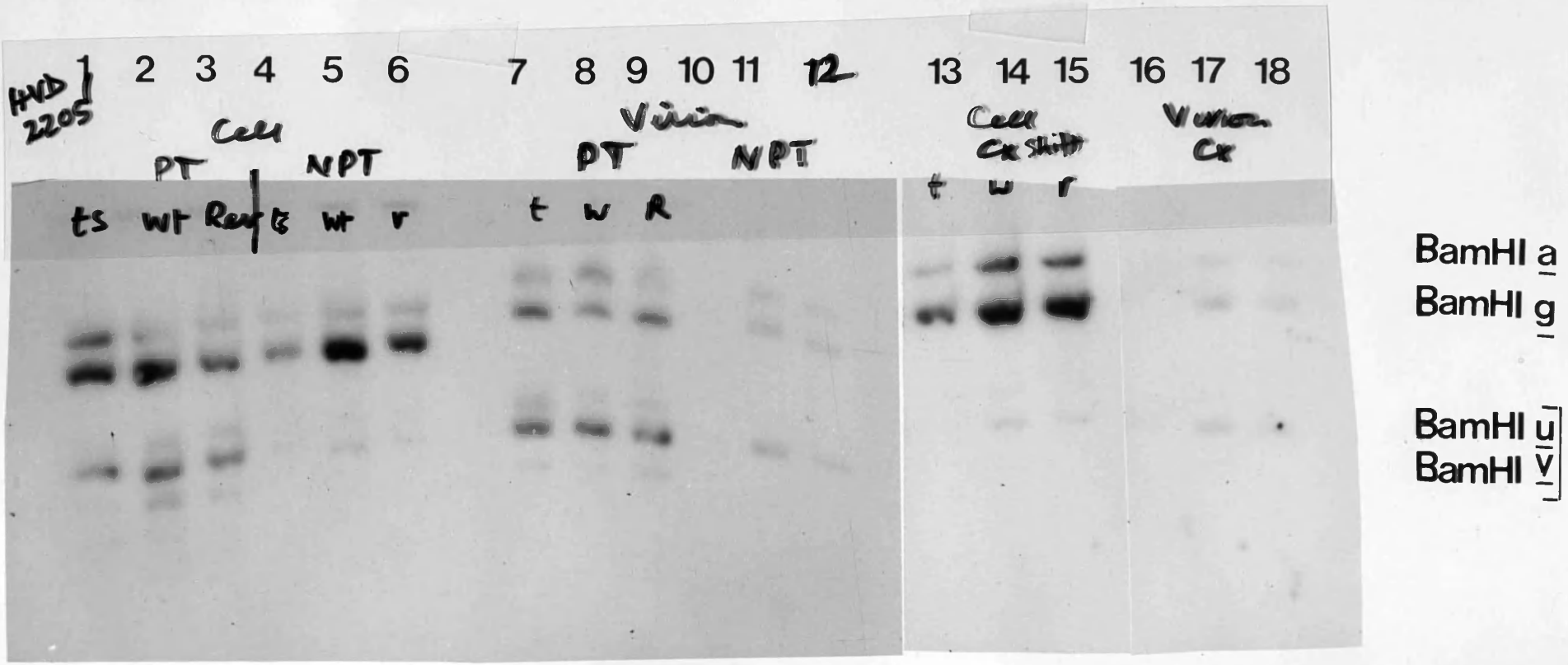
Figure 35

Processing of viral DNA in ts2205, WT HVD and HVD $^{+}$ts 2205 (MR-2)-infected cells

Cells were infected at the PT (31^{o}) or NPT (38.7^{o}) with ts2205, WT HVD or HVD $^{+}$ts 2205(MR-2) at a moi of 5pfu per cell, and harvested at 18h pi; alternatively cells were infected at the NPT and at 12h pi transferred to the PT in the presence of cycloheximide (200ug/ml).

1/20 total DNA and 1/10 encapsidated (DNase-resistant) DNA samples were digested with BamHI and the fragments separated by agarose gel electrophoresis. The DNA was transferred onto a nitrocellulose membrane and hybridised to [^{32}P]-labelled HVD pBamHI a and pGZ1 (HG52 BamHI g). An autoradiagram was then made of the Southern blot.

ts2205, HVD WT, HVD $^{+}$ts 2205(MR-2) total cell DNA (PT), tracks 1-3 respectively; ts2205, HVD WT, HVD $^{+}$ts 2205(MR-2) total cell DNA (NPT) tracks 4-6 respectively; ts2205, HVD WT, HVD $^{+}$ts 2205(MR-2) encapsidated DNA (PT) tracks 7-9 respectively; ts2205, HVD WT, HVD $^{+}$ts 2205(MR-2) encapsidated DNA (NPT) tracks 10-12 respectively; ts2205, HVD WT, HVD $^{+}$ts 2205(MR-2) CHX-treated cell DNA tracks 13-15 respectively; ts2205, HVD WT, HVD $^{+}$ts 2205 (MR-2) CHX- treated encapsidated DNA, tracks 14-16 respectively. Tracks 13-16 were transferred to a separate nitrocellulose membrane, and are shown at a longer exposure than tracks 1-12.


HVD 2205
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Cell
PT
NPT
Virion
PT
NPT
Cell
Cx Shift
Virion
Cx
ts wt Rev ts wt r t w R t w r
BamHI a
BamHI g
BamHI u
BamHI v

hybridised to [^{32}P]-labelled HVD p (BamHI a) and pGZ1. Both HVD WT and ts^{+} 2205 (MR-2) viruses produced large quantities of viral DNA at the PT (31^{o}) and NPT (38.7^{o}), and by densitometric analysis 15.3% WT HVD and 22.6% ts^{+} 2205 (MR-2) DNA, synthesised at the NPT, was encapsidated. In contrast, ts2205 failed to package significant quantities of viral DNA at the NPT, and almost all of the ts2205 total-infected cell DNA synthesised at the NPT was in the form of "endless DNA". This information in summarised in Table 11.

To determine whether the effect of the mutation could be reversed upon temperature downshift, virus-infected cells incubated at the NPT, were transferred to the PT at 12h pi, with the addition of cycloheximide to prevent any further protein synthesis. Cells were incubated for a further 6h at the PT, and harvested. The viral DNA was then examined as described above. Only background levels of packaged ts2205 DNA were detectable on extended exposures of film to the blot (Figure 35), less than 2% of ts2205 DNA packaged at the PT (Table 11), suggesting that the ts defect in ts2205 is not reversible.

3.20 Analysis of ts2205-infected Cell Polypeptides

Polypeptide profiles of WT HVD-, ts^{+} 2205 (MR-2)- and ts2205-infected cells incubated at the PT or NPT were

Table 11

Densitometric analysis of Southern blots of total virus-infected cell DNA and encapsidated (DNase-resistant DNA)

The amount of DNA synthesised or encapsidated by each virus is represented by the amount of [^{32}P]-labelled probe hybridising to the BamHI a fragment.

Total virus DNA is presented as a percentage of the DNA synthesised by HVD WT virus at the PT (1), NPT (2) or following shiftdown from the NPT to the PT in the presence of cycloheximide (3). In each case synthesis of HVD DNA is taken as 100%.

Encapsidated DNA at the PT or NPT is presented as a percentage of the total virus-infected cell DNA synthesised at the PT (4) or NPT (5) or on shiftdown from the NPT to the PT in the presence of cycloheximide (6), for HVD WT, ts^{+}2205 and ts2205.

Table 11

	HVD %	ts + 2205 %	ts2205 %
1. PT	100.0	76.1	186.5
C 2. NPT	100.0	67.1	50.3
3. Shift	100.0	57.0	28.7
4. PT	29.9	36.4	10.3
V 5. NPT	15.3	22.6	<1.0
6. Sh	6.1	4.7	<2.0

analysed to determine if Vmw65 was synthesised by ts2205 at the NPT, and to determine, from the mobility of Vmw65, if HG52 BglII i and ts13 DNA fragments carrying the Vmw65 gene had been recombined into the ts^{+} 2205 (MR-2) and ts2205 viruses respectively.

Mock-infected cells, or cells infected with the WT HVD, ts^{+} 2205 (MR-2) or ts2205 were incubated, as described previously, at 31^{o} or 38.7^{o}, labelled with [^{35}S]-methionine for 1h at various times pi, harvested, and the radiolabelled polypeptides analysed by SDS PAGE.

The polypeptide profiles of all three viruses were very similar, but at the PT and the NPT ts2205 synthesised a Vmw65 polypeptide which migrated slightly faster than the WT HVD or ts^{+} 2205 (MR-2) species (Figures 36 and 37).

3.21 Electron Microscopic Analysis of ts2205-, ts^{+} 2205 (MR-2)- and WT HVD-infected cells grown at the PT (31^{o}) and NPT (38.7^{o})

To confirm that ts2205 had a lesion affecting encapsidation of viral DNA at the NPT, thin section preparations were made of ts2205-infected cells grown

Figure 36

Autoradiogram of electrophoretically separated polypeptides from mock- and virus-infected cells incubated at the PT

Cells were labelled with [^{35}S]-methionine from 4-5 (tracks 1-4), 6-7 (tracks 5-8) and 7-8 (tracks 9-12)h pi and harvested. Protein samples were analysed on a 9% single concentration SDS-polyacrylamide gel (SDS-PAGE).

mock-infected (MI) cell polypeptides (tracks 1,5,9);
HVD-infected cell polypeptides (tracks 2,6,10);
ts2205-infected cell polypeptides (tracks 3,7,11);
ts^{+}2205-infected cell polypeptides (tracks 4,8,12).

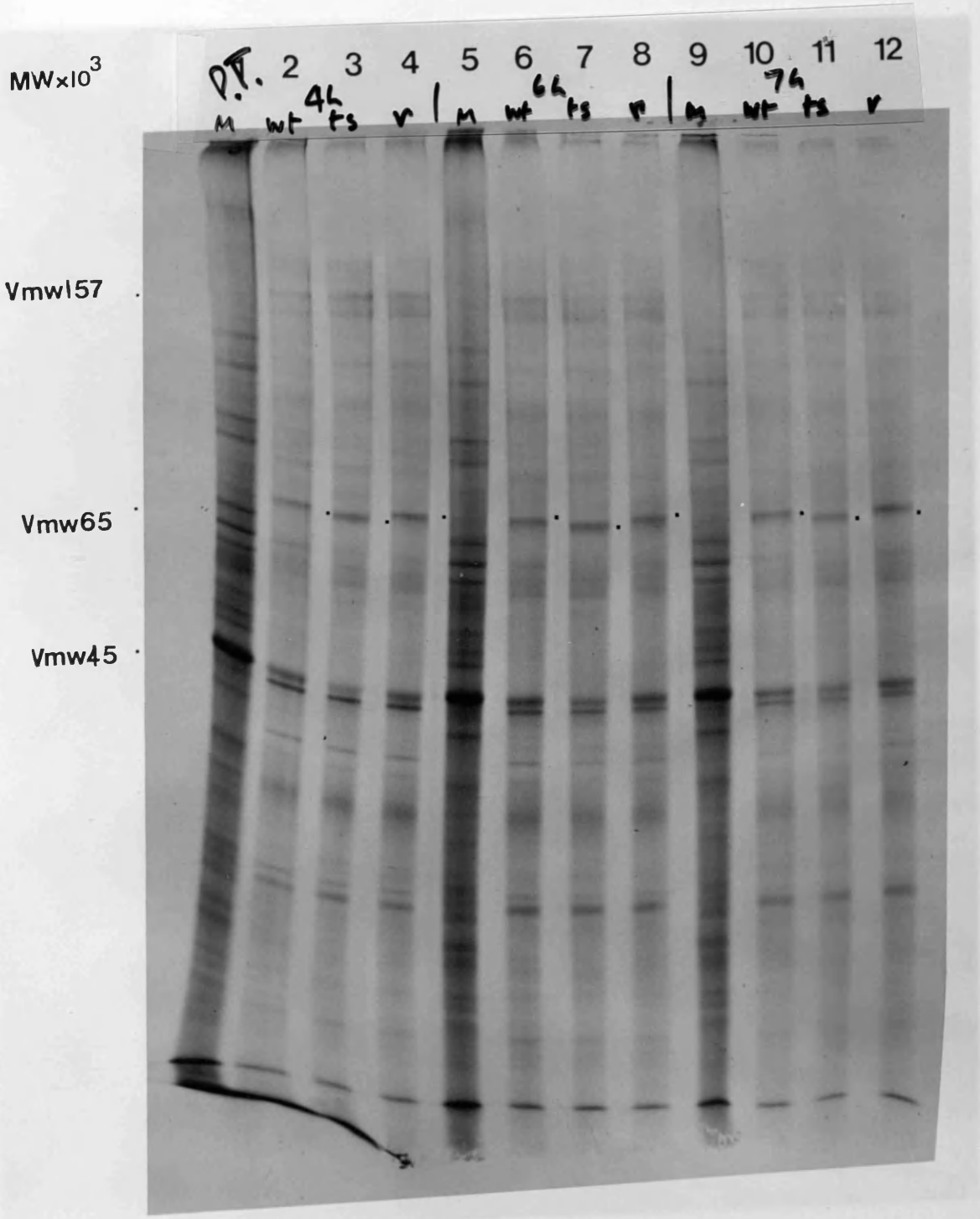

MW×10³
P.T.
2
3
4
5
6
7
8
9
10
11
12
M
wt
4h
ts
r
M
wt
6h
ts
r
M
wt
7h
ts
r
Vmw157
Vmw65
Vmw45

Figure 37

Autoradiogram of electrophoretically separated polypeptides from mock- and virus-infected cells incubated at the NPT

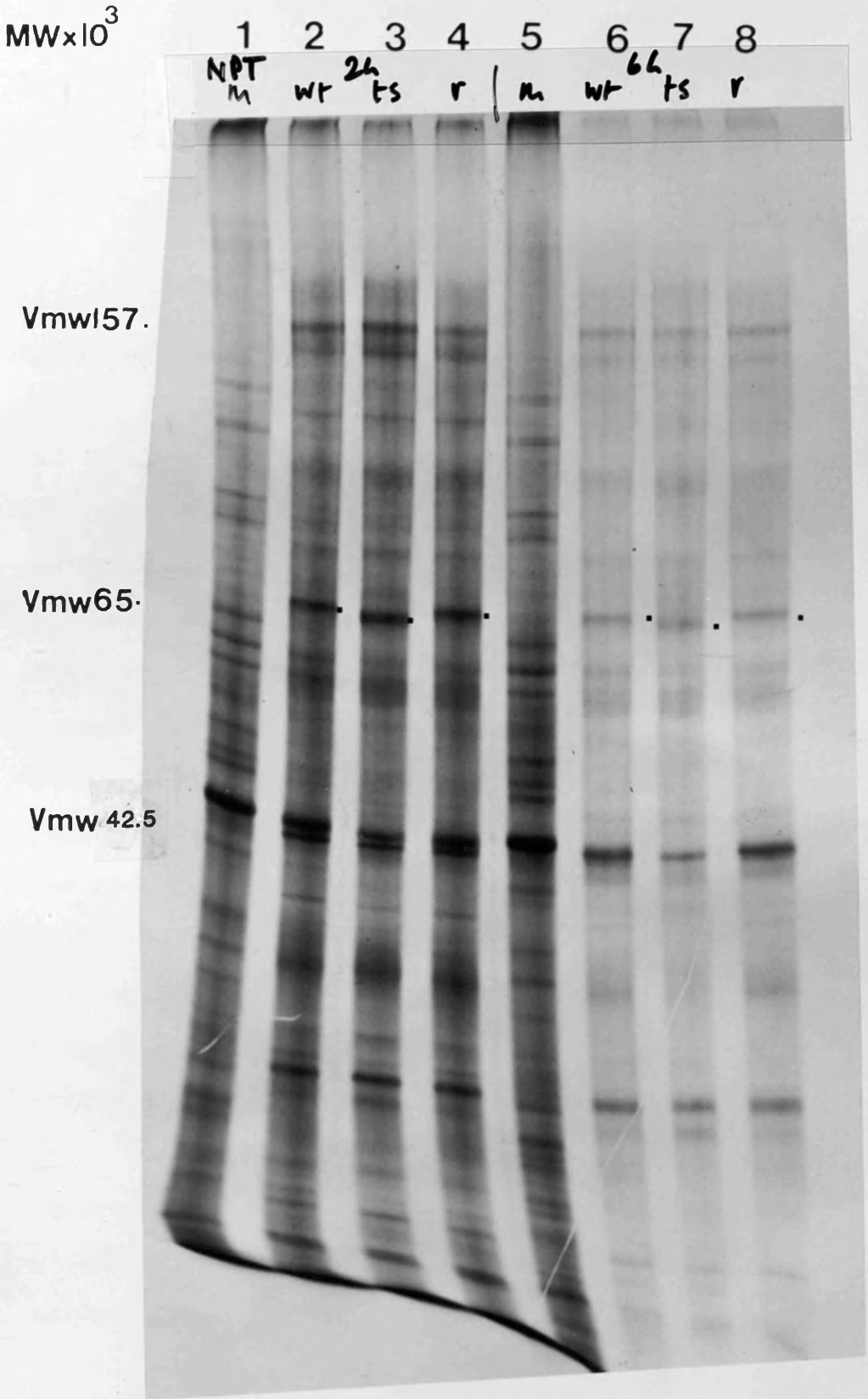
Cells were labelled with [^{35}S]-methionine from 2-3h (tracks 1-4) and 6-7h pi (tracks 5-8) and harvested. Protein samples were analysed on a 9% single concentration SDS-polyacrylamide gel.

mock-infected (MI) cell polypeptides (tracks 1,5);

HVD-infected cell polypeptides (tracks 2,6);

ts2205-infected cell polypeptides (tracks 3,7);

ts^{+}2205(MR-2)-infected cell polypeptides (tracks 4,8).


MW x 10^3
1
2
3
4
5
6
7
8
NPT
m
wt
24
ts
r
m
wt
66
ts
r
Vmw157.
Vmw65.
Vmw 42.5

at the PT and NPT, and compared under the electron microscope with WT- or ts$^+$ 2205 (MR-2)- infected cell samples.

At the PT, similar number of capsids were made by all three viruses, and large quantities of full capsids and virions were observed (Figure 38). At the NPT, WT HVD and ts$^+$ 2205 (MR-2) virus-infected cell nuclei contained similar numbers of dense-cored, partially cored and empty capsids. Larger amounts of enveloped virus particles were identified at later times in cells infected with these viruses (18-22h pi), compared with samples harvested at 12h pi. In contrast, very few full capsids (approximately 1 full capsid per 10-15 infected cell nuclei) and only one enveloped particle (in 30 infected cells examined), were found in ts2205-infected cells, even at 22h pi, and nearly all the capsids produced by ts2205 were partially cored. (Figure 39). There are two explanations for the presence of low numbers of full capsids in ts2205-infected cells at the NPT: the mutation, which clearly affects virus DNA encapsidation, could be slightly leaky, and permit a low level of DNA encapsidation. Alternatively, the full capsids could be due to low numbers of ts$^+$ revertants in ts2205-infected cells.

Figure 38

Electron microscopic analysis of virus-infected cells grown at the PT

Cells were mock infected (MI) or infected at a moi of 5pfu virus per cell with WT HVD, ts 2205(MR-2), ts2205 and incubated for 18h at the PT. Cells were then harvested and embedded in epon resin. Thin section preparations of infected cells were examined under the electron microscope. The bar represents 0.5um

1, ts2205-infected cells; 2, HVD-infected cells; 3, ts 2205(MR-2)-infected cells; 4, MI cells.

Empty capsids

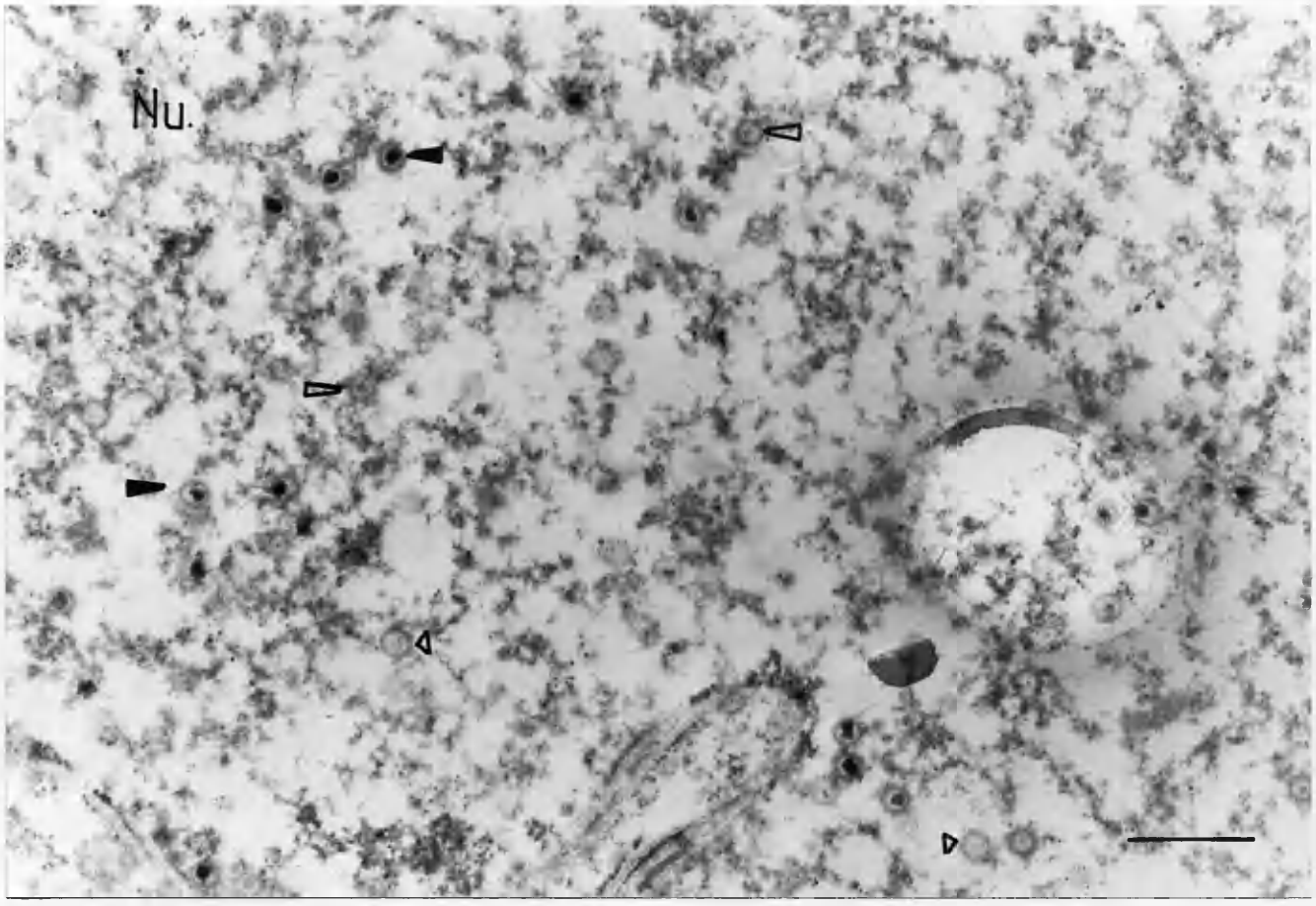
Partially cored capsids

Dense-cored capsids

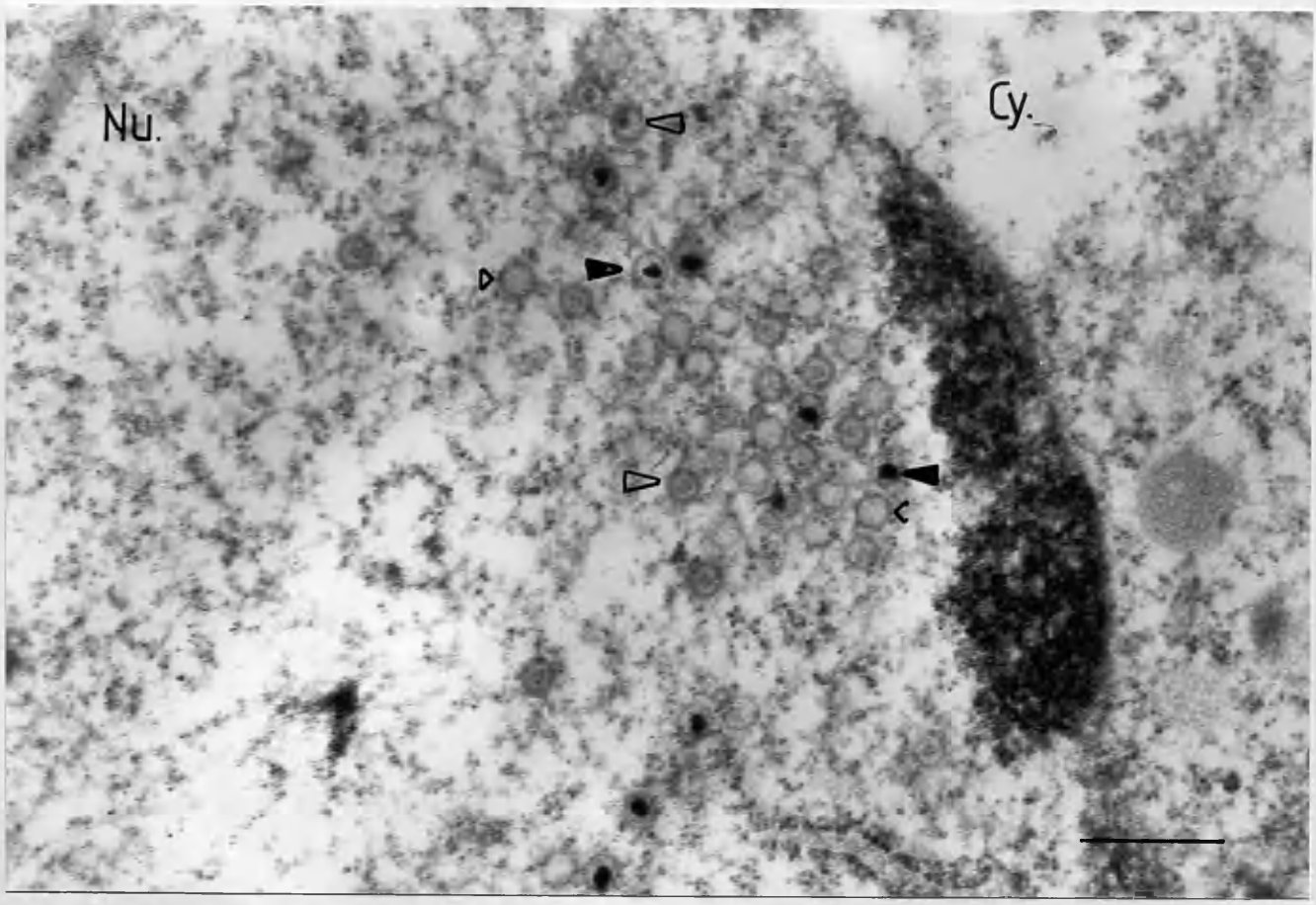
Virions

Nu: nucleus; Cy: cytoplasm

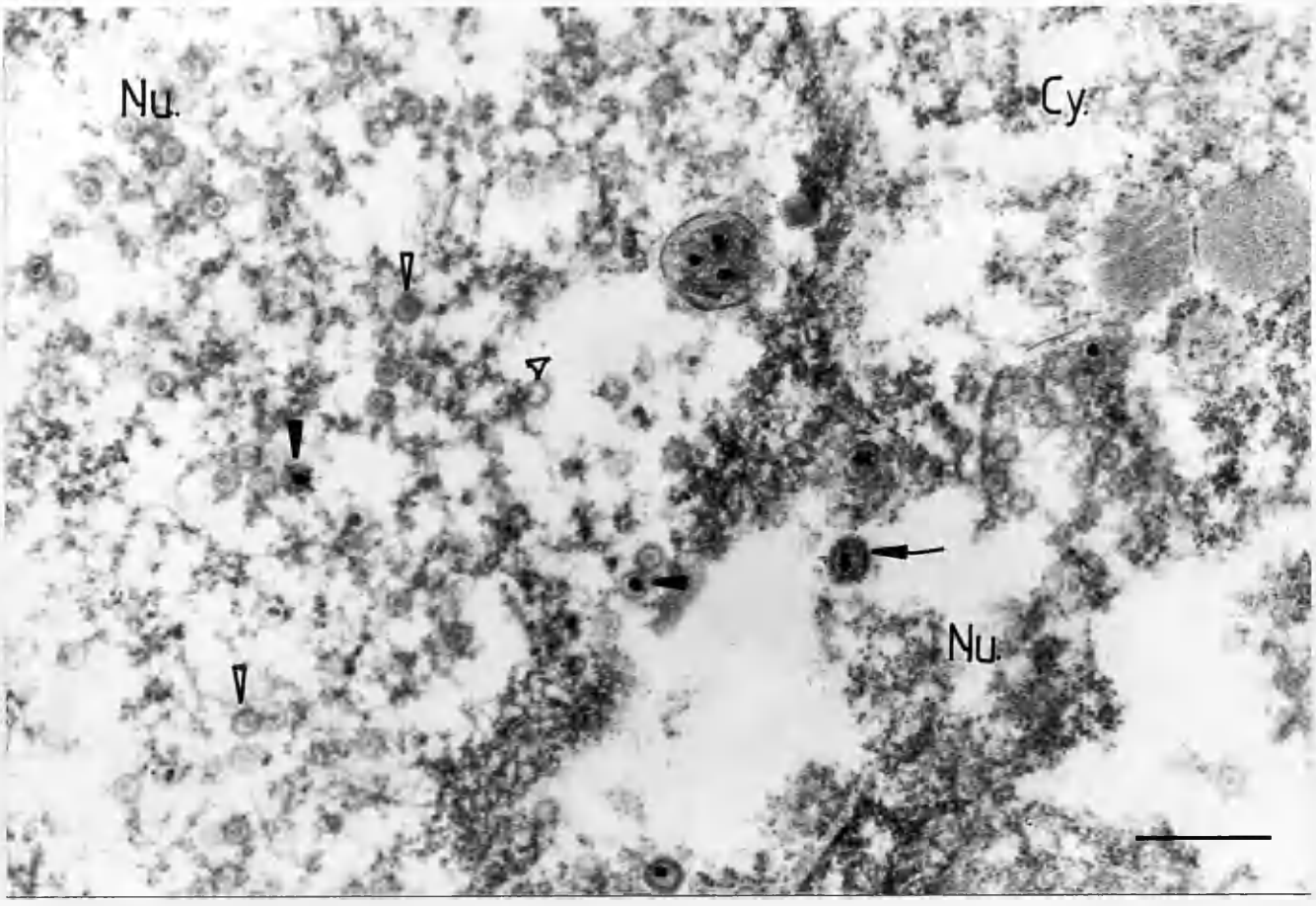
1



2



3



4

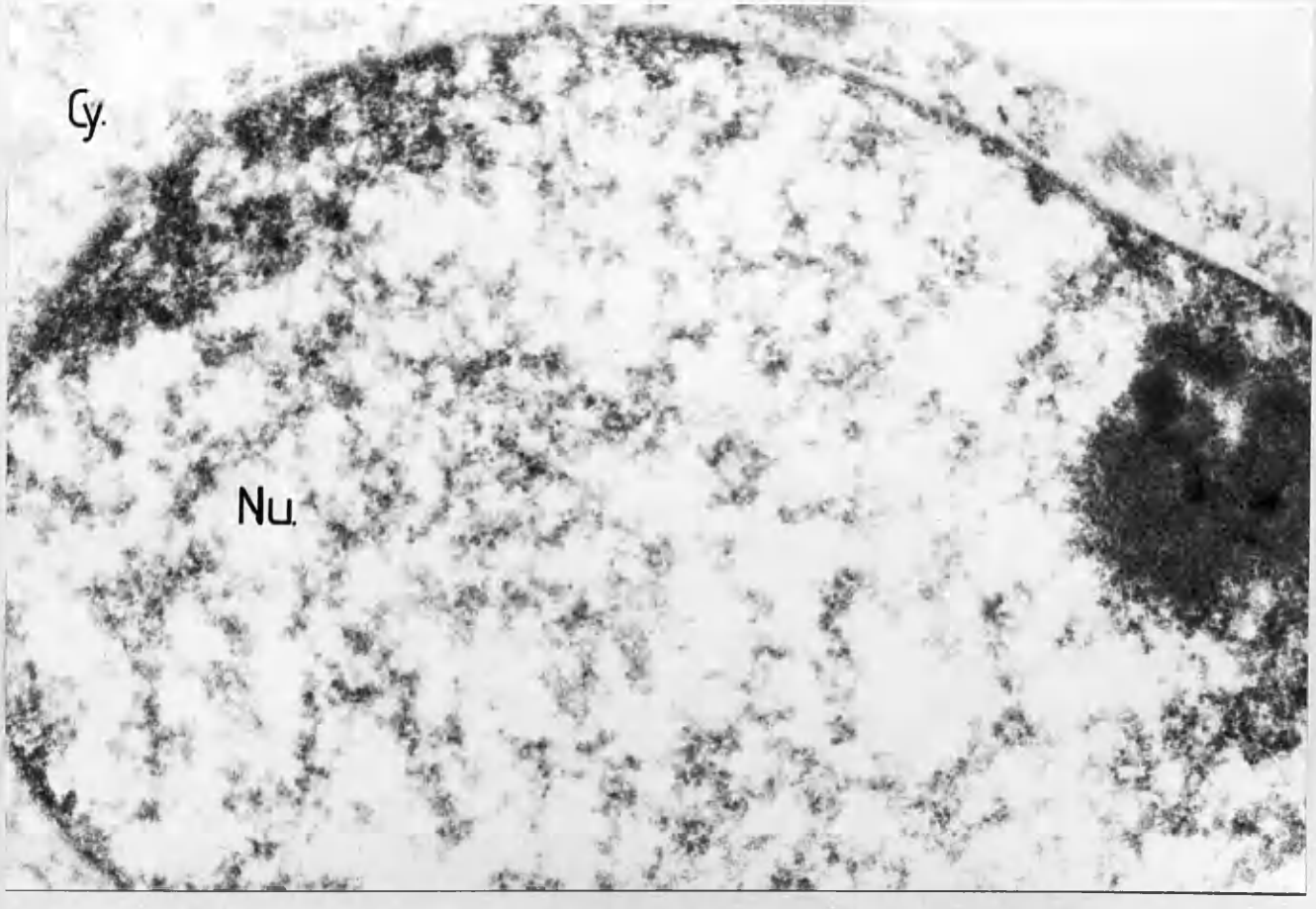


Figure 39

Electron microscope analysis of virus-infected cells grown at the NPT

Cells were infected with WT HVD, ts 2205(MR-2) or ts2205 at a moi of 5pfu virus per cell, and incubated at the NPT for 18h. Cells were then harvested, and embedded in epon resin. This section preparations of virus-infected cells were examined under the electron microscope. The bar represents 0.5um

1,2 ts2205-infected cells; 3, HVD-infected cells; 4, ts 2205-infected cells.

Empty capsids

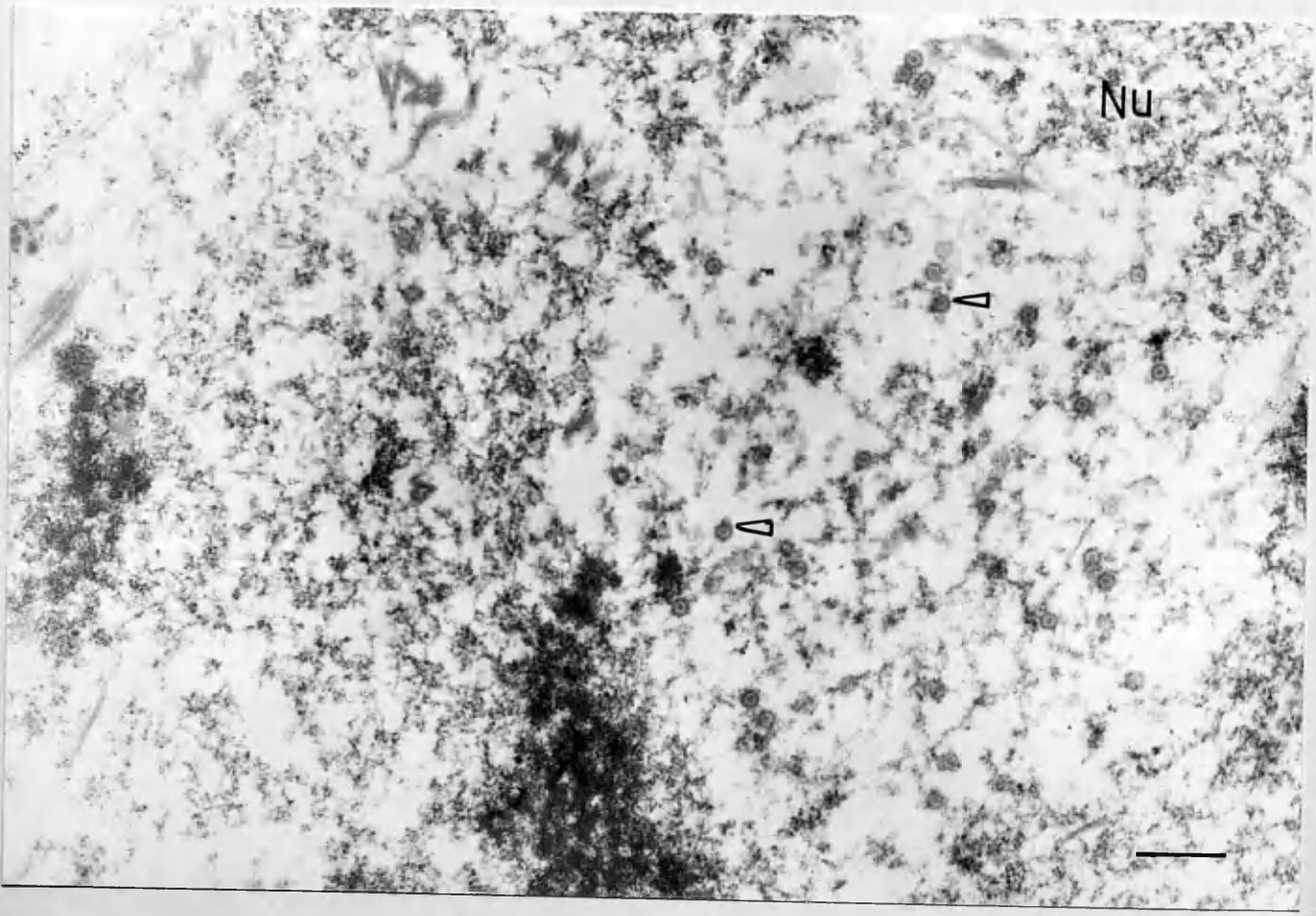
Partially-cored capsids

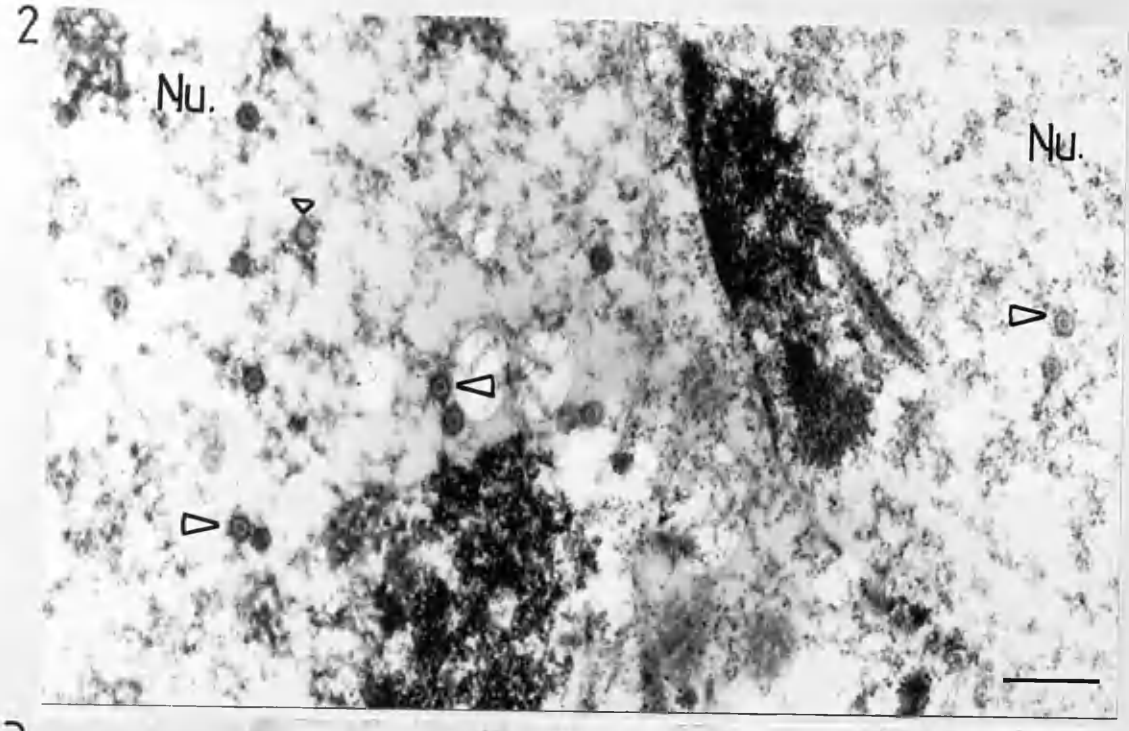
Dense-cored capsids

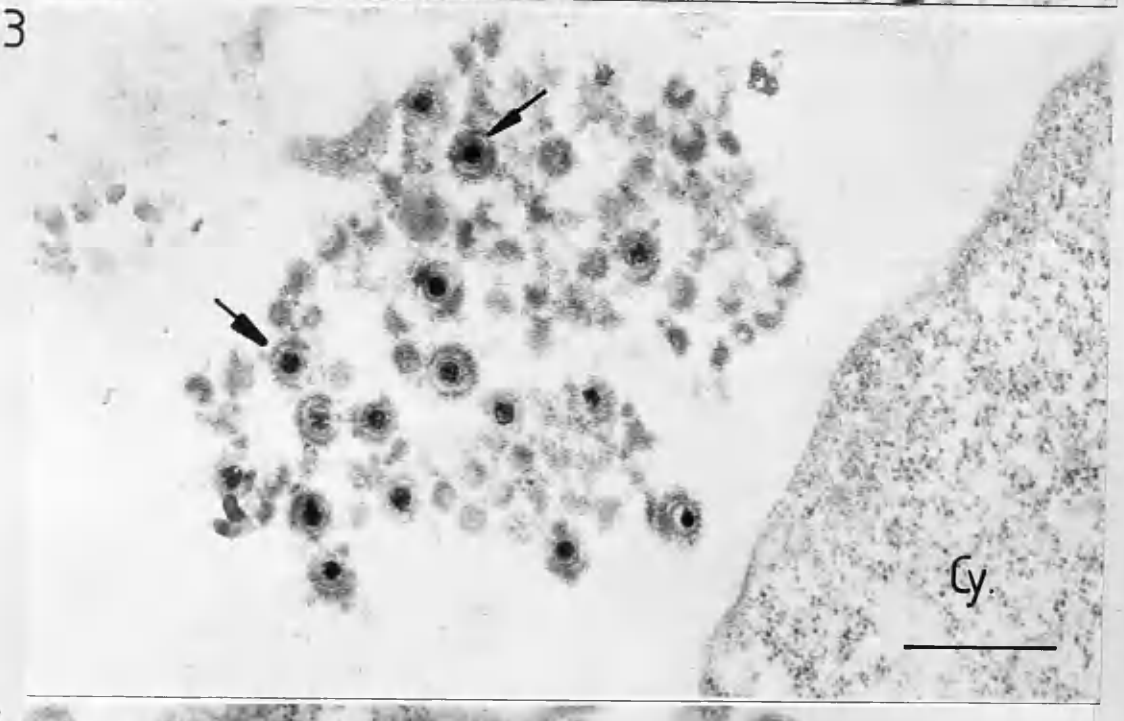
Virions

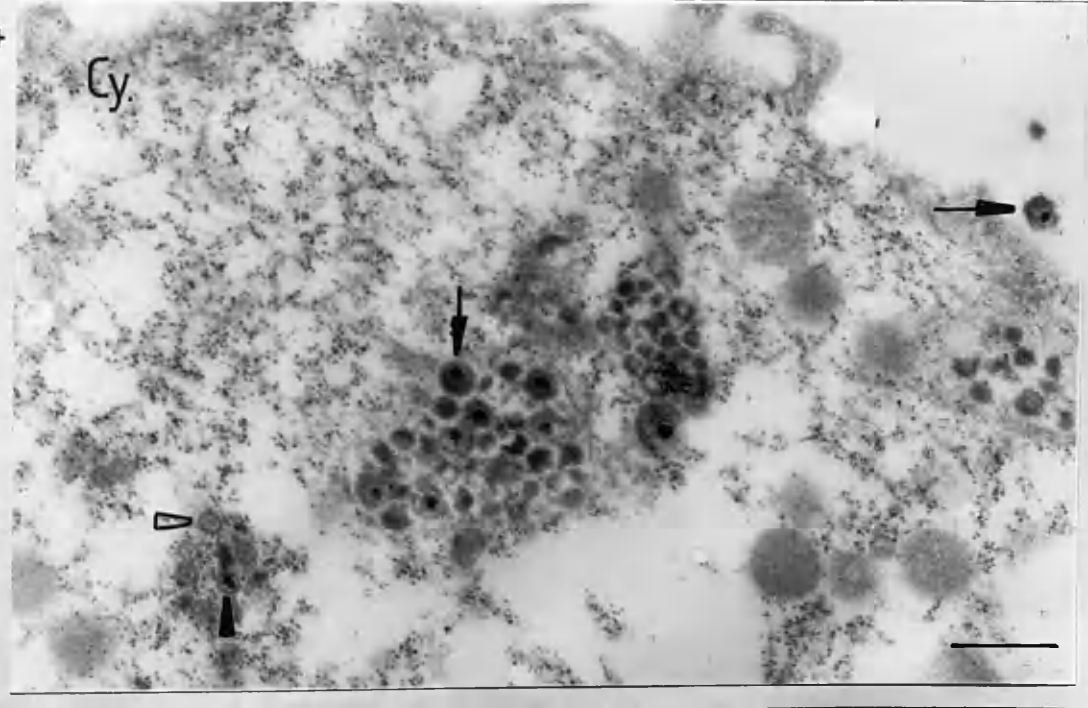
Nu: nucleus; Cy: cytoplasm

1




2
Nu.
Nu.


3
Cy.


4
Cy.

Virus-infected cells incubated at the NPT were also transferred to the PT in the presence of cycloheximide, at 12h pi, to determine if the defect was reversible. These cells were incubated for a further 6h at the PT, harvested, and then embedded in epon resin. Examination of thin sections of virus-infected cells confirmed that the lesion in ts2205 was not reversible (Figure 40), and that the lesion affected encapsidation of virus DNA.

The proportion of full, partially cored and empty capsids, and virions was determined from thin section preparations of infected cells under the electron microscope. Thirty cells were examined, and the results (Table 12) confirmed observations that ts2205 fails to encapsidate significant quantities of viral DNA at the NPT.

3.22 DISCUSSION

The tegument of HSV has been described as amorphous structure which interfaces with the virus capsid and envelope. Whilst some tegument proteins have been identified, the function of this structure as a whole, and the role of its component polypeptides have remained elusive.

Figure 40

Reversibility of the encapsidation defect of ts2205

Cells were infected at the NPT with WT HVD, ts^+ 2205(MR-2) or ts2205 at a moi of 5pfu virus per cell, and incubated for 12h. Infected cells were then transferred to the PT in the presence of 200ug/ml cycloheximide to prevent further protein synthesis, and incubated for 6h. Cells were harvested at 18h pi and embedded in epon resin. Thin section preparations of virus-infected cells were examined under the electron microscope. The bar represents 0.5um

1,2 ts2205-infected cells; 3, HVD-infected cells; 4, ts^+ 2205-infected cells.

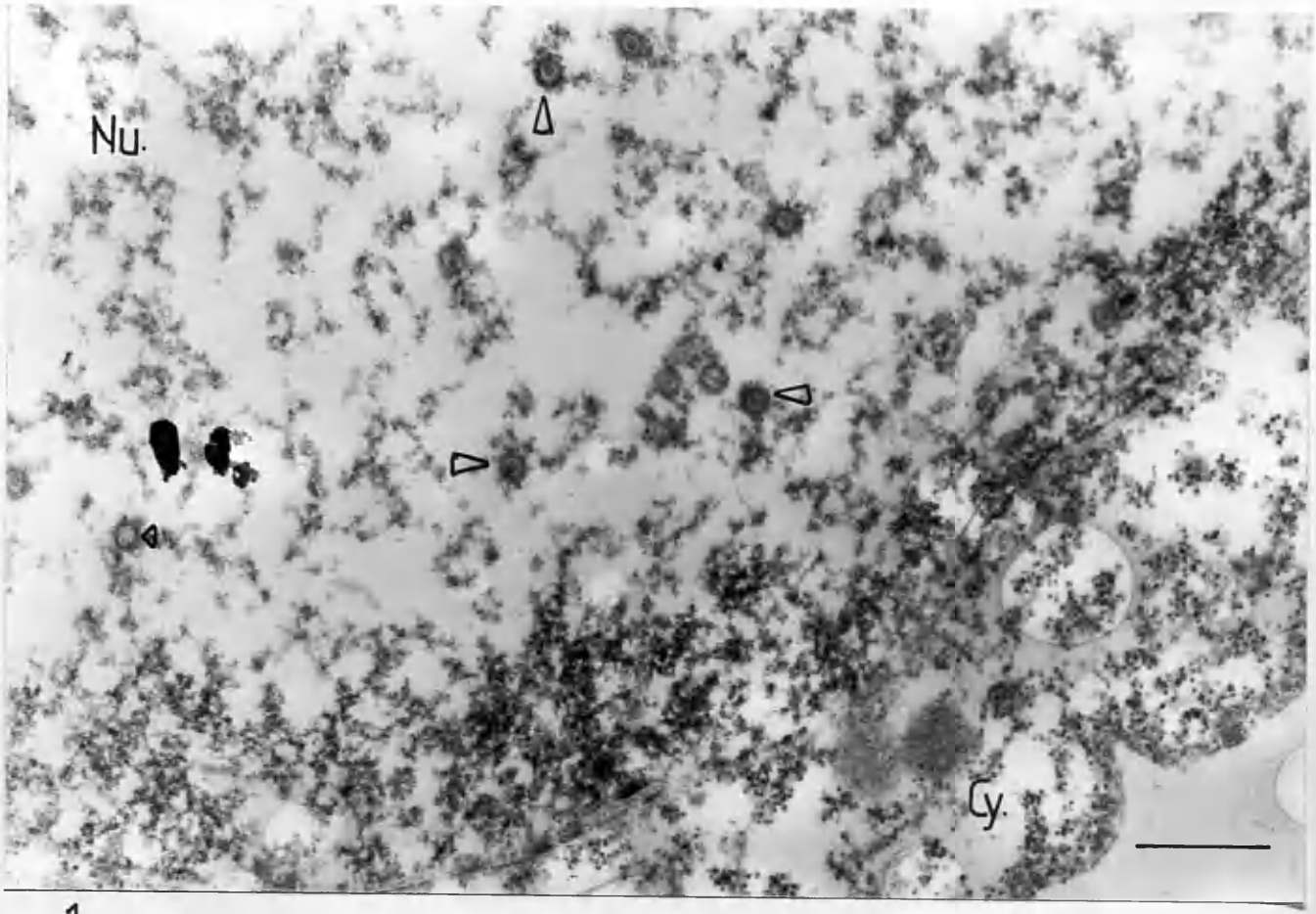
Empty capsids

Partially-cored capsids

Dense-cored capsids

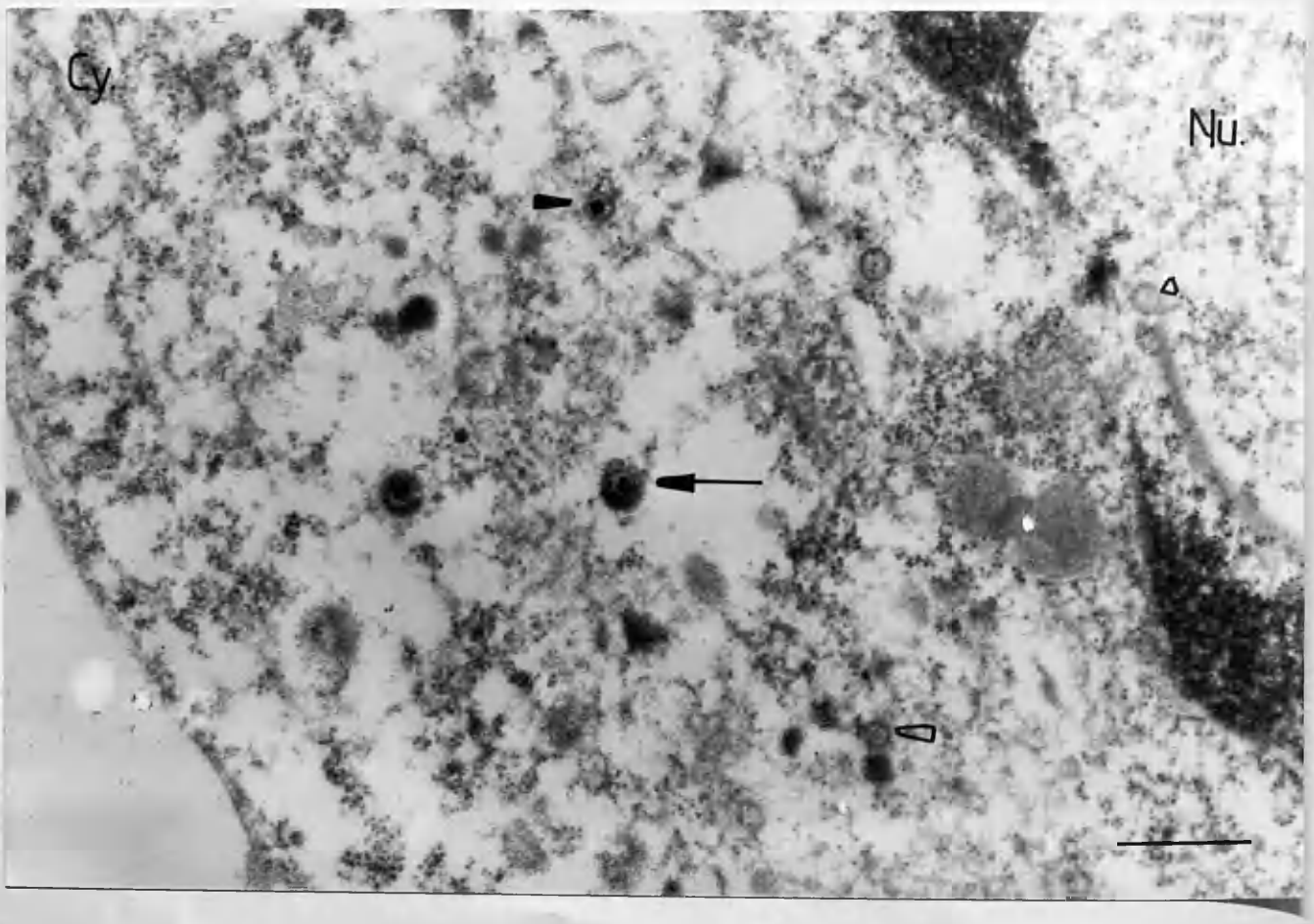
Virions

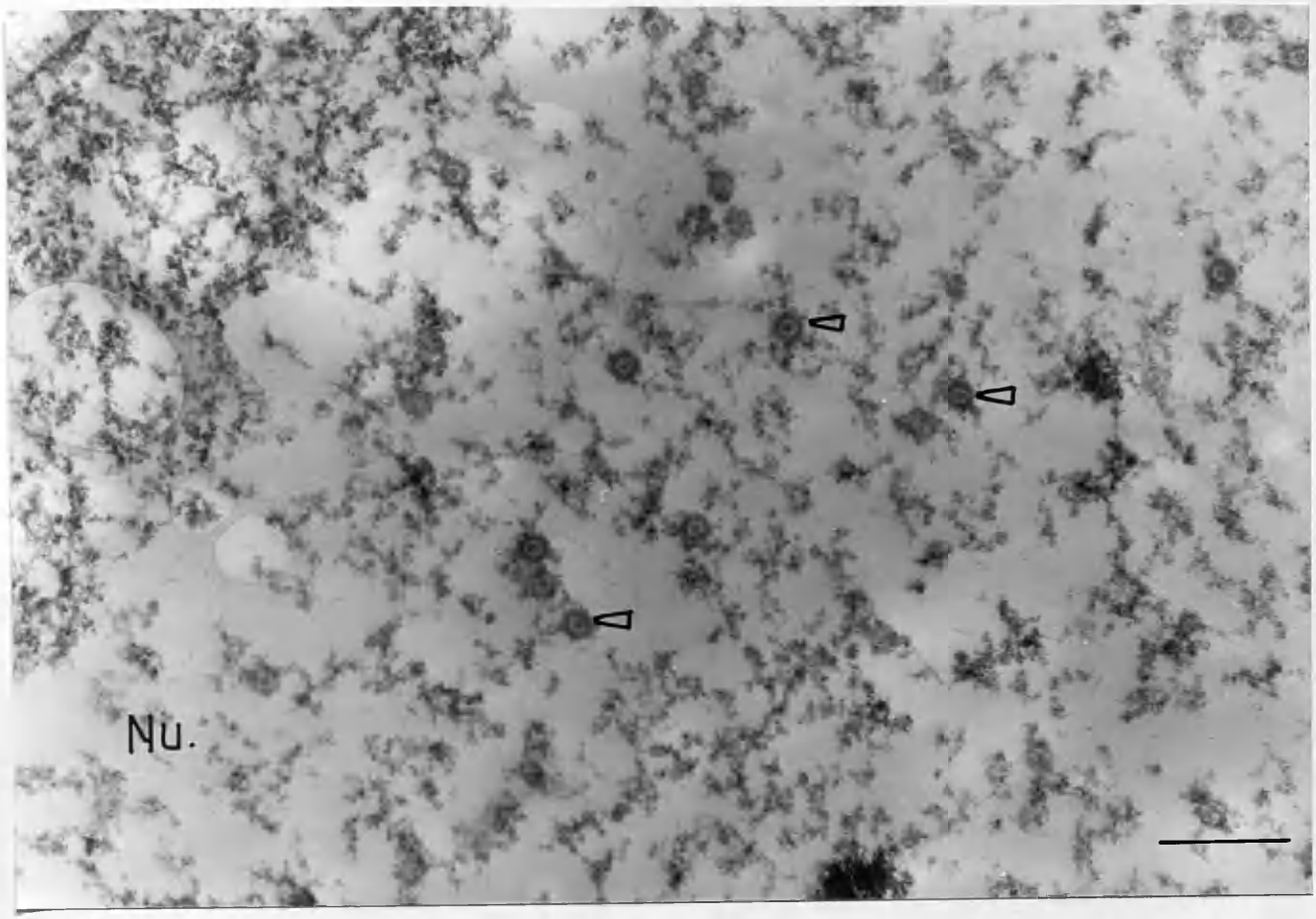
Nu: nucleus; Cy: Cytoplasm



1

3





2

4

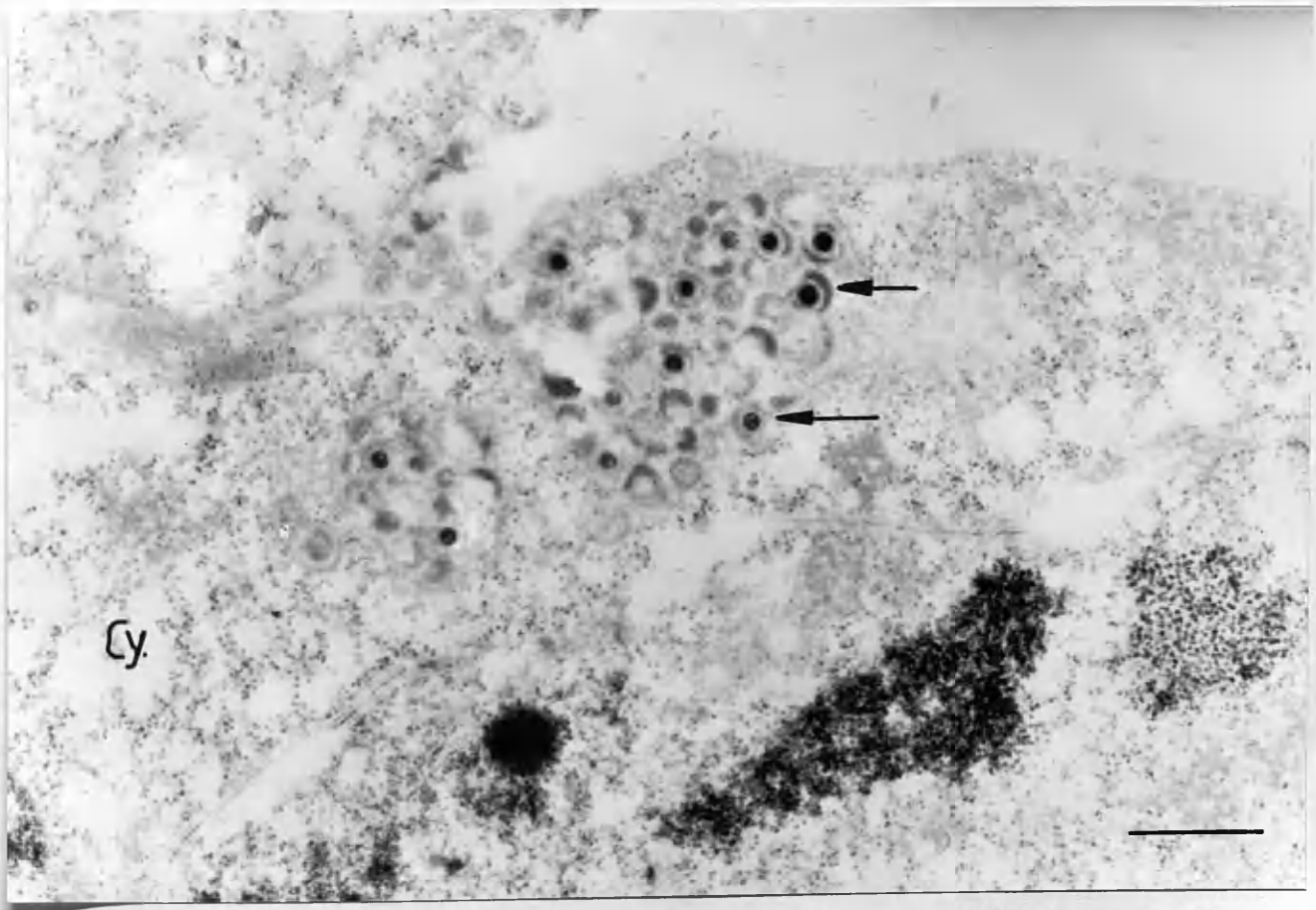


Table 12

Estimation of the proportion of each capsid type in virus-infected cells

The proportion of dense-cored, partially-cored and empty capsids in the nuclei of cells infected at the PT or NPT with WT HVD, ts2205 or ts^{+}2205(MR-2) was determined from examination of thin section preparations of cells under the electron microscope. 30 cells were screened. The results are presented as (1) average number of each capsid type per nucleus, and (2) % capsid type per nucleus.

Samples were examined at 16, 18 and 22h pi at the NPT, and at 18h pi at the PT.

Samples were also examined at 18h pi, following a temperature shift from the NPT to the PT at 12h pi in the presence of cycloheximide (CHX).

The proportion of capsids in the nuclei of cells infected at the NPT with HG52 or ts2203 is also shown.

Table 12(1)

Capsids per nucleus

	Partially Cored	Empty	Dense Cored	Virions	Av. No. particles/ nucleus
PT 18h pi					
ts2205 + ts 2205(MR-2)	25.06	4.72	34.39	5.0	59.17
	28.12	5.50	27.50	8.1	69.72
HVD	30.10	10.24	27.0	9.8	77.14
NPT 16h pi					
ts2205 + ts 2205(MR-2)	9.60	0.33	0.13	0.00	10.07
	8.00	0.70	2.50	0.17	11.37
HVD	8.63	1.00	2.83	0.23	12.70
NPT 18h pi					
ts2205 + ts 2205(MR-2)	12.73	0.26	0.40	0.00	13.40
	7.50	0.40	1.20	4.00	13.10
HVD	15.60	1.00	9.10	2.70	28.40
HG52	77.20	1.50	3.50	0.50	82.67
ts2203	53.00	0.90	1.60	0.20	85.70
NPT 22h pi					
ts2205 + ts 2205(MR-2)	23.90	1.80	0.20	0.00	25.90
	16.17	0.40	5.67	2.83	25.07
HVD	15.60	0.50	6.30	2.30	24.70
CHX:(18h pi)					
ts2203 + ts 2205(MR-2)	29.10	1.80	0.70	0.10	32.50
	19.50	1.60	4.70	2.80	28.60
HVD	9.60	0.70	2.70	2.70	15.70

Table 12(2)

% capsids/nucleus

	Partially Cored	Empty	Dense Cored	Virions
PT 31 18h pi				
ts2205 + ts 2205(MR-2)	42.32	7.98	41.22	8.45
	40.62	7.95	39.33	11.70
HVD	38.94	13.45	34.93	12.68
NPT 16h pi				
ts2205 + ts 2205(MR-2)	95.36	3.31	1.32	0.00
	70.26	6.14	21.93	1.46
HVD	67.98	7.87	22.31	1.84
18h pi				
ts2205 + ts 2205(MR-2)	95.02	1.99	2.99	0.00
	57.25	3.05	30.53	9.16
HVD	54.93	3.52	32.04	9.50
HG52	93.39	1.81	4.23	0.60
ts2203	95.15	1.62	2.87	0.36
22h pi				
ts2205 + ts 2205(MR-2)	92.28	6.95	0.77	0.00
	64.49	1.60	22.61	11.30
HVD	63.16	2.02	25.51	9.31
CHX(18h pi)				
ts2205 + ts 2205(MR-2)	89.54	8.00	2.15	0.31
	68.18	5.59	16.43	9.79
HVD	61.15	4.46	17.20	17.20

The results from characterisation of ts2205 showed that a major structural component of the tegument, Vmw65, has an essential role in the encapsidation of viral DNA. Although initial work on the HG52 mutant, ts2203, failed to determine whether Vmw65 was important for nucleocapsid formation or virus envelopment, the analysis of ts^+ revertants of ts2203 was extremely useful for providing information about the mutation in the Vmw65 gene.

3.22.1 Ts^+ Revertants of ts2203

The five independently isolated revertants of ts2203, ts^+ 2203rev1....rev5, fell into two categories.

1. Those that induced a Vmw65 which retained the altered electrophoretic mobility of ts2203 Vmw65 on SDS polyacrylamide gels.

2. Those that synthesised Vmw65 which comigrated with the WT polypeptide on an SDS polyacrylamide gel.

The conclusion, therefore, is that there is a single mutation in the Vmw65 gene of ts2203 which is responsible for the ts phenotype and altered electrophoretic mobility of Vmw65.

The reason for the alteration in apparent MW of the polypeptide specified by ts2203 is not known. There may be a defect in the processing of Vmw65 although the results of H.Moss (personal communication) suggest that the mutant polypeptide is still phosphorylated. It is also not clear whether the aberrant electrophoretic mobility of the mutant Vmw65 is responsible for the temperature-sensitive phenotype, or whether it is a secondary effect of the amino acid substitution. Since ts2203 induced an altered Vmw65 at both 31°C and 38.5°C, the aberrant polypeptide does not appear to significantly affect virus growth at the PT.

The ts lesion may cause a change to the tertiary structure of Vmw65 which affects the ability of the polypeptide to interact with other tegument or capsid proteins. Alternatively, ts2203 may synthesise a Vmw65 which is unstable at the NPT. Although this is an unusual situation, some ts mutations have been found to introduce instability into virus polypeptides, for example, there is a ts lesion in the PRV mutant ts1 which affects the stability of the major capsid protein (Ladin et al., 1980, 1982). Nevertheless, as will be discussed later, this is unlikely to be the case with ts2203 Vmw65.

There are several explanations for the isolation of ts+ revertants with an aberrant Vmw65 ability. A change in the DNA sequence at the site of the ts lesion would convert the amino acid back to the WT, or substitute a similar type of amino acid. In the latter situation, such a revertant may or may not regain the electrophoretic mobility of WT Vmw65. It is also possible that a "second-site" reversion or mutation, either elsewhere in the Vmw65 gene, or in another gene could also allow the mutant to grow as well as WT virus at the NPT. In this case, since the virus would retain the original ts lesion, it would still produce a Vmw65 with an altered electrophoretic mobility at the PT and NPT. If such a mutation was present in another gene, then this would suggest that WT Vmw65 interacts with this gene product.

3.22.2 Analysis of the Defect in ts2205

The defect in ts2205 was reversible on temperature shift-down of infected cells from the NPT to the PT in the presence of cycloheximide. This could be due to an instability of the mutant protein produced at the NPT making it susceptible to degradation. However, as the mutant Vmw65 was detectable at late times in infection at the NPT by SDS PAGE of ts2205-infected cell extracts, and by immunoprecipitation of ts2203-infected

cell extracts with a Vmw65-specific antibody, any degradation of the polypeptide at this temperature must be minimal, suggesting that the gene product is stable at 38.5°. A more likely alternative is that the polypeptide produced at the NPT has an altered, non-functional conformation which fails to resume the WT conformation upon temperature shift-down to the PT in the presence of cycloheximide, a protein synthesis inhibitor. Thus, the ts virus would fail to cleave and encapsidated DNA under these conditions unless the block in protein synthesis was removed.

3.22.3 Temperature-sensitivity of HSV-1 and HSV-2 DNA Encapsidation

From the analyses of viral DNA encapsidation, it is clear that HSV-2 strain HG52 packaged less DNA than HSV-2 strain HVD25766 at 38.5° and that both viruses encapsidated less DNA at this temperature than at 31°. Addison (1986) found that HSV-1 strain 17 was also slightly ts at 38.5° for DNA encapsidation, and that at higher temperatures the ts effect was increased. These results suggest that HSV-1 and HSV-2 both have thermolabile polypeptide(s) involved in packaging virus DNA, and that this effect can vary between strains of HSV.

3.22.4 Ts Lesions in other Herpesvirus Genes which Effect Encapsidation of Viral DNA

A number of HSV-1 ts mutants which have defects in the packaging of viral DNA have been isolated (Figure 41), and these fall into at least 5 different complementation groups. These mutants include ts1204 and ts1208, which each have a defect in the U_L gene (U_L 25) specifying a 62,000 MW polypeptide (Addison et al., 1984; D.J.McGeoch, personal communication), ts1201, which has a mutation in the gene encoding ICP35 (U_L 26)(Preston et al., 1983), ts1203 which has a lesion in a gene specifying an 85,000 MW (U_L28) polypeptide (Addison, 1986), and ts1233 which has a mutation in a gene encoding a 14,000 MW polypeptide (U_L 33) (M.Al.Kobasai, personal communication). In addition, two other mutants, tsN20 (Schaffer et al., 1973), and ts247 (Pancake et al., 1983) which probably have defects in encapsidation of DNA have also been described. These viruses, however, have not been well characterised. Recent results (M.Al Kobasai, personal communication) suggest that the lesion in tsN20 maps in a different gene to the one in which the ts1233 lesion lies, probably within U_L32.

PRV packaging mutants have been identified in at least

8 different complementation groups (Ladin et al., 1980; Ben Porat et al., 1982; Ihara et al., 1982; Ladin et al., 1982)(Figure 41). Little is known about the products of the genes in which these mutants have defects. The PRV mutants ts109, tsJ and ts1 fail to accumulate the 35,000 MW assembly protein at the NPT, but it is not clear whether these viruses have mutations in this polypeptide. It should be noted that although these viruses have essentially colinear genomes, the region between mu 0.1 and 0.4 is inverted (Davison and Wilkie, 1983) so that some of the HSV and PRV mutants will probably have lesions in analogous genes.

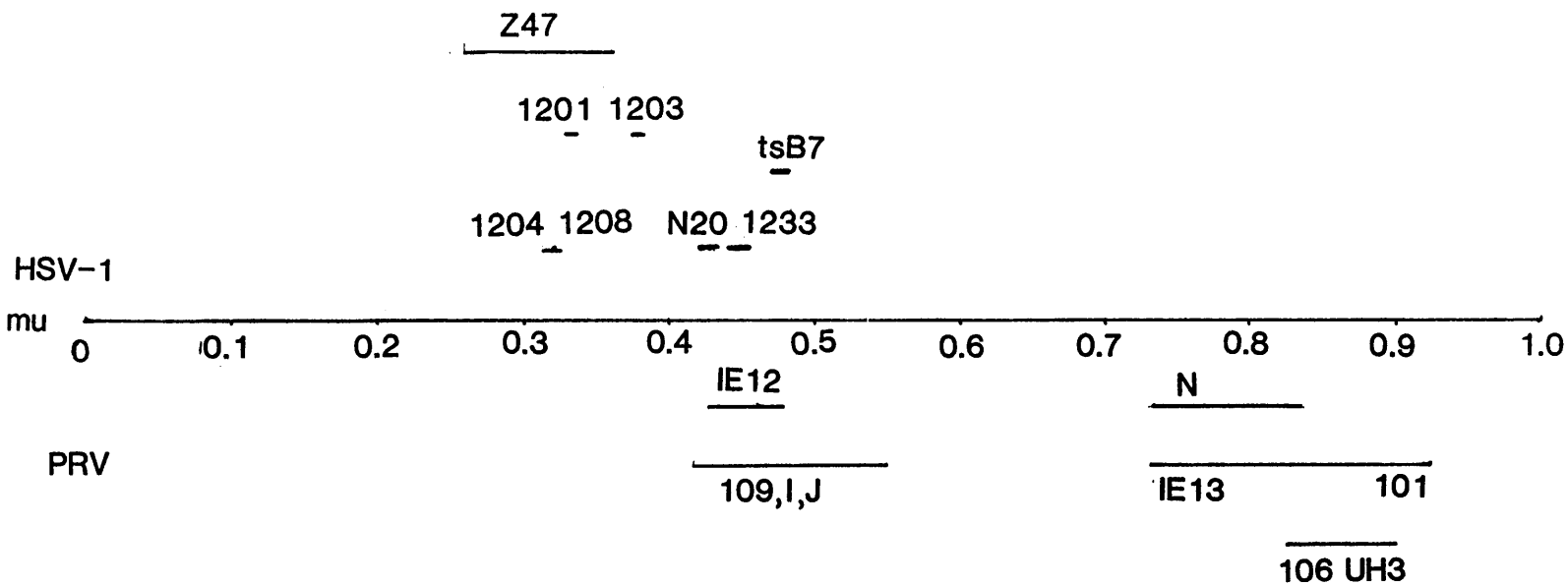
Although it is likely that both PRV and HSV-1 mutants have lesions in capsid or tegument proteins, with the exception of ICP35, the gene products in which the mutations lie have not been assigned to components of the virion. Interestingly, whilst the HSV Vmw65 tegument protein is clearly involved in packaging viral DNA, there is no evidence to suggest that the high MW tegument protein, Vmw273, is required for viral DNA packaging. Instead, information from analyses of ts mutants indicates that this polypeptide is involved in uncoating of viral DNA, and possibly in envelopment (Batterson et al., 1983; Batterson and Roizman, 1983;

Figure 41

Map locations of ts mutations of HSV-1 and PRV which affect the ability of virus to package DNA at the NPT

Also included is the map location of the lesion in tsB7, which lies within Vmw273 tegument protein and prevents the virus from being uncoated at the NPT.

Further details and references are given in the text.


Z47
1201 1203
tsB7
1204 1208 N20 1233
HSV-1
mu
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
IE12
N
PRV
109,I,J
IE13
101
106 UH3

V.Preston, personal communication).

3.22.5 Methods of Encapsidation of Viral Nucleic Acid

Two general models for encapsidation of viral nucleic acids have previously been described in Chapter 1. That is (a) a capsid forms about the replicated DNA or RNA and (b) the genome is inserted into a preformed capsid shell. Whilst the weight of evidence suggests that HSV DNA is packaged into a preformed capsid (Perdue et al., 1976; Ladin et al., 1980; Preston et al., 1983) this is not the case for all viruses. In analysing the role of Vmw65 and the virus tegument in HSV DNA encapsidation, it is perhaps worthwhile examining the means by which other viruses package nucleic acid.

The ds DNA bacteriophages have been extensively analysed for their pathways of capsid assembly, DNA cleavage, and encapsidation, and these processes are analogous to those of HSV in many ways. These viruses include lambda (λ), T1, T4, P22, ØX174 and Ø29, all of which produce concatemers of DNA either by rolling circle DNA replication, or by recombination (Takahashi, 1974; Tye et al., 1974; Bastia et al., 1975; Ritchie and Joicey, 1978); the head to tail multimers are

cleaved to approximately unit-length monomers and packaged. A number of packaging models have been proposed for these viruses. Whilst it was initially suggested that the DNA condensed and a protein shell was built around it (Kellenberger et al., 1958). Luftig et al, (1971) carried out in vivo density shift experiments on T4 phage which indicated that in fact, preformed proheads packaged the DNA. This was shown conclusively in an in vitro packaging system using λ (Kaiser et al., 1975, 1976). Furthermore, empty capsids were shown to be precursors of full capsids (Laemmli and Favre, 1973; Hohn and Hohn, 1974; Kerr and Sadowski, 1974; Kaiser et al., 1975). Similar proposals have been made for packaging of herpesvirus DNA. Perdue et al. 1976 suggested that DNA was packaged into a preformed capsid on the basis of a study of different capsid types in EHV infected cells. This model was shown to be in agreement with results from the analysis of ts mutants of PRV and HSV (Ladin et al., 1980; Preston et al., 1983) which failed to package DNA.

Phage λ initiates cleavage and packaging of DNA by means of a specific recognition step, and a virus encoded enzyme, the terminase, plays a major role in the overall process. The terminase recognises a cos

nicking and binding sites

site on the genome, which is composed of the two regions (cosN and cosB), and binds both to cosB and to a preformed phage prohead, initiating a site specific single strand nick on the DNA to begin packaging. The terminase then scans the genome and completes the packaging process by a second site specific cleavage at a cosN site (Emmons, 1974; Becker et al., 1977; Feiss et al., 1983)(Figure 42). Cos cleavage terminating packaging of one genome can also serve as an initial cos site for packaging of the next genome (Emmons, 1974) and during this process the terminase remains bound to the genome (Feiss et al., 1985).

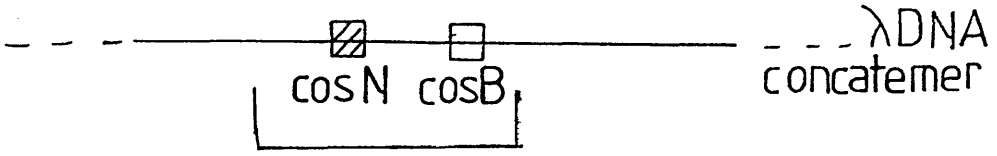
In some phages, for example, T1 and P22, packaging is initiated by a site-specific ds cleavage event at a unique pac site on the genome (Tye et al., 1974). Packaging then proceeds, without further sequence specifity, by sequential excisions of headfuls of the DNA of variable size, producing permutations of the genome (Jackson et al., 1978; Ramsay and Ritchie, 1983).

On the HSV genome certain sequences and processes are analogous to parts of these phage packaging systems. The a sequence plays a role similar to that of the pac or cos sequences and there is such a region in the a

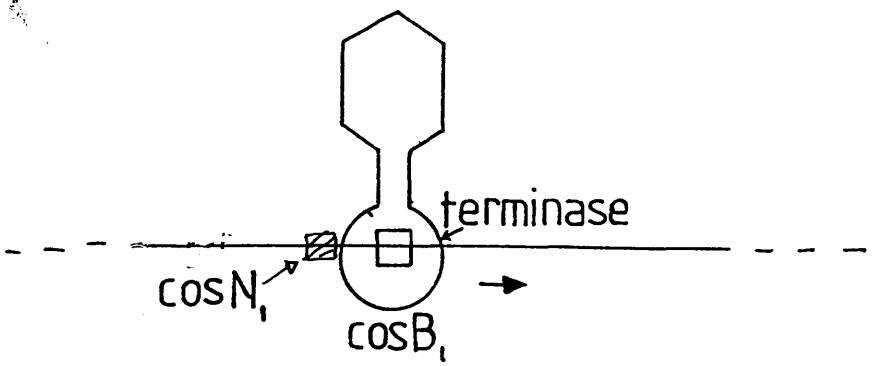
Figure 42

Schematic representation of the phage λ DNA packaging system

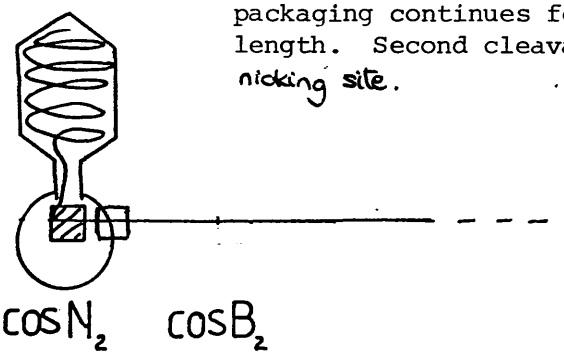
see text for details



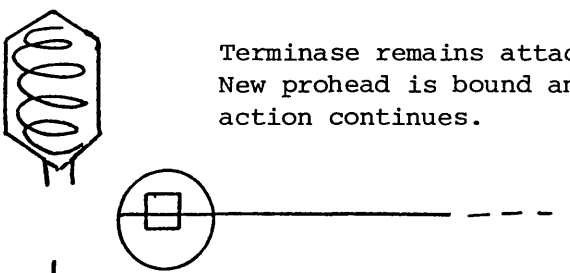
Phage prohead plus scaffolding proteins and terminase bind to concatemer at cosB site of cos.



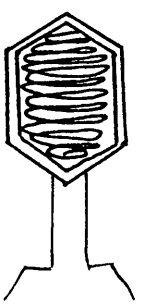
Cleavage and initiation of packaging; packaging continues for a genome's length. Second cleavage at cosN, the nicking site.



Terminase remains attached to DNA. New prohead is bound and processive action continues.



Full prohead stabilised; loss of scaffolding proteins.



Mature phage particle.

sequence of HSV-1 which has homology to HSV-2, EBV and VZV sequences, and is thought to represent a pac sequence (Deiss et al., 1986), although the evidence suggests that in HSV this is not the cleavage site, and thus it differs from the pac site of phage.

Site-specific ds cleavage mechanism, the theft and modified theft models (Varmuza and Smiley, 1985; Deiss et al., 1986) have been proposed for HSV. Indeed, in the simplest possible HSV cleavage system direct ds cleavage of viral DNA at a double a sequence, followed by a repeat cleavage at a similar double a sequence a genome's length from the initial cleavage site would fit the known packaging requirements. In addition, the staggered nick repair mechanism which is similar to the cleavage system has been suggested for HSV (Varmuza and Smiley, 1985). In this model ss cleavage is induced by a packaging complex which might be composed of immature capsids.

Studies on λ have also shown that phage DNA passes through, or makes contact with, a "connector structure" at the base of the prohead (Serwar, 1976; Coombs and Eiserling, 1977), and in vitro analyses of phage packaging have suggested that it is an active process

since ATP is required as a form of energy (Syvanen and Yin, 1978; Poteete et al., 1979).

Phage DNA makes contact at, and may interact with, the inner wall of the capsid, then coils inwards due to its stiffness and charged nature (Earnshaw and Harrison, 1977), becoming packaged as a superhelical toroid (King et al., 1973; Earnshaw et al., 1976). Following packaging, phage DNA is stabilised within the capsid by the addition of polyamines (Earnshaw and Casjens, 1980). The loss of "scaffolding proteins" from the unstable intermediate structure results in the formation of a mature phage head (Bjornsti et al., 1983, Guo et al., 1986).

HSV DNA encapsidation has many parallel aspects to these stages of encapsidation and maturation of phage DNA. p40, a low processed form of the ICP35 family may be analogous in some ways to the scaffolding proteins. This polypeptide appears to be intrinsically involved in DNA packaging, but whilst it is closely associated with empty capsids, it is absent from full capsids (F.J.Rixon, personal communication). The reasons for the alteration in p40-capsid association are unknown, but stabilisation of a mature full HSV capsid through the dis-association of p40 cannot be excluded.

However, p40 is again detectable in the mature virion. In addition, polyamines are found in the HSV capsid (Gibson and Roizman, 1971), and these may stabilise the newly packaged viral DNA which is wound about a cylindrical core. Finally, the core undergoes condensation to produce the toroidal HSV DNA structure (Furlong et al., 1972; Perdue et al., 1976).

Despite the similarities in packaging models, there is clearly a limit to the comparisons that can be made between HSV and the ds DNA bacteriophages since the viruses differ in many ways apart from that of the host cell which they infect. For example, structurally, the ds DNA phages have tails and tail fibres, and lack an envelope or tegument structure comparable to that of HSV. However, it is interesting that these viruses follow the same basic mechanisms of DNA encapsidation; that is, the site-specific cleavage of DNA, and the packaging of a genome into a preformed prohead or capsid shell. In contrast, a number of animal viruses have been shown, or are believed, to form a capsid shell about replicated DNA or RNA.

This may be the case with adenovirus, a non-enveloped DNA virus. It was originally proposed that insertion of adenovirus DNA and core proteins into preformed

capsids was by means of a specific recognition signal 290-330bp from the left hand end of the genome (Weber, 1976; Hammerskjold and Winberg, 1980; Philipson, 1984). Weber et al. (1985), however, found that virion assembly was dependent on concurrent DNA synthesis and Nicolas et al. (1983) have shown that inhibitors of DNA synthesis also inhibited adenovirus assembly, suggesting that virions arise from assembly of capsids around replicated virus genomes. A similar mechanism is believed to be employed by SV40. SV40 replicated DNA is present as nucleoprotein complexes (NPCs), and is then processed to form young, and mature virions, although the mechanism for this maturation remains unknown (Cosa Prados and Hsu, 1979; Garber et al., 1980; Blasquez et al., 1983).

The Rhabdoviruses are a group of non-segmented, negative strand RNA viruses, which include the animal pathogens, rabies and vesicular stomatitis virus (VSV)(Wagner, 1975). The replicated RNA in these viruses is encapsidated by a single major structural protein, termed N, which becomes associated with the RNA strand as a single molecule by means of a recognition signal sequence in the first 18 bases of the 5' leader. N protein molecules then coat the

genome 3' to the initiation point forming the ribonucleoprotein (RNP)(Emerson, 1985). Processed proteins L and NS enter the RNP, and a matrix (M) protein becomes externally associated with the RNP. The M protein may be thought of a structurally analogous to the HSV tegument, and is believed to interact with the glycosylated protein (G) to promote envelopment (Newcomb and Brown, 1981). A similar matrix is present in the influenza virion between the lipid envelope and nucleoprotein (Schule, 1972) and again the M protein has been suggested to be involved in the process of envelopment rather than encapsidation. It is interesting, however, that in some preparations of influenza-infected cells a space can be observed between the matrix and envelope (Nermut, 1972), perhaps indicating that the matrix may not be exclusively associated with envelopment, although other data suggests that the M protein may penetrate into the lipid bilayer (Lenard et al., 1974).

An alternative system of replication and encapsidation is found on examination of the poxviruses, which include vaccinia. These are large ds DNA viruses that multiply exclusively in the cell cytoplasm, and are therefore in a very different situation to that of DNA viruses such as the herpesviruses which replicate in the nucleus.

The immature pox virion is composed of a nucleoprotein fibrous matrix inserted into a spherical lipoprotein bilayer, or envelope, synthesised de novo in the cell cytoplasm, whose curvature is controlled by the precise attachment of spiracles (Peters, 1956; Westwood et al., 1964; Dales and Pogo, 1981). The virion is then matured by an internal reorganisation resulting in a dumbell-shaped core containing DNA, and an outer region containing two lateral bodies, surrounded by the envelope, where external spiracles are lost or replaced by surface tubular elements. Thus the immature spherical particle is converted to a brick-shaped mature virion (Sarov and Joklik, 1973).

3.22.6 The Location and Role of the Tegument in Herpes Simplex Virus

The tegument, possible because it is difficult to isolate, and is an ill-defined structure, has not been studied in detail for any herpesviruses. Information on the structural composition of the tegument of HSV has been reported only in a review by Roizman and Furlong (1974) and from this report Vmw65 cannot be exclusively associated with either the virus capsid or the envelope.

In one set of experiments described in this article, the envelope and tegument proteins were removed from HSV-1(F) virions by treatment either with the non-ionic detergent NP40 or with a solution of NP40, deoxycholate, and urea. A measurement of the attachment of residual proteins to the capsid was then made on the assumption that the relative recovery of proteins reflected their proximity and strength of binding to the capsid. In a second series of experiments, virions were externally labelled by means of tritiated borohydride reduction of Schiff's bases formed between pyridoxal phosphate and the protein amino group in the surface of the virions. The basis of this work was that extent of labelling should be inversely proportional to the distance from the surface of the virion (Roizman and Furlong, 1974). The results from these experiments are shown diagrammatically in Figure 43. The data derived from treatment of virions with NP40 suggests that Vmw65 is associated with the envelope, whereas results from experiments in which the extrinsic labelling technique was used indicate that Vmw65 is more closely associated with the capsid (Figure 43).

Gibson (1981) examined CMV Colburn strain capsids, purified from virus-infected cells, treated with NP40

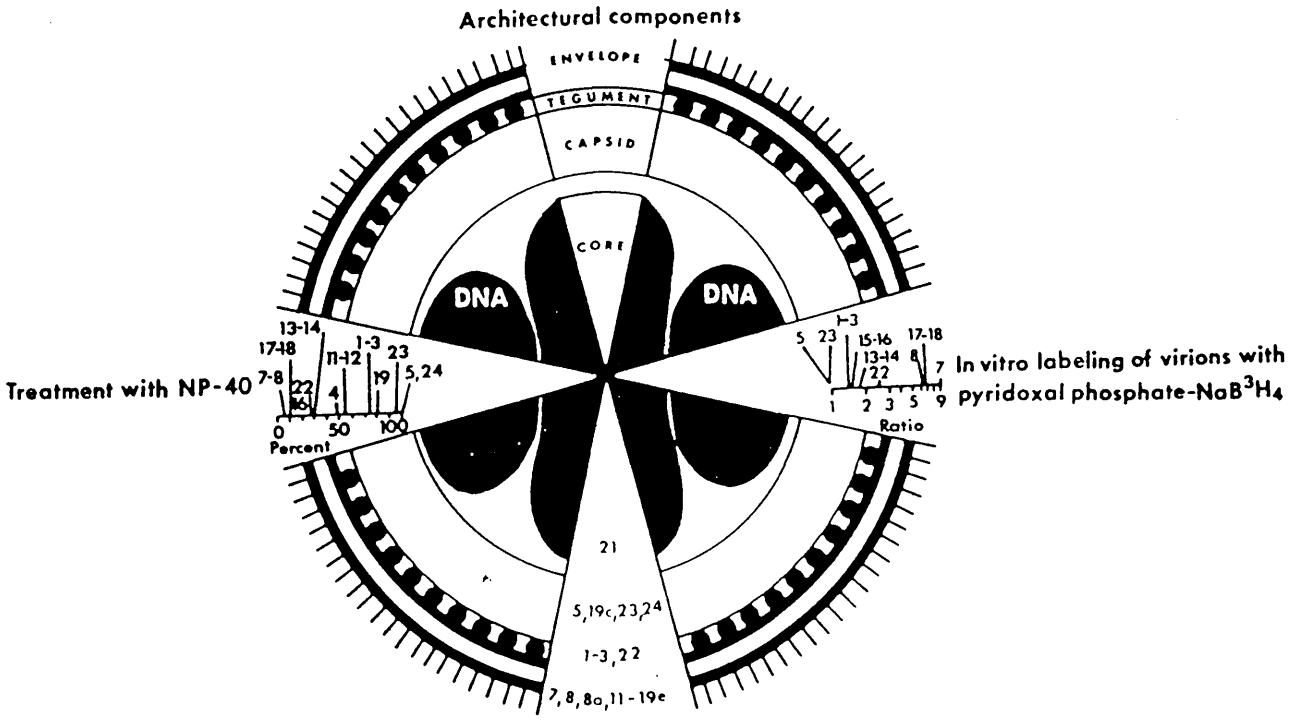
Figure 43

Schematic diagram of the HSV-1 strain F herpesvirion

(a) Possible order of polypeptides in envelope and tegument of the virion based on the relative efficiency of stripping from the virion by the nonionic detergent NP-40. Polypeptide number is plotted in terms of percentage remaining attached to virion after extraction with detergent.

(b) Possible order of polypeptides in the envelope and tegument of the virion based on relative accessibility of polypeptides to labelling by tritiated borohydride reduction of the Schiff's base formed between the polypeptide and pyridoxal phosphate. Polypeptide number is plotted from the ratio of ^{3}H-external label donated by tritiated borohydrate and ^{14}C-label from ^{14}C-amino acid mixture added to the culture medium.

Taken from Roizman and Furlong (1974).



VP	24	5	23	19	1-3	11-12	4	13-14	22	16	17-18	7-8		
Vmw	23	155	37	55	273	100	175	82/81	38	65	gD	gB-gC		
					248									

and dounce homogenised. In the most complex of three capsid forms he identified a 205,000 MW polypeptide analogous to the HSV Vmw273 tegument polypeptide (Spear and Roizman, 1972; Gibson and Roizman, 1972), and a phosphorylated 66,000 MW protein which was thought to interface the viral nucleocapsid and envelope, and therefore is probably equivalent to HSV Vmw65. The data indicates that the CMV counterpart to Vmw65 is associated with the capsid rather than the envelope, although Gibson (1981) had suggested that the tegument structure interacts with glycoprotein to promote virus envelopment.

The results presented in this thesis do not exclude the possibility that the tegument, or indeed Vmw65, has a role in envelopment, but on the basis of this work, a model for the involvement of Vmw65 in the packaging of HSV DNA is proposed in which Vmw65 binds to the virus capsid prior to packaging of DNA.

This model makes the assumption that a capsid-tegument complex must be formed in order for cleavage and packaging of viral DNA to proceed. A ts lesion in Vmw65 would prevent the production of such a complex at the NPT, and viral DNA would be neither cleaved nor packaged until synthesis of functional Vmw65 was

allowed to proceed at the PT. The model does not anticipate direct involvement of Vmw65 in the encapsidation of DNA, since the protein has been definitively shown to lack any DNA binding properties, but it does not preclude the involvement of other tegument proteins in such a role. Whilst this mechanism does not exclude the possibility that Vmw65 or the tegument as a whole can promote envelopment of the capsid, perhaps by an attraction between certain

glycoproteins and the tegument, the possibility that multiple functional forms of Vmw65 exist in the virion cannot be excluded at this stage. In this case, differently processed forms of the proteins might function in encapsidation, and in envelopment and the trans-induction of HSV IE gene expression during the next round of infection.

3.23 FUTURE DIRECTIONS

The work on ts2205 strongly suggests that the tegument protein Vmw65 is important for viral DNA encapsidation. To prove conclusively that the ts lesion in ts2205 lies within the Vmw65 gene, it will be necessary to sequence the region in the ts2205 genome where the mutation has been mapped by marker rescue, and to compare it with the WT sequence. It will also be interesting to

analyse the Vmw65 gene in each of the five independently isolated ts+ revertants to find out whether the base pair change has occurred at the site of the ts mutation or elsewhere, and to determine the nature of the amino acid substitution; such a study may identify interacting regions of Vmw65.

The immunofluorescence study indicated that Vmw65 was located in the nucleus of both WT- and mutant virus-infected cells. It is important to find out whether Vmw65 is present at the inner nuclear membrane or if it is associated with a particular type of capsid. This information would be of great value in elucidating the role of Vmw65 in encapsidation of viral DNA and in determining the reason for the defect in ts2205-infected cells at the NPT.

Vmw65 can be located by immune electron microscopy, using either the available monoclonal antibodies, which recognise different epitopes on Vmw65, or an antiserum raised against an oligopeptide specific to a portion of Vmw65.

The development of a cell-line expressing WT HSV Vmw65 would allow the isolation of host range mutants with defects in Vmw65. The advantage of this method of

mutant isolation is that a range of defined mutations in Vmw65 can be introduced into the virus. The phenotype of any mutant can thus be examined to determine the function of Vmw65. It will be interesting to find out whether, as suggested by Gibson (1981), this polypeptide is required for virus envelopment as well as for the encapsidation of viral DNA, and trans-activation of IE gene expression.

CHAPTER 4

4. ANALYSIS OF HSV-1 Vmw65: A STRATEGY TO IDENTIFY THE FUNCTIONAL DOMAINS OF AN HSV GENE

4.1 Introduction

Post et al (1981) and Batterson and Roizman (1983) showed that HSV IE transcription is strongly stimulated by a trans-acting structural component of the virion located outside the nucleocapsid, since trans-activation of IE gene transcription was detectable in infections at the NPT with tsB7 (a ts mutant which fails to uncoat at the NPT). Subsequently, Campbell et al. (1984) identified this polypeptide component using a novel application of the cotransfection and transient expression assay.

In the experiments of Campbell et al. (1984), cloned DNA fragments, spanning the HSV-1 genome, were transfected into cells together with an HSV-1 IE-TK chimaeric plasmid. The plasmid pTKN2 (Figure 44) consisted of the TK gene under the control of 5' upstream regulatory regions of IE175; these included the target sequences for stimulation by the virion component, the TAATGARATTC consensus, represented in IE175 by TAATGAGATGC (Murchie and McGeoch, 1982; Mackem and Roizman, 1982a, 1982b; Whitton and Clements, 1984a), and GA-rich flanking regions (Preston et al., 1984; Bzik and Preston, 1986), extending to 331bp upstream from the IE175 mRNA start site. Cells were harvested at 16h post-transfection, and cell extracts assayed for stimulation of TK expression from pTKN2. The gene sequences encoding the trans-inducing factor

Figure 44

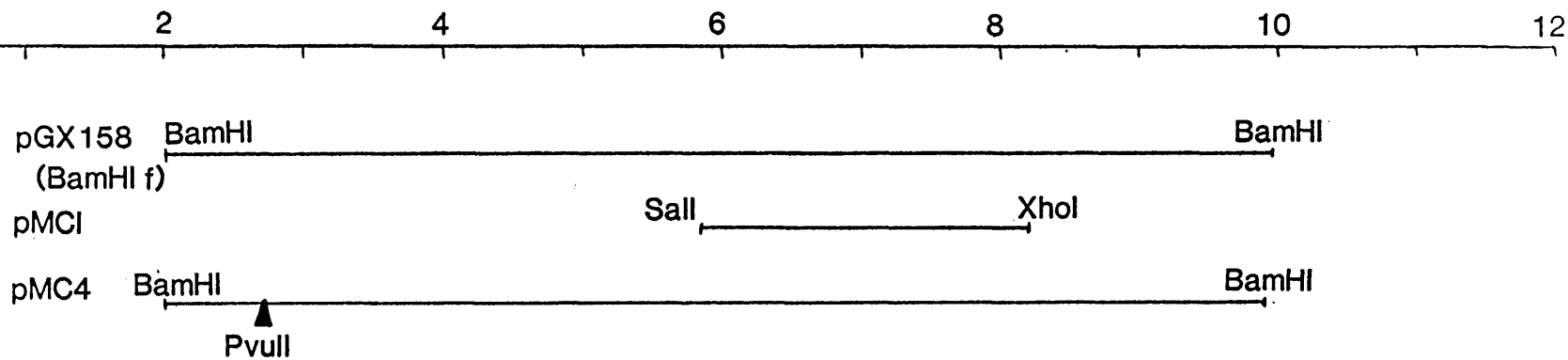
a. Location of trans-activating sequences in HSV-1

Transient expression assays were used to locate trans-acting stimulatory sequences in HSV-1 to the EcoRI i fragment. Sequences were isolated to BamHI f (pGX158), and shown to be contained within pMC1, which contains a SalI-XhoI fragment of BamHI f cloned into pUC9.

pMC4 is pGX158 (BamHI f), with an EcoRI 8bp insertion at a PvuII site outside the stimulatory sequences of pMC1.

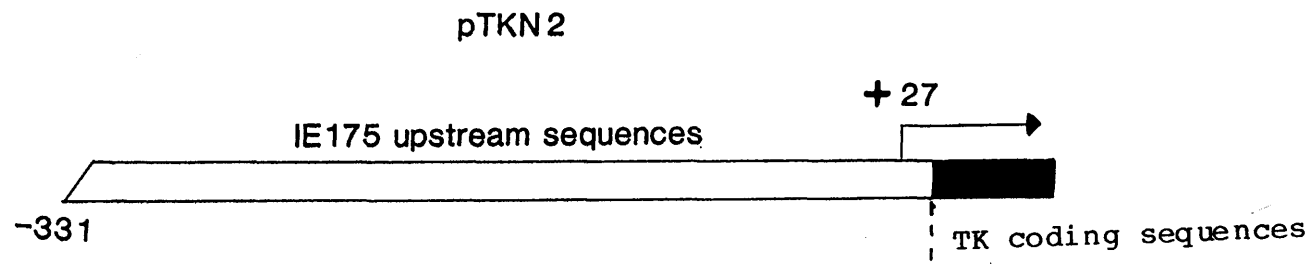
b. pTKN2 is a chimaeric plasmid containing the upstream sequences of the gene encoding IE175 responding to Vmw65 stimulation, to -331bp. These were linked to the coding sequences of the HSV-1 TK gene. The data are taken from Campbell et al. (1984).

a.



b.

Chimaeric TK plasmid structure



were thus mapped to a portion of BamHI f, and subcloned into the pUC9 vector as a 2.7kb SalI-XhoI DNA fragment (pMC1) (Campbell et al., 1984), Figure 44.

pMC1 contains sequences which encode a 1.9kb mRNA, flanked by noncoding regions from adjacent genes whose polypeptide products have approximate MWs of 33,000 and 72,000 (Dalrymple, 1986). The 1.9kb mRNA encoded a 65,000 MW polypeptide (Hall et al., 1982) and hybrid arrested translation experiments and immune precipitations, using a monoclonal antibody, MA1044, identified this as the tegument protein, Vmw65 (Campbell et al., 1984). Subsequent sequencing of BamHI f confirmed that only a single complete open reading frame existed within pMC1 (M Dalrymple, 1986), and a MW of 54,342 was predicted for the HSV-1 strain 17 Vmw65 protein (Dalrymple et al., 1985). The HSV-1 strain F gene encoding Vmw65 has also been sequenced (Pellet et al., 1985).

In order to demonstrate that in vitro stimulation of TK expression from an IE-TK chimaeric plasmid was due to the expression of the Vmw65 polypeptide, the Vmw65 gene coding sequences were interrupted by the insertion of an 8 base pair EcoRI DNA oligonucleotide linker (GGAATTCC) at a PvuII site. This procedure disrupted the normal reading frame, and no stimulation of TK expression could be detected on cotransfection of this altered plasmid into cells with pTKN2. Similarly, an EcoRI DNA oligonucleotide linker was inserted into the

cloned HSV-1 BamHI f fragment at a PvuII restriction endonuclease site outside the pMC1 (Vmw65) region, producing a plasmid termed pMC4. This insertion had no effect on stimulation of IE transcription in vitro. Campbell et al. (1984)

4.2 Analysis of Vmw65

The tegument polypeptide, Vmw65, has a dual role in the virion; as a structural polypeptide which is required for packaging of viral DNA, and as a trans-inducing factor for IE gene transcription. To study the relationship between structure and function, the gene was analysed for functional domains within the coding sequences of Vmw65.

This process involved the mutagenesis of cloned HSV-1 Vmw65 (pMC1) by the insertion of 6 base pair XbaI oligonucleotide linkers, at positions throughout the DNA coding sequence of the gene. Thus, in frame insertion mutants would be produced. Mutagenised plasmids could be analysed for: (1). The ability to stimulate IE gene transcription, by assaying for stimulation of expression from an IE-TK chimaeric plasmid in a transient expression assay (2). The ability to rescue the ts lesion in ts2203 (intertypic marker rescue). The failure to perform either function could then be interpreted as the disruption of an essential function domain. However, the retention by a mutagenised plasmid, of the normal ability to rescue ts2203, could also be indicative of recombination events between the site of the insertion in pMC1, and

the site of the ts lesion in ts2203, resulting in the production of a WT recombinant virus. Therefore, the ability to rescue ts2203 did not exclude the possibility of a disrupted functional domain within the plasmid.

4.3 Strategy for Insertional Mutagenesis

The procedure is summarised in Figure 45.

4.3.1. Preparation of Linear pMC1 DNA

pMC1 DNA was restricted with a restriction endonuclease which cleaves at multiple sites within pMC1, in the presence of ethidium bromide (EtBr), an intercalating agent which preferentially binds to linear molecules rather than to supercoiled DNA, preventing further digestion of DNA. Preliminary experiments determined the optimal concentrations of EtBr and enzyme required for partial digestion to give maximal recovery of linear pMC1 DNA (Figure 46), and a bulk preparation of linear DNA was then made.

DNA fragments were separated by agarose gel electrophoresis, and linear molecules eluted from the gel slices (Section 2.5.2). DNA was extracted with saturated butan-2-ol to remove any EtBr and then purified.

4.3.2. Insertion of XbaI Oligonucleotide Linkers

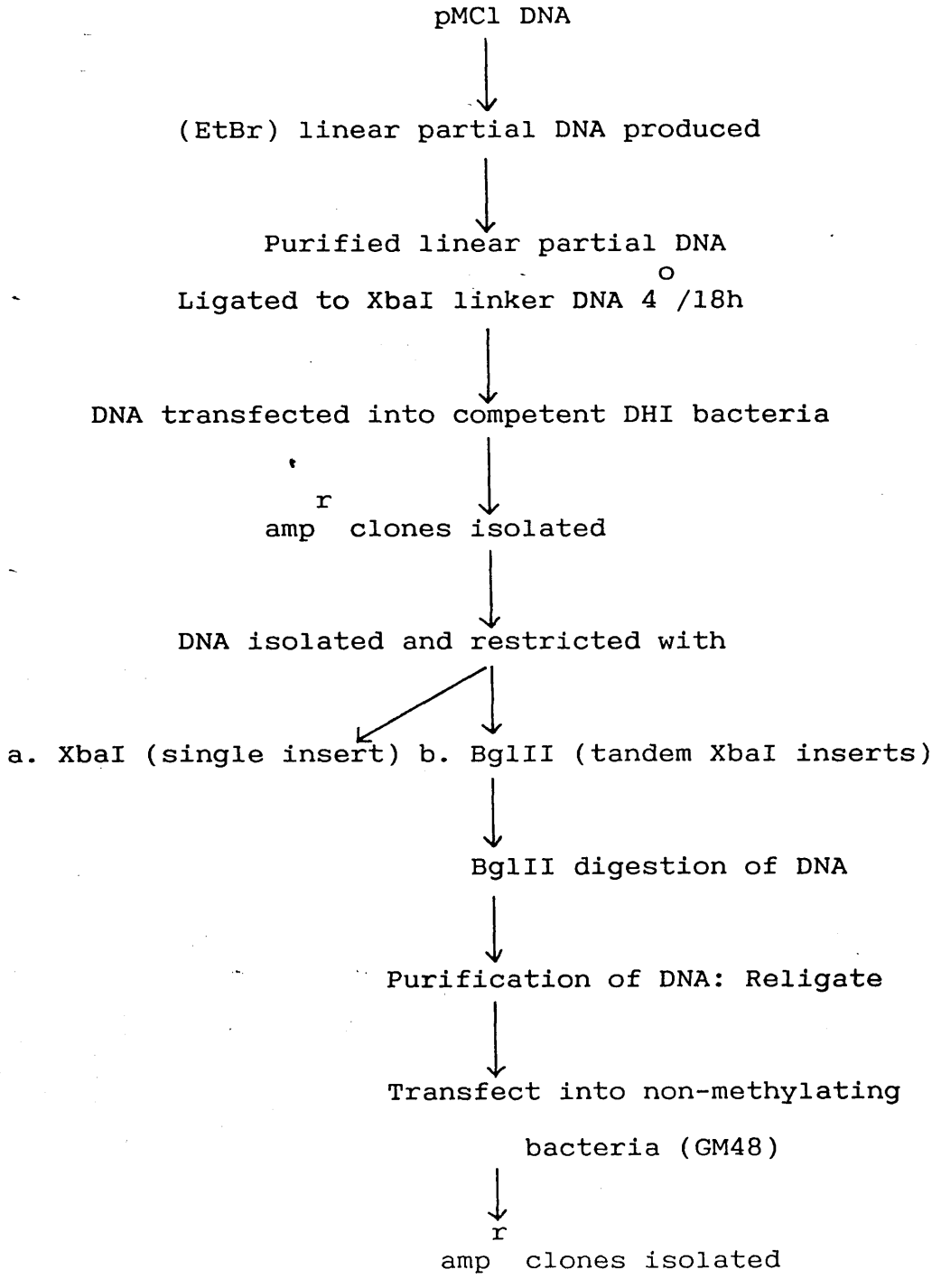
In order to define discrete functional domains of

Figure 45

Flow chart of the procedure for insertional mutagenesis of pMC1

Figure 45

Flow Chart: The Procedure for Insertional Mutagenesis of pMC1



Plasmid DNA can be restricted with XbaI, and BglII.

↓

Plasmid is restricted with XbaI, and religated

↓

A 6bp XbaI linker is now retained in all plasmids

↓

Insertion located within Vmw65 sequences (Figure 47)

↓

Precise location of insert (Figure 48)

↙ ↘

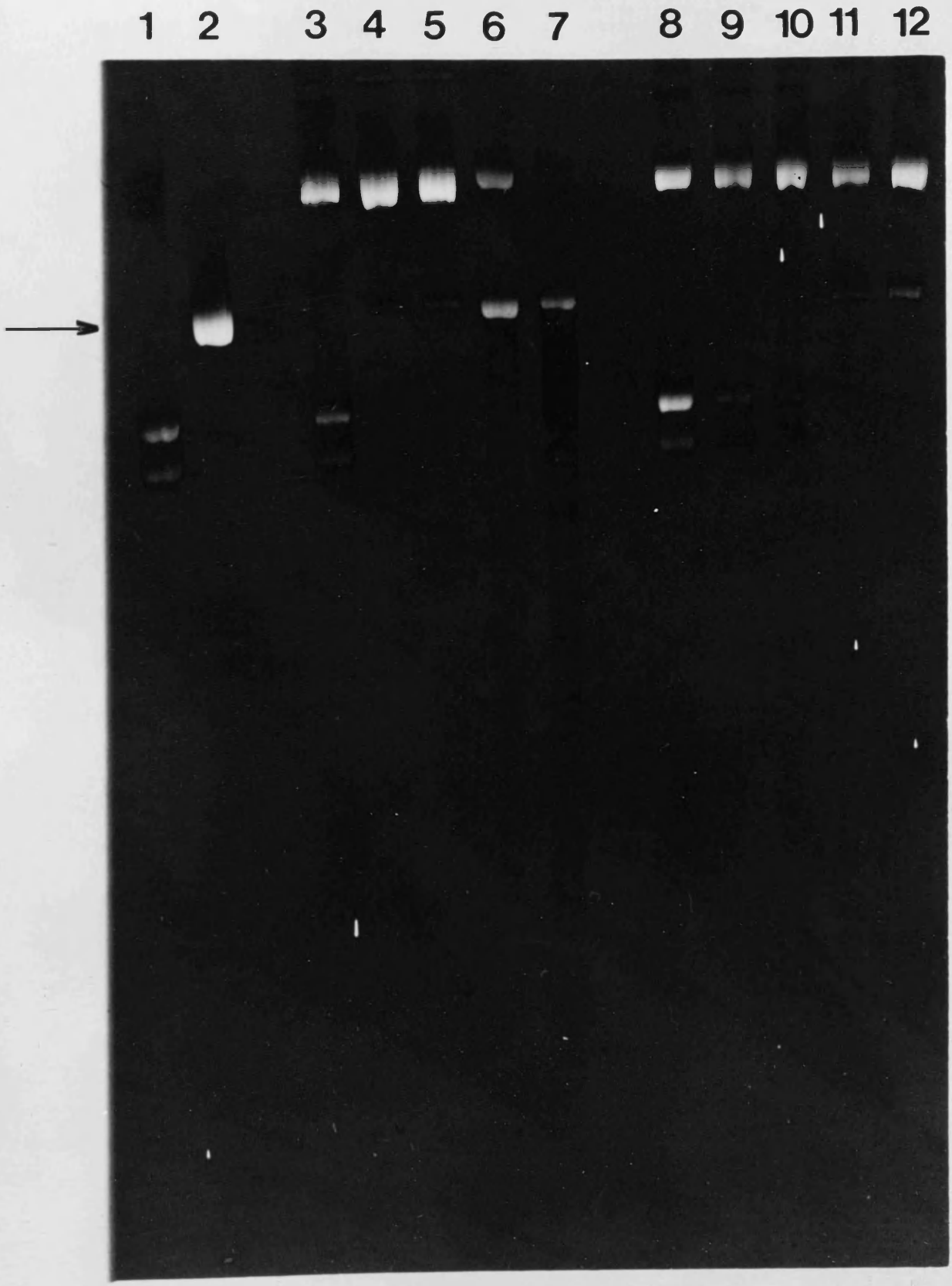
Transient expression assay (Table 14)

Marker rescue assay (Table 15)

Figure 46

Preparation of linear pMC1 DNA

Track 1: undigested pMC1 DNA; track 2: HindIII digested pMC1, linear DNA. pMC1 DNA (2ug) was digested with 2u HaeIII endonuclease at 37°C for 120min (tracks 3-7) or 60min (tracks 8-12) in the presence of 2.0ug EtBr (tracks 3,8); 1.0ug EtBr (tracks 4, 9); 0.5ug EtBr (tracks 5, 10); 0.25ug EtBr (tracks 6, 11); 0.125ug EtBr (tracks 7, 12). Samples were separated by agarose gel electrophoresis (1% agarose gel) and linear partial DNA identified against pMC1 linear DNA (arrowed).


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

the gene, it was essential to limit disruption to the Vmw65 sequence, so in these experiments 2 amino acid codons were inserted into the sequence. Synthetic 6bp oligonucleotide linkers used for this purpose were blunt-ended and phosphorylated, and were composed of the nucleotides (5'-TCTAGA-3'), a sequence which is recognised by XbaI restriction endonuclease and does not occur in pMC1. All mutagenised plasmids could, therefore, be screened for the presence of a unique XbaI site.

Insertions were made into blunt-ended sites generated by restriction endonucleases (RsaI, AluI, ThaI or HaeIII), but insertion of oligonucleotide linkers was only achieved at HaeIII endonuclease sites (GG'CC). Purified linear partial DNA was treated with phosphatase, and then ligated to XbaI 6bp linkers (at a molar ratio of 10-100 times that of the plasmid DNA) in a 10ul volume at 4°C for 16-18h in the presence of T4 DNA ligase. Following ligation, the DNA was transfected into competent E.coli DHI bacteria (Section 2.5.4), plated onto L-broth agar plus ampicillin, and incubated at 37°C. Ampicillin resistant clones were harvested and plasmid DNA extracted (Section 2.5.5).

4.3.3 Analysis of XbaI Insertions

Because the ratio of linker to plasmid DNA was

very high in the ligation mix, it was anticipated that multiple tandem linkers would become ligated into the HaeIII site. Under these conditions adenine residues in the sequence 5'-GATC-3', which is formed by tandem XbaI linkers (5'-TCTAGATCTAGA-3'), would become methylated by the bacterial host (dam methylation), and rendered insensitive to XbaI endonuclease digestion. All plasmids were, therefore, screened with XbaI (for any single linker insertions), and BglII, which recognises the sequence in a double XbaI insertion

(5'-TCTAGATCTAGA-3') (BglII; XbaI XbaI) but which is not affected by

dam methylation of adenine residues.

All plasmids isolated by this method contained at least 2 XbaI 6bp insertions, since they were digested by BglII alone. The DNA was, therefore, linearised with BglII, and purified with ethanol to remove the small fragments. DNA was then religated and transfected into non-methylating E coli GM48 bacteria (Marinus, 1973). Plasmid DNA was harvested from the amp^r bacterial clones, and could be restricted with XbaI enzyme. Plasmids were then digested with XbaI and religated, to produce samples with 6bp insertions.

4.3.4 Location of XbaI Oligonucleotides in the pMC1 Sequence

An EcoRI/EcoRV digestion separates the plasmid

vector and Vmw65 sequences present in pMC1 (Figure 47) and the further addition of XbaI to the digestion of mutagenised plasmids showed whether the insertion was in vector or coding sequences (Figure 47). Plasmids with gross deletions, or an insertion at a HaeIII site in the vector were discarded.

The location of the inserts was determined by digestion with pairs of restriction enzymes (ApaI and XbaI, PvuII and XbaI, SalI and XbaI or SmaI and XbaI), followed by separation of the fragments on a 16% polyacrylamide gel. The size of the fragment produced by a XbaI insertion was determined (Figure 48), and XbaI inserts were thus identified at HaeIII positions 5, 6, 11, 15 and 17 (Figure 49). These will be referred to as p5, p6, p11, p15 and p17. In addition, an XbaI oligonucleotide was inserted into pMC4 (Figure 44), at a unique PvuII site within the Vmw65 gene.

pMC4 was restricted with PvuII, and XbaI linkers ligated to the DNA as previously described. On analysis, a single XbaI linker was found to be inserted into pMC4, thus destroying the remaining PvuII site (Figure 48). This plasmid was termed pP2. The location of all the insertions are given in the map of pMC1 in Figure 49.

Figure 47

Location of XbaI inserts in Mutagenised pMC1 Plasmid DNA

Track 1 pMC1 DNA digested with EcoRI plus EcoRV: (larger fragment represents vector DNA and 5' non-coding flanking sequences of Vmw65, smaller fragment is Vmw65 plus 3' non-coding flanking sequences); Tracks 2-7, mutagenised plasmids digested with EcoRI/EcoRV/XbaI; Track 8, pTK1 digested with BamHI plus SmaI to provide size markers.

In plasmids 2-6 the Vmw65 fragment is reduced in size as XbaI insert is in Vmw65 coding sequences. Tracks 3, 4 location of smaller fragment is indicated by an arrow. Track 7, the insert has been made in the vector: this plasmid was therefore discarded.

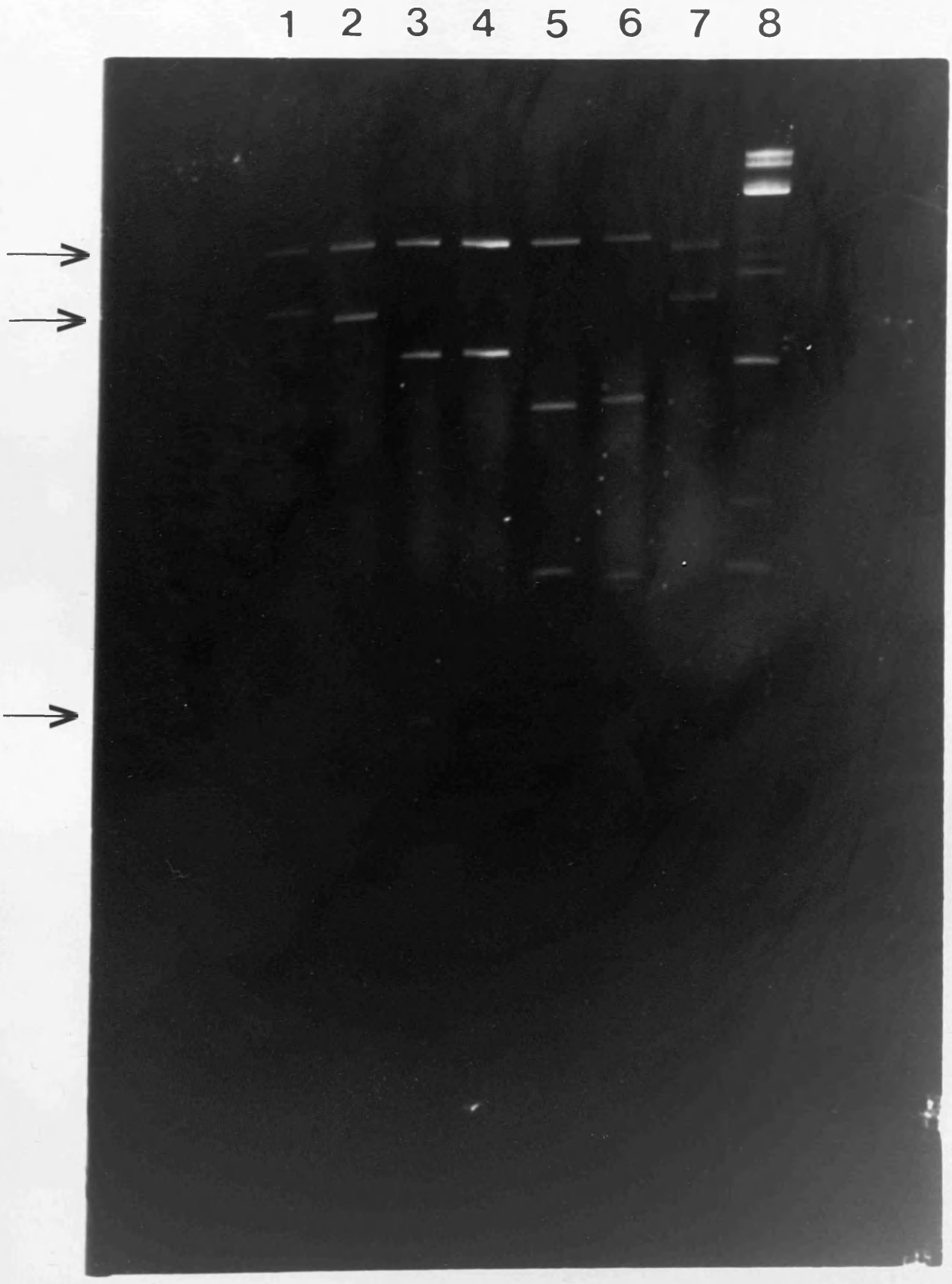

1 2 3 4 5 6 7 8

Figure 48

Location of XbaI oligonucleotide insertions in Vmw65 mutagenised plasmids

Mutagenised pMC1 DNA was digested with ApaI plus XbaI, PvuII plus XbaI, SalI plus XbaI or SmaI plus XbaI, and fragments separated on a 16% polyacrylamide gel. The presence of an XbaI site resulted in the production of a novel fragment, and the location of the insert could be determined from the size of this fragment relative to ApaI and PvuII sites, or SmaI and SalI sites in pMC1. The location of insertions is given in Figure 49.

1. U : undigested pMC1 DNA
L : linearised pMC1 DNA
1,2: insert at HaeIII site 17 [1: SalI, XbaI. 2: SmaI, XbaI]
4,5: Insert at HaeIII site 15 [4: SalI, XbaI, (where no novel fragment is produced) 5: SmaI, XbaI]
3: pAT size markers (HinfI, EcoRI]

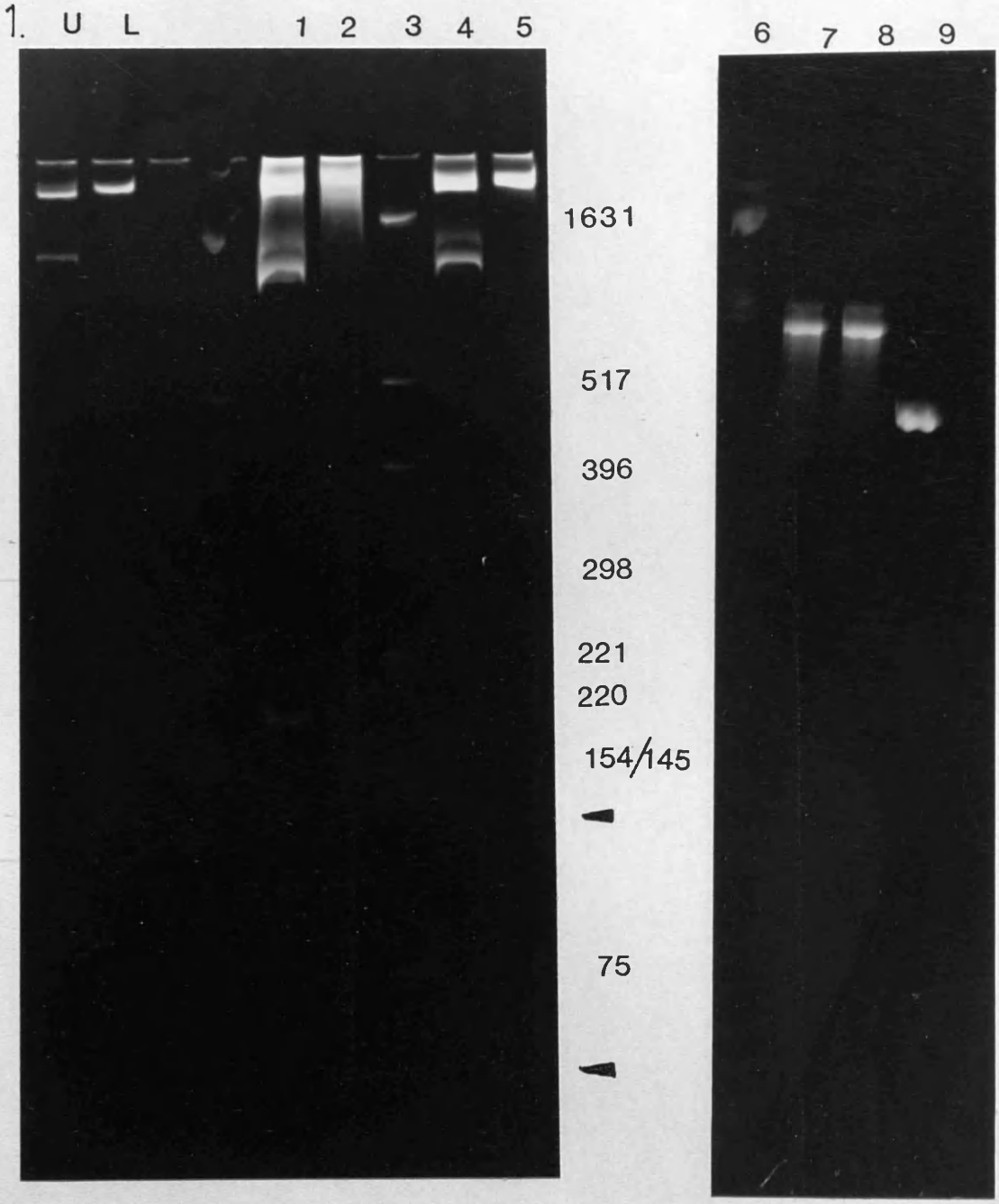
An XbaI insertion was made into pMC4 at a PvuII site in the Vmw65 coding region.

6: undigested pMC4 DNA
7: pP2 [XbaI]
8: pMC4 [linearised with PvuII]
9: pMC1 [linearised with HindIII]

Fragments were separated on a 1% agarose gel.

2. 1,2: Insert at HaeIII site 11 [1: ApaI, XbaI. 2: PvuII, XbaI]
4,5: Insert at HaeIII site 6 [4: ApaI, XbaI. 5: PvuII, XbaI]
7,8: Insert at HaeIII site 5 [7: ApaI, XbaI. 8: PvuII, XbaI]

3,6,9: PAT size markers [HinfI, EcoRI].


1.
U
L
1
2
3
4
5
6
7
8
9
1631
517
396
298
221
220
154/145
75

2.

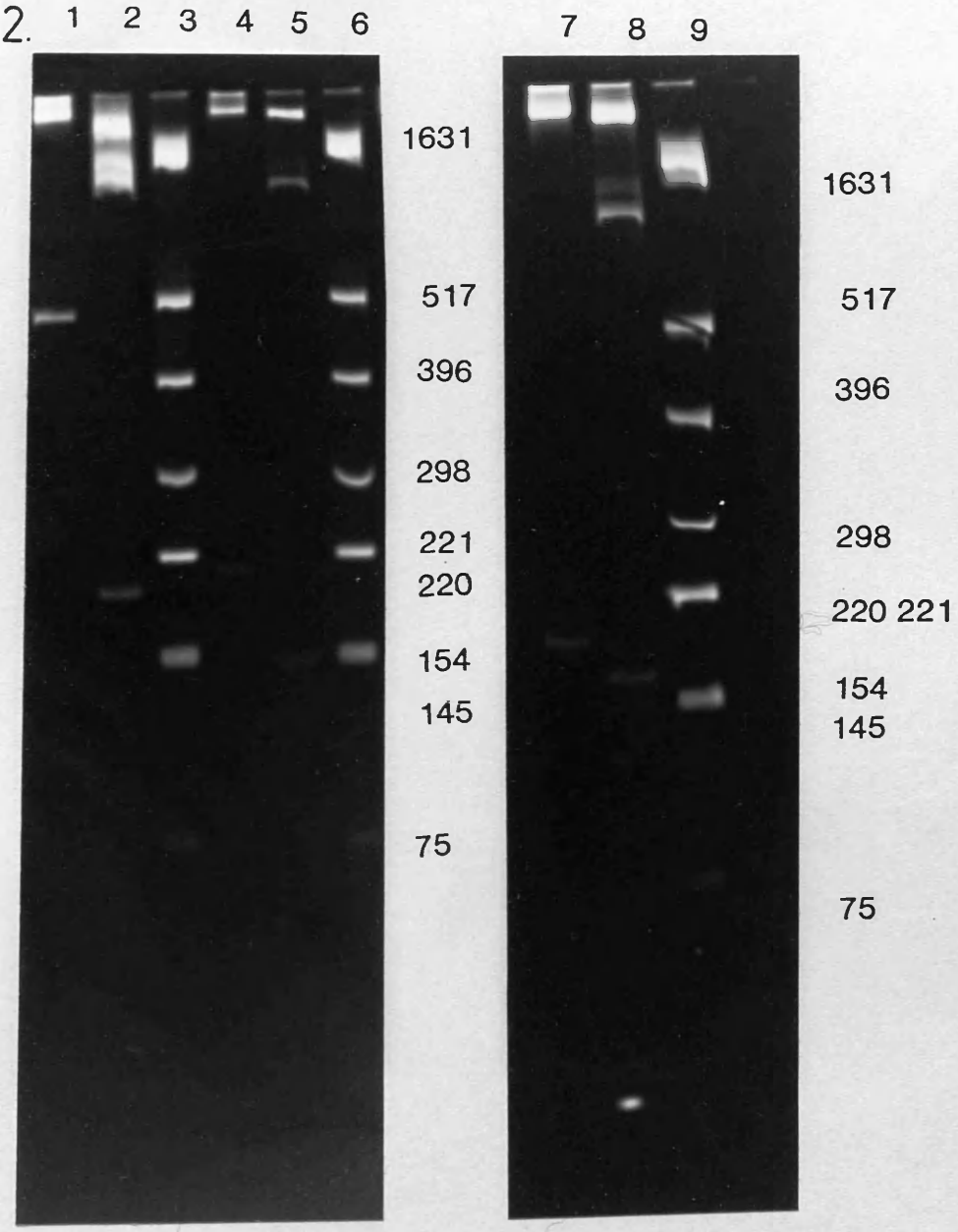
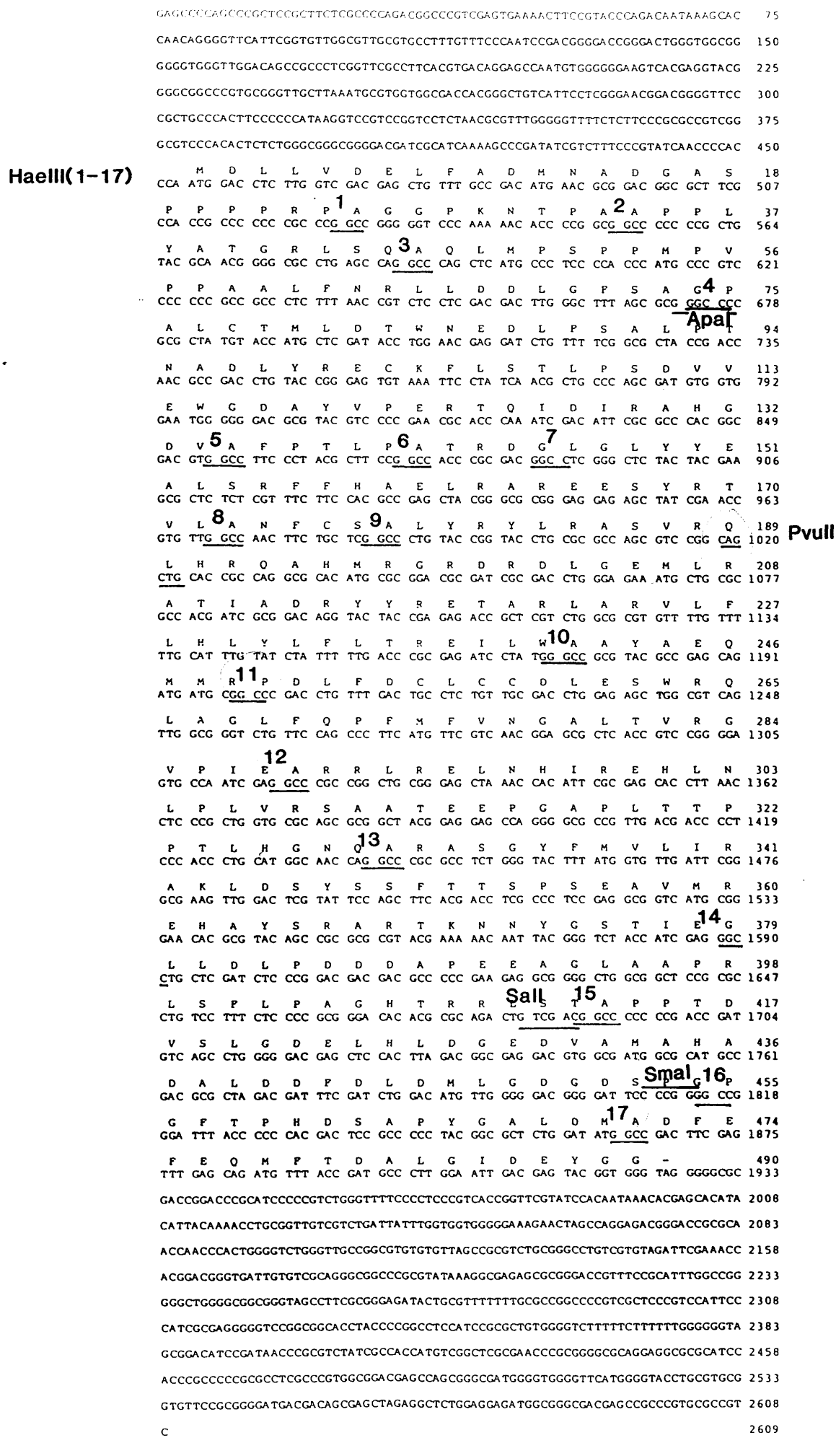

1 2 3 4 5 6
7 8 9
1631
517
396
298
221
220
154
145
75
1631
517
396
298
220 221
154
145
75

Figure 49

DNA Sequence and Amino Acid Sequence of pMC1

HaeIII sites are underlined, and numbered; HaeIII sites where an XbaI insertion was made are 5, 6, 11, 15 and 17. PvuII site where an XbaI insertion was made in pMC4 is P2.


HaeIII(1-17)
1
2
3
4
ApaI
5
6
7
8
9
PvuII
10
11
12
13
14
SalI
15
SmaI
16
17

4.4 Insertion of Amino Acid Codons

The insertion of the XbaI linker (TCTAGA) into the Vmw65 reading frame can result either in the addition of the amino acids serine and argine, leucine and asparate, or in the production of an amber termination codon (TAG) (Table 13). The insertions at positions 5, 6, 15 and 17 produced termination codons, which would generally preclude the synthesis of any functional polypeptide. However, there is a possibility that the insertion of such a codon close to the normal termination site would have a limited effect on polypeptide function. Interestingly, therefore, the XbaI insert at p17 is just 59bp upstream of the 'normal 'stop-codon'.

4.5 Functional Analysis of pMC1

Plasmids were analysed for the disruption of functionally essential domains within Vmw65.

4.5a. Analysis of domains of Vmw65 Important for Transcription Activation

WT pMC1 or mutagenised plasmid DNA was transfected into cells together with the chimaeric plasmid pTKN2, at 37°. Cells were incubated, and harvested after 24h; cell extracts were then assayed for TK activity (Section 2.6, Table 14). The results show that only p17 retained the WT Vmw65 ability to stimulate IE-TK expression. This suggests that pP2 affects an essential functional domain of Vmw65 which is

Table 13

Codon Changes in pMC1 Following Insertion of an XbaI Linker

Changes are described for plasmids p5, p6, p11, p15, p17 and pP2. Valine: V; alanine: A; threonine: T; glutamine: Q; leucine: L; serine: S; arginine: R; tryptophan: W; proline: P; phenylalanine: F; glutamate: E; methionine: M; stop codon:-

Locations of inserts are shown in Figure 49 (pMC1 sequence).

Table 13

Plasmid	pMC1 Sequence		Mutagenised Sequence			
	V	A	V	V	-	T
p5	GTG	GCC	GTG	GTC	TAG	ACC
	P	A	P	V	-	T
p6	CCG	GCC	CCG	GTC	TAG	ACC
	Q	L	Q	S	R	L
pP2	CAG	CTG	CAG	TCT	AGA	CTG
	R	P	R	S	R	P
p11	CGG	CCC	CGG	TCT	AGA	CCC
	T	A	T	V	-	T
p15	ACG	GCC	ACG	GTC	TAG	ACC
	M	A	M	V	-	T
p17	ATG	GCC	ATG	GTC	TAG	ACC

Table 14

Stimulation of pTKN2 Expression by Cotransfection with pMC1 or Mutagenised Plasmids

Cells were transfected with 1.0ug pTKN2, 1.0ug test plasmid and 1.0ug carrier DNA.

Stimulation represents the increase in expression of TK relative to pTKN2 (pTKN2 stimulation = 1.0).

The results of 2 experiments are given, and the value relative to pMC1 (where pMC1 stimulation = 100%) is also given.

Table 14

Plasmid	TK Stimulation from pTKN2			
	1.	2.	Mean Value	% of pMC1
pMC1	8.77	9.77	9.27	100%
p5	0.68	0.75	0.715	7.7
p6	0.65	0.85	0.750	8.1
p11	1.25	1.09	1.170	12.6
p15	0.70	0.86	0.780	8.4
p17	5.47	4.40	4.935	53.2
pP2	0.88	0.79	0.835	9.0

required for transcriptional stimulation, and that the sequences 3' to site 17 are not required for this function. The results obtained with p11 were less conclusive. Although Vmw65 activity was clearly diminished, some stimulation (approximately 13% of pMC1 levels) of IE-TK expression was consistently observed.

4.5b. Analysis of Structural Domains of Vmw65

The effects of the ts lesion in ts2203, which is located at the 5' end of the HSV-2 Vmw65 gene, and the marker rescue of this lesion by pGX158 (containing HSV-1 BamHI f DNA fragment) or pMC1, have been described in detail in Section 3.

Marker rescue of ts2203 was carried out, as previously described, at 31°C and virus progeny were screened at the PT and NPT for ts$^+$ virus.

The eop $\frac{NPT}{PT}$ of progeny virus is given in Table 15. From the results, p5, p6 and p15, all of which contain termination codons, failed to rescue ts2203. p11 also failed to rescue the lesion, suggesting that the XbaI linker in this plasmid is present within a functional domain for this property. Plasmids p17 and pP2 were both able to rescue the lesion. However, as described before, the recovery of ts$^+$ progeny is not complete proof that these inserts do not affect an essential domain, since WT recombinant viruses may have been produced without insertion of the oligonucleotide.

Table 15

Summary of Marker Rescue Experiments

ts2203 DNA and a 10 times molar excess of plasmid DNA was cotransfected into BHK cells, and progeny virus were titrated at the PT and NPT for ts^+ recombinant virus.

Rescue is calculated as eop $\left(\frac{38.5^{\circ}}{31^{\circ}}\right)$ x 100, and is indicated by + = rescue of lesion; - = no rescue.

This result is representative of a number of experiments.

Table 15

DNA Fragment or Plasmid	Eop x 100	Rescue
ts2203 (alone)	0.001	-
BglII i (HG52)	30.770	+
BglII i (ts13)	0.001	-
BamHI f (pGX158)	0.200	+
pMC1	0.080	+
5	0.0040	-
6	0.0100	-
P2	0.1890	+
11	0.0006	-
15	0.0070	-
17	0.1500	+

Therefore, failure to rescue ts2203 is more significant at this stage than the apparent ability to rescue the lesion.

4.5c. Hydropathicity Plot of Vmw65

Previous work (Dalrymple, 1986) demonstrated that the HSV-1 Vmw65 was highly charged and acidic, with significant proportions of β-pleated sheets, but that it had no extensive regions of hydrophobicity or hydrophilicity.

A graphical analysis of hydropathicity of Vmw65 and the mutagenised plasmids was carried out using the programme of Kyte and Doolittle (1982) to study the effects of newly inserted amino acids (Figure 50). Little change in hydropathicity was evident for any plasmid.

4.6 Functional Domains of HSV-1 Vmw65

The results of this analysis are given in Table 16, together with those obtained from a similar study in which 12bp BamHI insertions were made into HaeIII endonuclease sites of pMC1, resulting in addition of 4 amino acids (C.I.Ace, personal communication). In these experiments IE trans-activation by Vmw65 was assessed by cotransfection of plasmid plus an IE-CAT chimaeric plasmid, and subsequent CAT-assays. However, in all other respects, the procedures were the same as those described for XbaI insertional mutants.

Figure 50

Comparison of hydropathic profiles of pMC1 and mutagenised plasmids

Hydropathic profiles were predicted by the program of Kyte and Doolittle (1982).

Degree of hydrophobicity increases with distance above the mid-line; degree of hydrophilicity decreases with distance below the mid-line. Letters beneath the plots refer to amino acid residues.

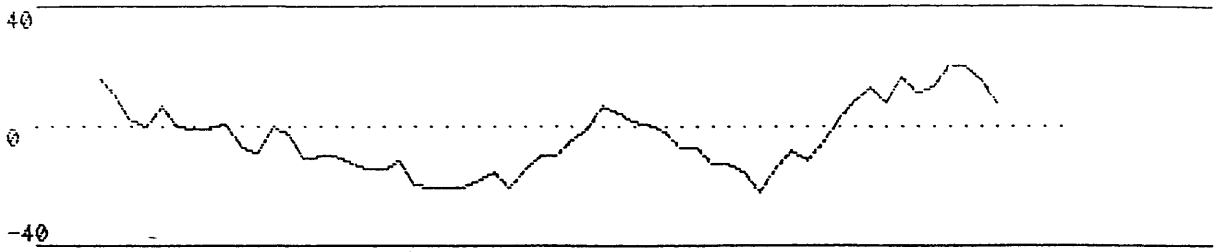
Parameters: group length = 9
shift = 1

pMC1 samples are given as representatives of the WT protein.

Hydropathicity

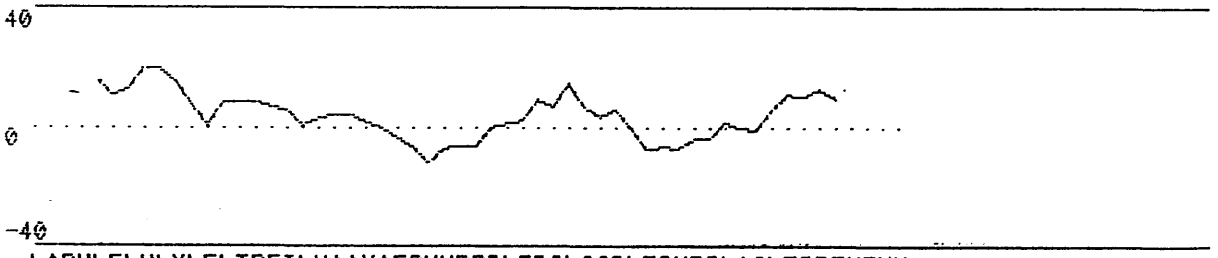
1. pMC1, 2. pP2 amino acids 169-250
3. pMC1, 4. p11 amino acids 226-281
5. pMC1, 6. p17 amino acids 449-490

1



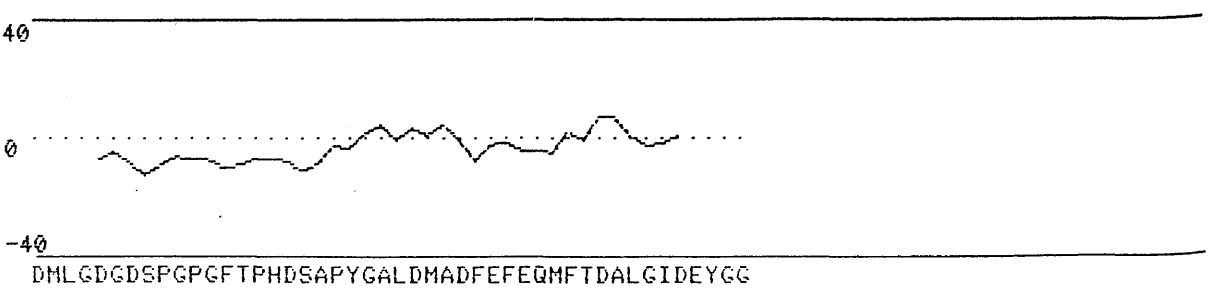
VLANFCSALYRYLRASVRQLHRQAHMRGRDRDLGEMLRATIADRYYRETARLARVLFLHLYLFLTR

3



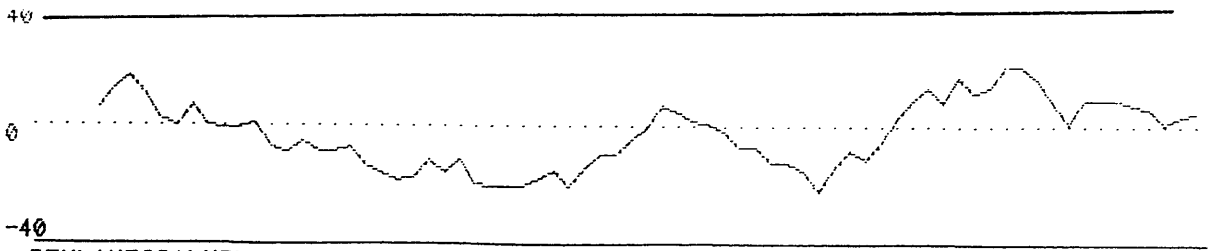
LARVLFLHLYLFLTREILWAAYAEQMMRPDLFDCLCCDLESWRQLAGLFQPFMFVN

5



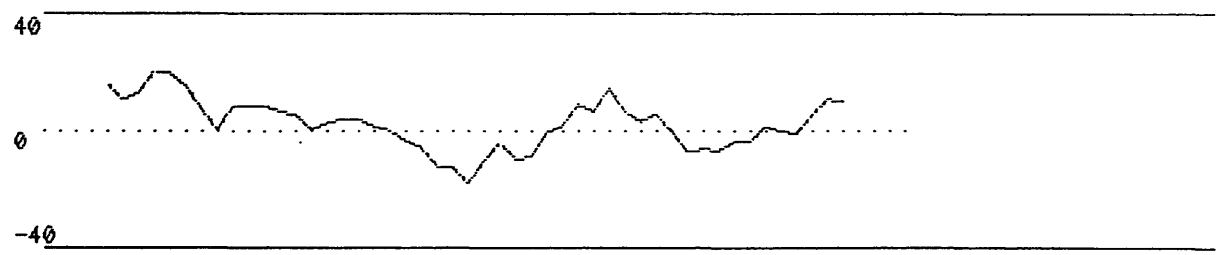
DMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG

2



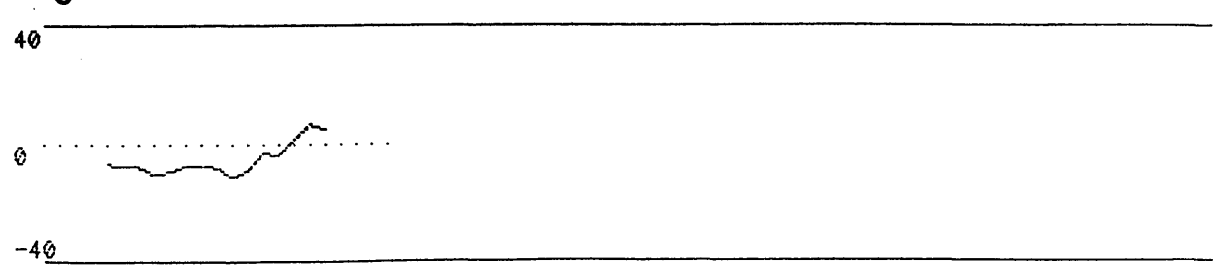
RTVLANFCSALYRYLRASVRQSRLHRQAHMRGRDRDLGEMLRATIADRYYRETARLARVLFLHLYLFLTREILWA

4



LARVLFLHLYLFLTREILWAAYAEQMMRSRPDLFDCLCCDLESWRQLAGLFQPFMF

6



GDSPGPGFTPHDSAPYGALDMV-

Table 16

Summary of insertional mutagenesis of Vmw65 (pMC1)

Insertions of 12bp (BamHI) or 6bp (XbaI) linkers were made at HaeIII sites (1-17 see Figure 49), and assessed for

1. Stimulation of TK or CAT expression from IE-TK or IE-CAT constructs and

2. For the marker rescue of ts2203.

(P2 insertion was made into pMC4, which encodes all of BamHI f).

Plasmids containing BamHI-linker insertions were supplied by Mr C I Ace.

Table 16

Insertions at HaeIII sites (1-17) within pMC1	Transcriptional Stimulation IE-TK(XbaI)	IE-CAT(BamHI)	Marker Rescue XbaI	BamHI
pMC1	+	+	+	+
2		+		+
4		+		-
5	-		-	
6	-		-	
7		+		+
8		-		-
9		-		-
PvuII	-	+/-	+	+
11	+/-	+	-	-
12		+		-
14		-		+
15	-	+	-	+
17	+	+	+	+

4.7 Predicted Secondary Structure of Vmw65

The insertion of amino acids and their effect upon the function of the Vmw65 protein can be related to their effect on the secondary structure of the protein. A prediction of the HSV-1 Vmw65 secondary structure was made using the Chou and Fasman computer program (Chou and Fasman, 1973), accepting only strongly predicted structures (Figure 51).

As expected, Vmw65 structure can be seen to have regions of hydrophilicity and hydrophobicity throughout the protein stucture. Also evident through the protein are strongly predicted α-helices and β-structures, as well as a number of reverse turns. In the majority of cases the insertion ofXbaI and BamHI inserts had little effect on the predicted stucture of Vmw65.

At p11, a 2 codon XbaI insertion altered the hydropathicity, whereas a 4 codon BamHI insert appeared to reduce the length of a strongly predicted α-helix (Figure 52).

At pP2, both inserts added hydrophilic sites to a region of weakly predicted structure.

At p17, a BamHI insert increased the hydrophilicity, however the XbaI insertion, producing a termination codon, eliminated a 3'- α-helical prediction (Figure 52).

Figure 51

Predicted secondary structure of pMC1

The predicted secondary structure of Vmw65 was determined using the Chou and Fasman computer program. Only strongly predicted structures were accepted:

Predicted α-helices, β-structures and reverse turns are indicated by α, β and r, respectively.

Hydrophobic and hydrophilic regions are indicated by O, h.

pMC1

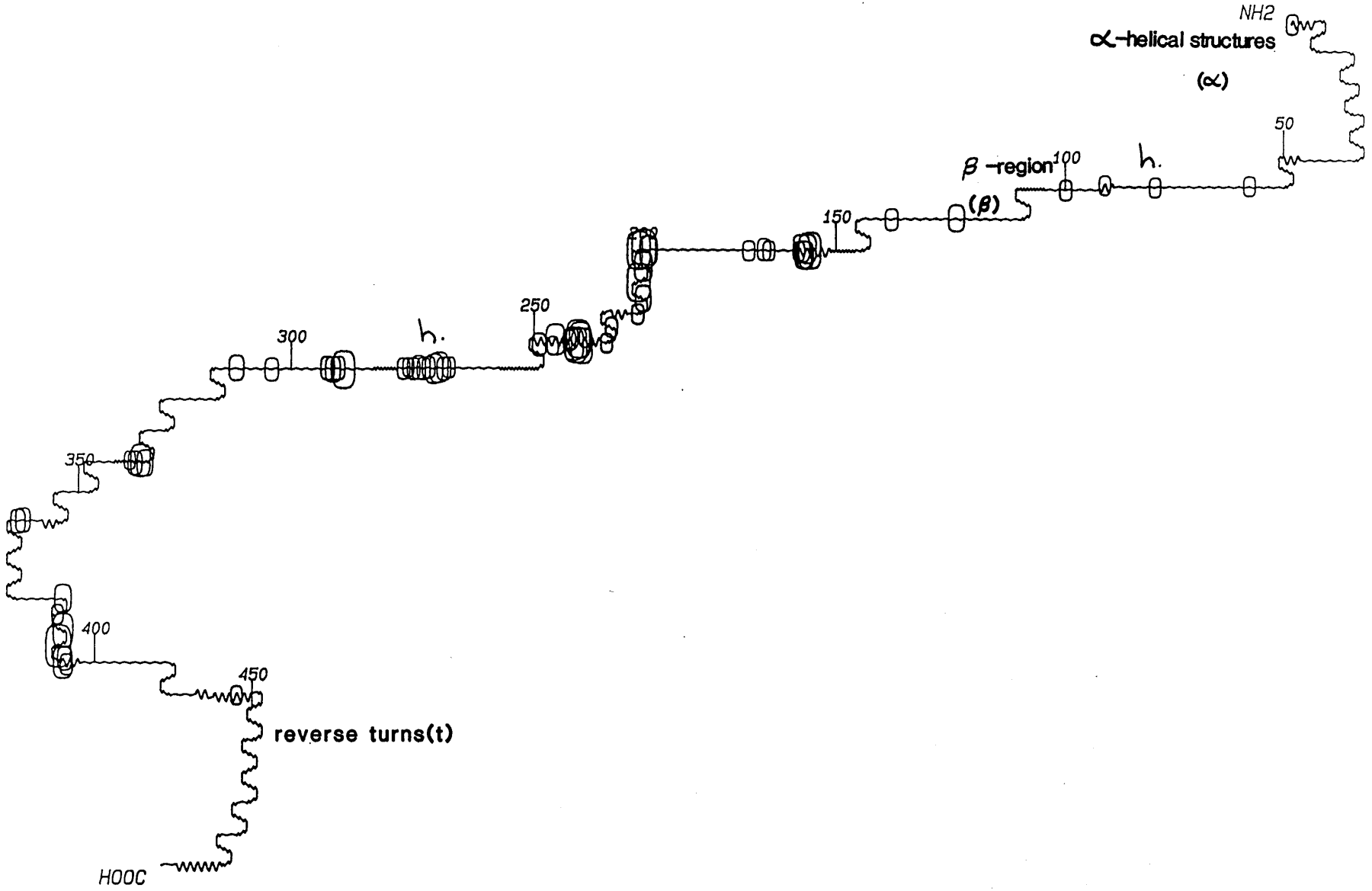

NH2
α-helical structures
(α)
50
β -region
100
(β)
150
250
300
350
400
450
reverse turns(t)
HOOC

Figure 52

Predicted secondary structures of mutagenised pMC1

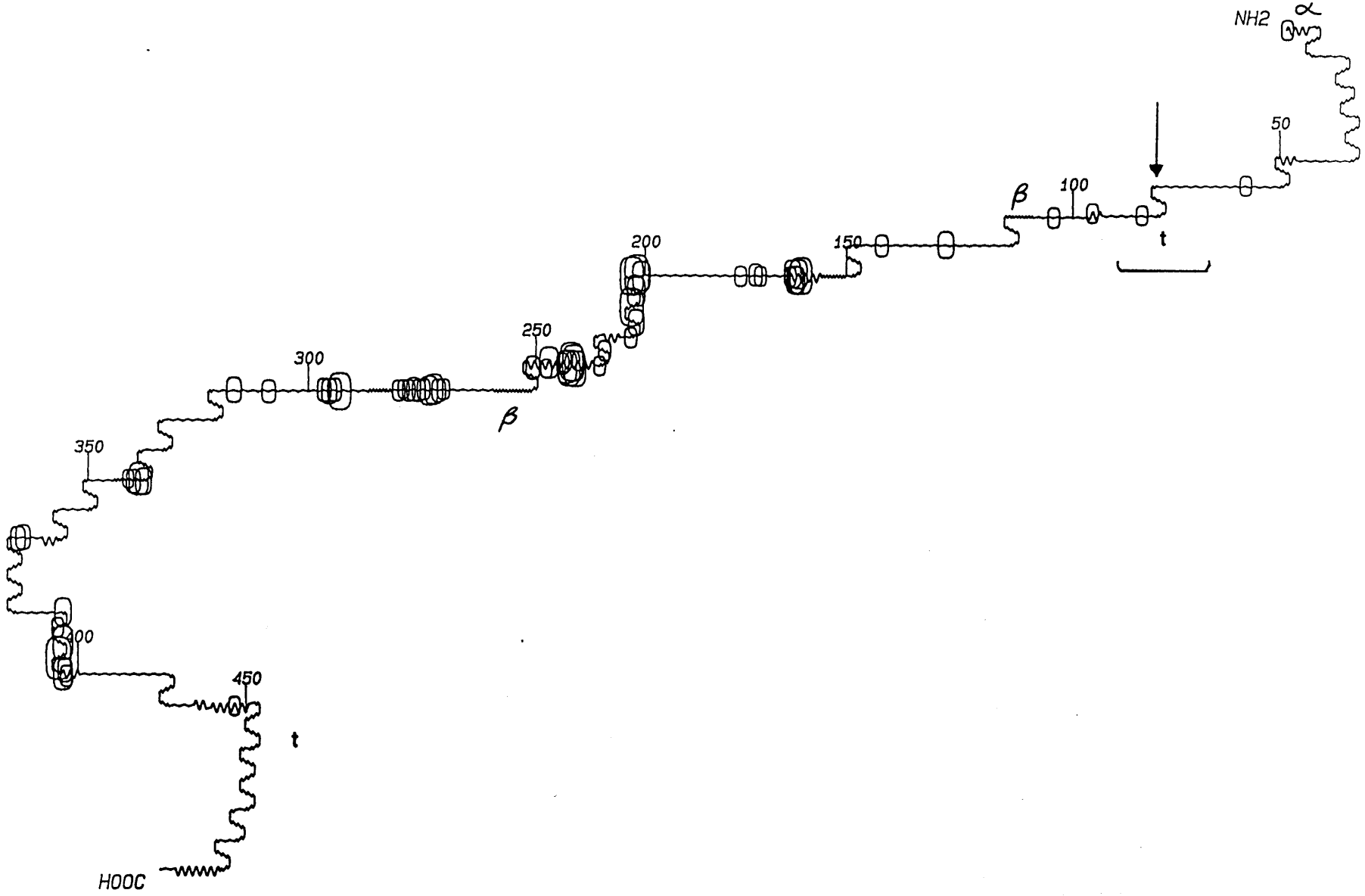
Structures predicted by the Chou and Fasman computer program for (1) p4 (BamHI); (2) p9 (BamHI); (3) p11 (BamHI) and (4) p17 (XbaI) are shown, and the approximate site of the insertion is indicated for each plasmid.

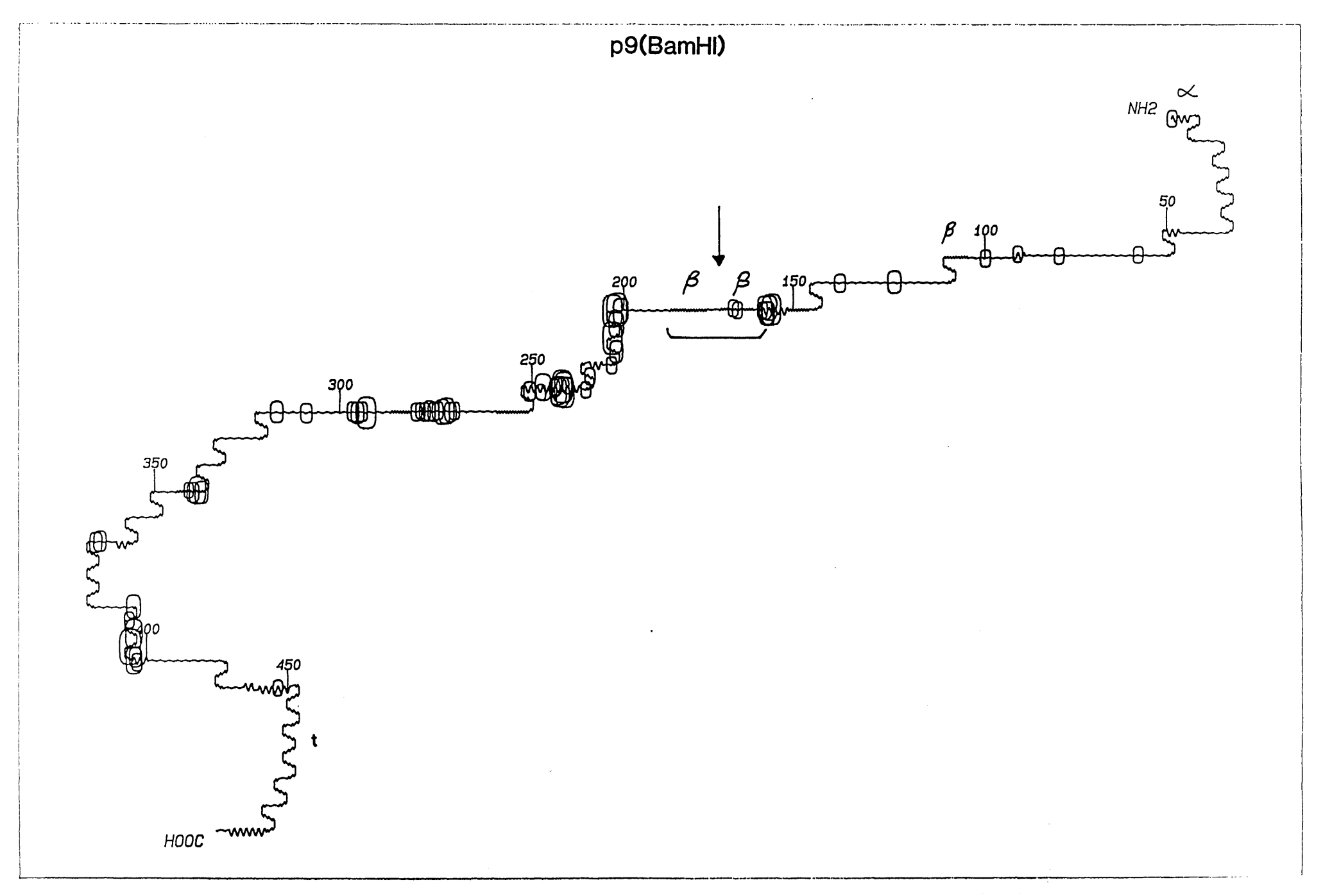
Hydrophobic and hydrophilic regions are indicated by O.

Predicted α-helical regions, β-structures and reverse turns are indicated by α, β, and r, respectively.

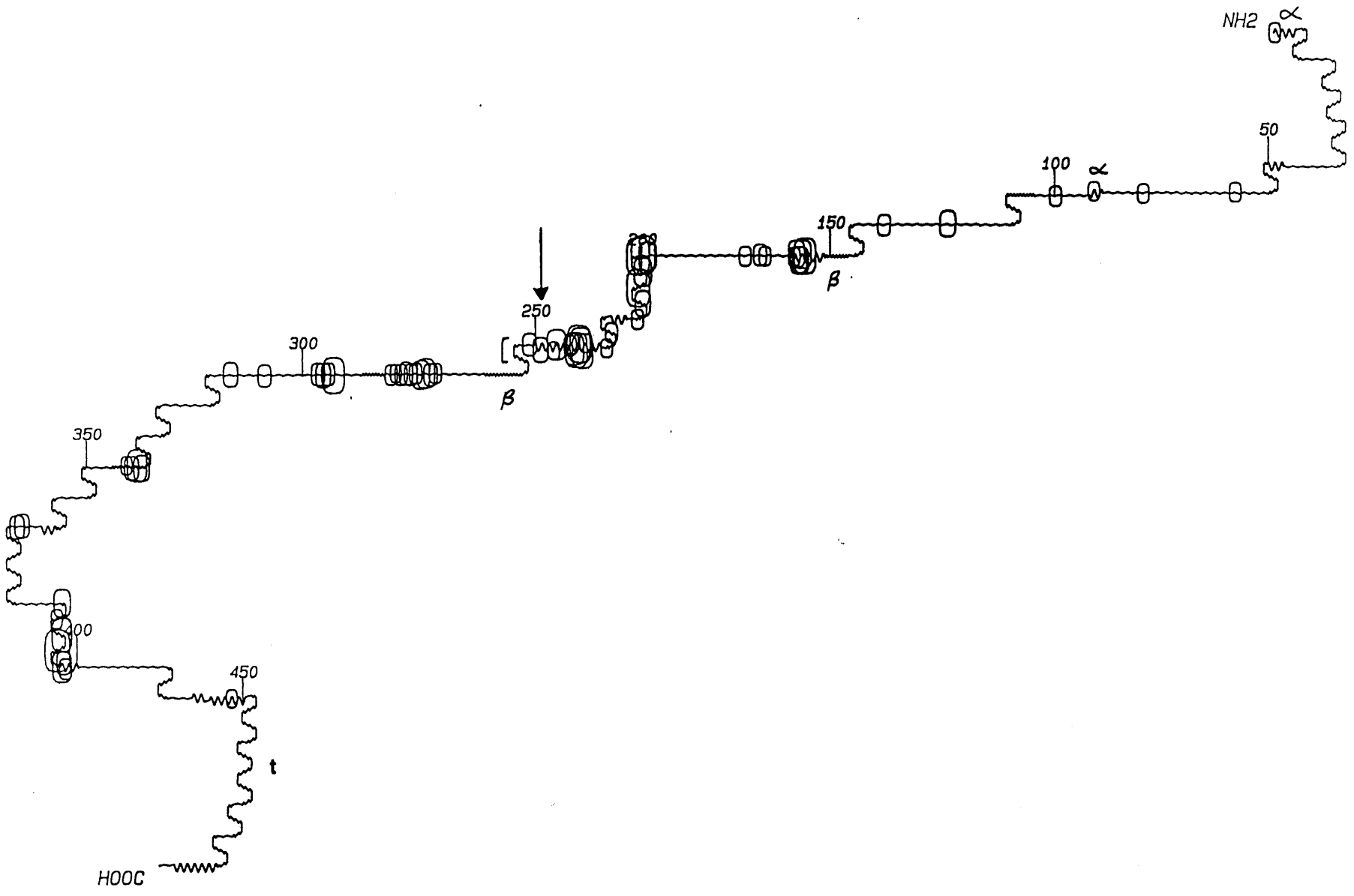
Plasmids containing BamHI-linker insertions were generously supplied by Mr C I Ace.

p4(BamHI)

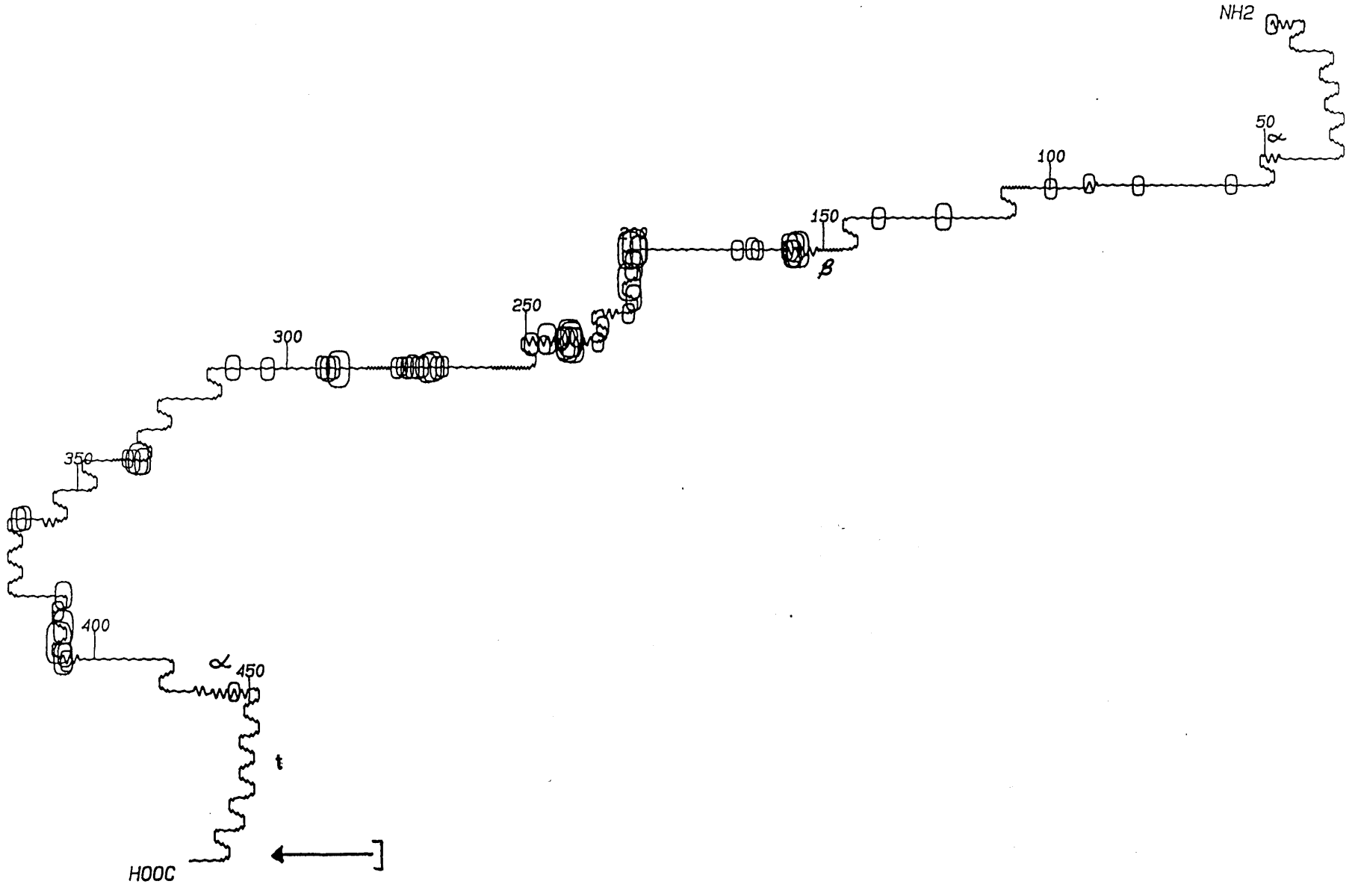



p9(BamHI)
NH2
α
50
β
100
β
β
150
200
250
300
350
400
450
t
HOOC

p11(BamHI)



p17(Xbal)



Of the remaining plasmids, BamHI insertions at p9 converted a region of weakly predicted β-structures to strongly predicted β-forms, and at site 4 a turn was predicted to be introduced into the secondary strcuture by the addition of a BamHI linker (Figure 52).

4.8 DISCUSSION

The section of work described here was undertaken in order to demonstrate the identification of essential functional domains in an HSV-1 gene encoding Vmw65, a tegument structural and transcriptional *trans*-activator of HSV IE gene expression.

4.8.1 Strategy for Mutagenesis

Early methods of DNA mutagenesis involved the essentially random action of chemicals to produce base changes in a specific DNA fragment. These methods have been superceded by advances in recombinant DNA technology, and site specific mutagenesis of cloned DNA is now well established as a method of manipulating gene structure and protein function (Shortle *et al.*, 1981).

For relatively small regions of DNA, gap misrepair mutagenesis (Shortle *et al.*, 1982),

oligonucleotide-directed mutagenesis (Hutchinson et al., 1978; Dalbadic McFarland et al., 1982) and linker scanning mutagenesis, whereby 10bp oligonucleotides containing a BamHI site were substituted for 10 nucleotides in the HSV TK promoter (McKnight and Kingsbury, 1982), are all methods that have been successfully applied to the analysis of DNA sequence and function. For mutagenesis of larger sequences, such an entire gene, these methods become unsuitable, as they are very labour intensive, and require the sequencing of large region of DNA. In such cases, synthetic oligonucleotides specifying a novel restriction enzyme site can be inserted into a restriction site, producing insertional mutations across the gene that may be identified by the presence of a unique restriction enzyme recognition site. However, in doing this a frameshift mutation or a termination codon may arise within the DNA sequence, thus resulting in the synthesis of a truncated protein (Heffron et al., 1978).

A refinement of this technique is the direct insertion of a known number of amino acid codons (2-4 codons) into a restriction enzyme recognition site, thus retaining the reading frame, and adding a new restriction site for screening and identification of the site of insertion. Defined regions within a gene can then be related to the function of the gene product.

The insertion of 3 or 4 amino acids can be disruptive to overall protein structure (Lobel and Goff, 1984). However, this can be remedied by minimising the size of the insertion, and using, for example, a 2-codon oligonucleotide linker (Boeke, 1981; Stone et al., 1984; Barany, 1985), as was done in the experiments described in this thesis.

Two and four codon insertions have now been made into pMC1 DNA at HaeIII-restriction endonuclease sites across the coding sequence of the gene, and no overall disruption was detected using the larger insertion. XbaI linkers were also ligated to AluI or ThaI-restricted linear partial pMC1, but no XbaI-positive clones could be recovered. The reason for this observation remains unclear.

In addition, the frequency of plasmids containing an XbaI insertion recovered from amp^r bacterial clones was never greater than 30% of the total number of extracted plasmids. Interestingly, under similar conditions, Lobel and Goff (1984) recovered 9 and 12bp insertions at frequencies varying from 10-50%, and C.I.Ace (personal communication) has also noted a similar frequency for BamHI linker insertions into pMC1 DNA.

The possibility that this low frequency was caused by the presence of an amp^r background of spontaneously

religated pMC1 was excluded by experimental controls, and DNA purification and ligation conditions were varied, but the frequency of recovery of plasmid DNA containing XbaI recognition sites was not increased. It is possible that the small size and high A+T content of the 6bp oligonucleotides makes them very susceptible to denaturation, and thus unavailable to DNA ligase.

The work presented in the previous section is a preliminary assessment of Vmw65 functional domains, since for a full analysis of this protein a greater number of mutants with insertions scattered throughout the coding sequence of the gene would be required. It should also be noted that this work was begun before the sequencing of Vmw65 was completed, and unfortunately it could not be anticipated that the insertion of XbaI linkers into HaeIII sites would result in the termination codon (TAG) being inserted into the reading frame at such a high frequency (11 out of a total of 17 HaeIII sites compared with an expected frequency of 1 in 3 sites). This situation was reflected in the recovery of plasmids, where 4 out of 6 successful insertions generated a termination codon.

4.8.2 Polypeptide Structure and Mutagenesis

An important point that has emerged from this study is that whilst Vmw65 has several domains affecting only one of its functions (at HaeIII sites, 4, 12 and 14), a

central region extending from HaeIII site 8 to the PvuII site, and possibly as far as HaeIII site 11, is required for the maintenance of both functions of Vmw65.

However, this analysis of Vmw65 gives no information as to the impact of such mutations on the spatial conformation, or tertiary structure of the protein which might alter the ability of Vmw65 to interact with other polypeptides. Whilst the determination of protein tertiary structure requires X-ray crystallography studies, the native conformation of the polypeptide is dependent upon the primary amino acid sequence, and numerous efforts have been made to predict the protein structure from the sequence, using computer programs designed for this purpose. Early predictive studies concentrated on the proteins, myoglobin and haemoglobin, which are composed of 80% α-helices and 0% β-structures. As a consequence, the presence of α-helices was overestimated in subsequent predictions. In later work, Chou and Fasman (1978) made a statistical survey of 15 proteins, and identified the potential for α-helix or β-sheet conformation of all 20 amino acids, arranging them in a hierarchical order. In addition, these workers formulated rules for the folding of secondary structures with an estimated accuracy of 70-80% (Chou and Fasman, 1978).

Using this method, amino acids can be predicted to be strong, weak or indifferent "formers" of α-helices or β-sheets, or alternatively, "breakers" of these conformations. For example, glutamate has a particularly high potential to form an α-helix, whilst its β-sheet potential is as a strong "β-sheet breaker". Using this information, the potential of a particular region to form a helix or β-sheet can be estimated by averaging the potential of residues in this portion of sequence. The predicted conformations of the secondary structure of a protein are:

1. α-helical structures, which are initiated by 4 out of 6 residues having a high potential for helix formation, and which have characteristic intra-chain hydrogen bonding.

2. β-sheet structures, where 3 β-former residues can initiate a parallel or antiparallel chain of β-sheets, and inter-chain hydrogen bonds are formed.

3. Coil regions and β-turns (or reverse turns). Coil regions are made up of groups of residues which are not predicted to form α-helices or a B-sheet, and may be composed of β-turns. β-turns are structures in which the polypeptide chain folds back on itself by nearly 180°, giving a protein, such as Vmw65, its globularity.

It must be remembered, however, that these predications are limited in their application, and that in a given protein the true secondary structure, rather than a predicted one may not conform to the "rules" governing conformational structures.

However, within these limitations, the formation of predicted secondary structures are a useful and interesting means of analysing the possible effects of mutations on protein structure, in conjunction with their effects on protein function.

It might be expected that the regions most susceptible to mutation would be those where the inserted amino acids disrupt intra- or inter-chain interactions, such as hydrogen bonding. In pMC1, the only insertion to make a strong alteration to the predicted structure of the sensitive central domain was a BamHI insertion at HaeIII site 9, where the addition of [arginine: isoleucine: arginine: alanine] enhanced a weakly predicted B-structure over a region of approximately 30 residues (Figure 52), thus resulting in a strong computer prediction of two regions of β-sheets. Another small alteration was evident at HaeIII site 11, where a BamHI insert reduced the predicted length of a region of α-helices. Interestingly, however, the XbaI insert at this site had no apparent effect on the predicted structure of pMC1, but unlike the larger

BamHI insert, affected the ability of Vmw65 to stimulate IE expression. Perhaps this is an indication of the sensitivity of this domain to mutations, since although no strongly predicted changes to the secondary structure were evident from the XbaI insertions at PvuII or HaeIII 11 sites, or from the BamHI insertions at HaeIII 8 and PvuII sites, there were significant changes to the functioning of the polypeptide. This suggests that the conformation of this region is directly essential for Vmw65 function, or alternatively, that a change in this domain affects the configuration of other regions of the polypeptide, such that normal protein functions cannot be fulfilled.

Other functional domains were identified at HaeIII sites 4 and 12, where the marker rescue ability was affected, and at HaeIII site 14, where Vmw65 stimulation of IE gene expression was lost. HaeIII site 4 is in a weakly predicted region of β-turns, and the BamHI insertion resulted in the stronger prediction of a β-turn. HaeIII site 14 is in a region of reverse turns, and HaeIII site 12 is in a weakly predicted α-helix structure: neither of these regions was detectably altered in structure by a BamHI insertion.

Finally, the insertion of a BamHI linker or a termination codon (XbaI insert) at HaeIII site 17 had no effect on protein function, although the latter

insertion resulted in the loss of the predicted α-helix at the 3' terminus of the protein (Figure 52). This, plus the fact that a BamHI insertion at HaeIII site 2 had no effect on function suggests that the 3' terminus, and possibly the 5' terminus may be of little functional significance. It is interesting to speculate that regions of increasing functional importance may be retained towards the centre of the structure, and perhaps the protein folds itself to conserve such areas, which by these results would lie approximately between amino acids 150 and 250 in Vmw65.

4.8.3 CONCLUSIONS

Vmw65: Summary and Future Developments

Recent advances in the study of Vmw65 have contributed significantly towards understanding the function of this protein. Firstly, in addition to its role as a structural component of the virus tegument, functional Vmw65 has been shown to be essential for encapsidation of viral DNA (this have been discussed in detail in Section 3). Secondly, Vmw65 is now known to be the HSV *trans*-activator of IE gene expression, increasing IE mRNA synthesis by 5-10 fold *in vitro*.

This transcriptional aspect of Vmw65 function, which appears to be unique for an HSV polypeptide, is not retained by all other herpesviruses. Campbell and Preston (1987) have analysed the upstream sequences of

the PRV major IE gene, and whilst expression was stimulated by HSV Vmw65, no increase in expression was observed in cells transfected with PRV IE or HSV IE genes or upon addition of, UV-irradated PRV, suggesting that PRV does not contain a functional TIF. However, in comparison, the evidence suggests that the betaherpesvirus, HCMV, encodes a TIF which stimulates HCMV but not HSV IE transcription, and that the HCMV major IE gene may be induced by the HSV trans-activator, Vmw65 (Spaete and Mocarski, 1985; Stinski and Roehr, 1985). Whilst these viruses lack the TAATGARATTC sequence in IE upstream sequences, they share versions of a small repeated element which has homology to the TAATGARATTC consensus.

In contrast to these viruses, VZV IE upstream regions lack any obvious sequences to TAATGARATTC, but this virus encodes a 46,000 MW polypeptide, which apart from lacking the final 80 amino acids, has an almost colinear in amino acid structure to HSV Vmw65.

This raises the question as to the functional significance of the 80 carboxyl terminal amino acids in HSV Vmw65. Whilst their removal (as in p15), resulted in the loss of any protein function, the removal of the final 20 amino acids (in p17) had no discernible effect on Vmw65 function. It would be interesting, therefore, to determine the minimal amino acid requirement for

Vmw65 function, and compare its predicted secondary structure with that of the VZV 46K polypeptide, thus identifying any predicted structural differences which might be related to the ability of Vmw65 to interact with the TAATGARATTC sequences of IE regulatory sequences.

Further work is also required to analyse VZV IE upstream sequences for *trans*-activation by VZV 46K and/or HSV Vmw65, and also to determine the effect of VZV 46K on HSV IE gene expression. If VZV does *trans*-activate IE gene expression, it probably does so via sequences differing from the TAATGARATTC motif, and therefore the introduction of mutations into the VZV 46K sequence at equivalent points to essential functional domains of Vmw65, might provide further evidence concerning functional differences between these proteins.

Preston *et al.* (in press) have already begun to elucidate the means by which HSV-1 Vmw65 stimulates HSV IE gene expression, despite the inability of this protein to bind to DNA (Marsden *et al.*, 1987). It has been shown that purified Vmw65 or HSV-infected cell extracts form a novel complex with cellular polypeptides, and that this complex interacts specifically with DNA fragments containing the TAATGARATTC consensus element. The binding of cellular

components to TAATGARATTC has also been noted by Pruijn et al. (1986), who identified the adenovirus replication factor, nuclear factor 3 (NF-3) binding site, and demonstrated its similarity to the TAATGARATTC consensus sequence. Kristie and Roizman (1987) have shown that cell polypeptides from both infected and uninfected cells bind to TAATGARATTC. However, the protected region identified by DNaseI protection studies differed from that identified by Preston et al. (in press). The evidence suggests that whilst a number of cellular proteins, or different protein complexes have the ability to bind to TAATGARATTC, Vmw65 forms a complex with polypeptides which are not normally bound to DNA. This novel complex can then act at the TAATGARATTC sequence to stimulate IE gene expression.

The availability of the insertional mutants of pMC1 may be of significant use in the further analysis of Vmw65 function, once the location and sequence composition of the mutations is confirmed. A particularly interesting aspect will be the study of mutants which partially retain the trans-activating properties of Vmw65 [p11 (XbaI); pPvuII (BamHI)]. The fact these mutants retain some trans-activating properties suggests that the protein cannot fully interact with the cellular component, or that the complex does not efficiently interact with the TAATGARATTC sequence. This might be

detectable, either as a decrease in the production of IEC, or, if Vmw65 interacts with a number of proteins, by a change in size of the IEC. Alternatively, the location of the insertion may result in the protein complex binding to DNA, but prevent efficient binding of, for example, the polymerase to the IEC.

These proposals could also be applied to tr- mutants, since complete failure to trans-activate IE genes may not necessarily preclude formation of the IEC, but may result in the production of a non-functional complex. In addition, this experimental approach may be applicable to those herpes viruses such as HCMV which perhaps encode a trans-activating protein, but do not appear to act on HSV IE genes. Further study using the above methods might demonstrate (1). whether a complex of some sort is formed with a TIF produced, for example, by HCMV or VZV and (2). where this might act other than at the TAATGARATTC element.

In conclusion, therefore, although a number of important advances have been made, the means by which Vmw65 acts on the DNA sequence is far from clear, and this, and the method by which Vmw65 associates with cellular polypeptides, represents an interesting and expanding area of study. A more complete analysis of the functional domains of Vmw65 will be important in elucidating the means by which this gene control system operates.

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