CONSTRUCTION AND CHARACTERISATION OF A HERPES SIMPLEX VIRUS MUTANT DEFICIENT FOR VMW65-MEDIATED STIMULATION OF IMMEDIATE EARLY GENE TRANSCRIPTION

by

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SUMMARY

Herpes simplex virus (HSV) gene expression can be classified into three phases, immediate early (IE), early and late. The 5 IE genes are the first to be transcribed after infection and the products (IE polypeptides) are essential for early and late gene expression. A distinctive feature of HSV gene activation is the stimulation of IE transcription by the late polypeptide Vmw65, the major tegument protein. The transinduction of IE transcription by Vmw65 is dependent on the *cis*- acting regulatory element TAATGARAT which is located in the promoters of all IE genes. Polypeptide Vmw65 interacts with cell factors, including the "octamer"-binding transcription factor, OCT-1, to form a complex (named IEC) which binds specifically to DNA sequences containing TAATGARAT and it is thought that IEC is an important mediator of IE gene transinduction. Thus, Vmw65 is involved in initiation of virus gene expression at a very early stage of infection. In addition to its role as an IE gene activator, Vmw65 is essential for virion assembly during the latter stages of infection and as a result large scale insertions or deletions in the polypeptide are not tolerated by the virus. The aim of the project was to investigate, by *in vitro* mutagenesis, the importance and relevance of Vmw65 as an IE gene transinducer in viral infection. The approach taken initially was to analyse the effect of small oligonucleotide insertions in cloned fragments of the gene, in an attempt to map domains within the polypeptide important for IE transinduction and for virion assembly.

A series of ten plasmids each with a 12 base pair, *BamHI* linker, insertion in the gene encoding Vmw65 of HSV-1 was constructed. The plasmids were analysed for their transinducing activity in transient cotransfection experiments, and mutant polypeptides synthesised *in vitro* were analysed for their ability to form IEC in gel retardation experiments. The ability of mutant polypeptides to be correctly assembled into virus particles was assayed by the insertion plasmids' ability to marker rescue a *ts*

virion assembly defective mutant of HSV-2 whose lesion resides in the Vmw65 homologue and which does not affect the transinducing function. It was found that approximately 50% of the insertions abolished the ability of Vmw65 to transinduce IE promoters and that those polypeptides defective in activity did not form IEC, and vice versa. This striking correlation between complex formation and stimulation of transcription is convincing evidence that IEC is an essential intermediate of IE transinduction. When the mutant plasmids were analysed for their virion assembly phenotype, again approximately 50% were non-functional but the regions important for virion assembly mapped to regions distinct from those required for transinduction.

One of the plasmid mutations which abolished transinduction but which had a wild-type (wt) virion assembly phenotype was introduced into virus by intra-typic recombination. A virus, named in1814, containing the linker mutation was isolated and its DNA characterised by Southern blotting. A distinctive feature of in1814 was its reduced ability to grow and produce plaques in tissue culture cells at low multiplicities of infection (moi). The plaquing efficiency of in1814 was cell-type dependent and was reduced by 100-fold in BHK and Vero cells and as much as 10,000-fold in HFL cells. The mutant virus produced equivalent numbers of virion particles as wt HSV-1 stocks and analysis of virus adsorption to cells and DNA migration to the nucleus showed that in1814 was able to enter cells efficiently and that the infectivity of the virus particles was normal. Therefore the impaired growth of in1814 is unlikely to result from a defect in virion structure. A "revertant" virus was constructed by marker rescue of in1814 with a DNA fragment containing a wt coding sequence of Vmw65. This rescued virus behaved as wt HSV-1, indicating that the phenotype of in1814 does not result from a second site mutation.

Vmw65 expressed by in1814 did not associate with cellular factors to form IEC and the virus was incapable of activating IE promoters that had been transfected into cells. The intrinsic level of IE gene expression by in1814 at high moi was reduced but not dramatically lower than wt

HSV-1 levels. The mutant virus expressed mRNA transcripts and polypeptides whose abundance was reduced by 4 to 5-fold for IE genes 1 and 2, and 2-fold for IE genes 4 and 5. However, the product of IE gene 3 was expressed in amounts comparable to those of wt HSV-1. In contrast to the situation for IE gene expression, the mutant virus synthesised early and late gene products to wt levels at high moi However, the relative levels of these proteins were dramatically reduced at low moi in in1814. The growth defect of in1814 at low moi was shown to result directly from underexpression of IE polypeptides since the plaqueing efficiency of the mutant increased when titrated on cells transfected with plasmids expressing the product of IE gene 1, Vmw110. The virulence of the mutant in mice was reduced to between 10^3 and 10^5-fold as measured by LD_{50} determinations, suggesting that the effect of Vmw65 in vivo may resemble its effect seen at low moi in tissue culture.

The observations suggest that Vmw65 is required in order to stimulate IE gene expression and this function is essential for productive infection under conditions of low multiplicity. At high moi the transinducing activity of Vmw65 becomes redundant, presumably because the basal level of IE gene transcription at high copy number is sufficient for virus growth. Although the growth of the mutant virus in tissue culture at high moi is unimpaired, the role of Vmw65 during in vivo infection is clearly important and it could therefore be regarded as a target for anti-viral drugs.

ABBREVIATIONS

Standard nomenclature for SI units of weights and measurements has been used. Abbreviations not defined in the relevant area of text are listed below.

A	adenine
aa	amino acid
Ac	acetate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
APS	ammonium persulphate
bp	base pairs
BHK	baby hamster kidney
BSA	bovine serum albumin
C	cytosine
^{14}C	radiolabelled carbon
CAM	chloramphenicol
CAT	chloramphenicol acetyltransferase
CH	cycloheximide
Ci	curie(s)
CMV	cytomegalovirus
cpe	cytopathic effect
cpm	counts per minute
CTP	cytidine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DATD	N,N'-diallytartardiamide
dl	deletion
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
EBV	Epstein-Barr virus
E. coli	Escherichia coli
EDTA	sodium ethylenediamine tetra-acetic acid
EtBr	ethidium bromide

g	gravity
G	guanine
GTP	guanosine-5'-triphosphate
h	hours
HC	host complex
HCMV	human cytomegalovirus
HeBs	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane
HFL	human foetal lung
HSV	herpes simplex virus
in	insertion
kb, kbp	kilobase(s), kilobase pair(s)
min	minute(s)
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
mw	molecular weight
N	unspecified nucleotide (A, G, C or T)
NP40	nonidet p40
NPT	non-permissive temperature
OD	optical density
ORF	open reading frame
ori	origin of DNA replication
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming unit
PIPES	piperazine-N,N'-bis(2-ethane sulphonic acid)
poly dI	polydeoxyinosinic acid
poly dC	polydeoxycytidylic acid
PRV	pseudorabies virus
PT	permissive temperature
R	purine
RNA	ribonucleic acid
RNAase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RR	ribonucleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
snRNA	small nuclear ribonuceic acid

SV40	simian virus 40
T	thymidine
TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylethylene diamine
TK	thymidine kinase
TPA	12-O-tetradecanoyl-phorbol-13-acetate
tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive
UTP	uridine-5'-triphosphate
UV	ultraviolet
v/v	volume/volume
Vmw	molecular weight of viral polypeptide in kilodaltons
VP	virion protein
VZV	varicella zoster virus
wt	wild type
w/v	weight/volume
Y	pyrimidine

INTRODUCTION

The subject of this thesis concerns the mutational analysis of the herpes simplex virus type 1 (HSV-1) immediate-early (IE) gene regulator, Vmw65, and the consequences of IE transinduction during virus infection. In the Introduction, a brief background to the biology of the HSV is given (section 1), with attention focused on the control of HSV-1 IE gene transcription (section 2). A review of transcription control mechanisms in other eukaryotic systems is also included (section 3).

1. THE HERPESVIRUSES

1.1 CLASSIFICATION

The family Herpesviridae is comprised of more than 80 species which infect a wide range of higher eukaryotic hosts (Roizman and Batterson, 1985). Membership of this family is based on four distinct morphological features:

(i) The core is an electron dense fibrillar spindle and is surrounded by the double-stranded DNA of the viral genome (Epstein, 1962; Furlong et al., 1972; Nazerian, 1974).
(ii) The capsid is icosahedral in shape, approximately 100nm in diameter and is composed of 162 capsomeres which surround the core (Wildy et al., 1960).
(iii) The tegument, an ill-defined layer of proteinaceous material, is located between the capsid and the envelope (Schwartz and Roizman, 1969; Morgan et al., 1968; Roizman and Furlong, 1974).
(iv) The envelope, surrounding the capsid and tegument, is derived from budding through the nuclear membrane and harbours numerous virus-encoded glycoprotein spikes (Morgan et al., 1959; Wildy et al., 1960; Asher et al., 1969; Spear and Roizman, 1972).

Although the herpesviruses as a family exhibit a very diverse array of biological properties, one characteristic common to all is the ability to persist in a

latent state in their hosts. Herpesviridae have been classified into three sub-families (alpha, beta and gammaherpesvirinae) based on criteria of host range, duration of reproductive cycle, cytopathology and characteristics of latent infection (Matthews, 1982; Roizman, 1982). The herpesviruses have also been classified on the basis of the arrangement of characteristic repeated DNA sequences within their genomes (Roizman et al., 1981; Roizman and Batterson, 1985).

(i) Alphaherpesvirinae can have a wide or narrow host-range in tissue culture. The reproductive cycle is short (less than 24h) and causes complete destruction of infected cells. Latent infection is frequently established in neuronal cells. Members of this sub-family include the human herpesviruses HSV-1, HSV-2 and VZV.

(ii) Betaherpesvirinae have a narrow host-range in tissue culture, frequently restricted to the species or genus of the natural host. Virus replication and lytic progression is slow, and infection results in an enlargement of cells. Latent infections are generally established in secretory glands, lympho-reticular cells and kidneys. HCMV is a member of this sub-family.

(iii) Gammaherpesvirinae have a limited host range which is usually restricted to the family or order to which the host belongs. Members of this family replicate only in T or B lymphocytes in vivo, and latent infection is frequently established in lymphoid tissue. This sub-family includes the human herpesvirus EBV.

1.2 PATHOGENICITY

HSV-1 and HSV-2 HSV-1 is widespread in the human population and causes vesicular lesions of the mouth, lips and nasal membranes ("cold sores"), ocular keratitis and occasionally more severe symptoms including encephalitis, particularly in immunocompromised individuals (Smith et al., 1941; Gallardo, 1943; Rawls, 1985). HSV-2 is the agent of

sexually transmitted genital herpes. The two viruses are closely related and some overlaps in their clinical manifestations exist (Whitley, 1985). HSV-1 and HSV-2 often establish latency in trigeminal and sacral ganglia respectively, with periodic recurrence of lytic infection leading to outbreaks of lesions at peripheral sites (Klein, 1982; Knox et al., 1982; Hill, 1985).

VZV VZV is the causative agent of chickenpox (varicella), a childhood disease resulting from a primary infection, and shingles (herpes zoster), a localised vesicular condition which occurs in adults and appears to be caused by reactivation of latent VZV (Weller, 1958; Gelb, 1985).

HCMV HCMV infects the majority of the human population, usually resulting in a mild or subclinical condition. However, infection in neonates is sometimes associated with neurological damage (Alford and Britt, 1984). HCMV infections also cause complications in immunosuppressed individuals, particularly in organ transplant or blood transfusion patients (Ho, 1982). Outbreaks of HCMV infection also occur in patients with acquired immune deficiency syndrome (AIDS). In addition, HCMV has been implicated in the development of cervical cancer, since HCMV DNA can be detected in a small proportion of biopsies from patients with cervical intraepithelial neoplasia (Fletcher et al., 1986).

EBV EBV infects B lymphocytes and is the causative agent of infectious mononucleosis. The virus is also associated with Burkitt's lymphoma, nasopharingeal carcinoma and lymphomas of immunosuppressed individuals (Neiderman et al., 1976; Miller, 1985).

HHV6 HHV6 was first isolated from immunosuppressed patients, although it can infect T cells in vitro and is linked to specific childhood illness (Salahuddin et al., 1986).

1.3 INTERACTIONS BETWEEN HOST CELL AND VIRUS

1.3.1 Latency

An important property of HSV is the ability to establish latency and coexist with its host in a non-infectious state (Stevens and Cook, 1971; Baringer and Swoveland, 1973; Galloway et al., 1979; McLennan and Darby, 1980). After infection at peripheral skin sensory nerve cell sites, HSV-1 travels intra-axonally down nerves to ganglionic neurones. The latent state is characterised by the prolonged association of the viral genome in host neuronal tissue, during which time no infectious virus can be isolated (Hill, 1985). The definition of latency is an operational one based on experiments performed on animals in vivo: infectious virus cannot be recovered from latently infected nervous tissue following homogenisation but can be recovered following cultivation of explanted tissue in vitro (Wildy et al., 1982). The role of the peripheral nervous system in harboring latent HSV was first postulated by Goodpasture (1929) based on studies using rabbits. Stevens and Cook (1971) demonstrated that HSV established long-term "silent infections" in mice by recovering latent virus after cultivation of sensory ganglia explanted 4 months after the primary infection. Latent HSV was first isolated from the trigeminal ganglia of humans taken at post-mortem by Baringer and Swoveland (1973). McLennan and Darby (1980) identified latent HSV ts mutants in the neuronal cell bodies of mice that reactivated at the PT but could only develop viral antigen (detected by immunoflorescence) at the NPT. The study of ts mutants by others has revealed that viral DNA synthesis may be irrelevant in the establishment of latent infection in mice since some DNA positive and DNA negative mutants are equally able to establish latent infections, although some mutants of both categories were latency negative (Subak-Sharpe et al., 1974; Marsden et al., 1976; Stevens, 1981; Clements and Subak-Sharpe, 1983).

Using solution hybridisation techniques, Puga et al. (1978) were the first to detect HSV specific DNA in sensory ganglia from latently infected mice. Latent HSV genomes exist in a form other than unit length, linear DNA

and it is not clear whether the viral molecules are maintained in circular or concatemeric forms (Rock and Fraser, 1983; Efstathiou et al., 1986). The latent DNA does, however, appear to exist in an episomal state rather than being integrated in cellular DNA (Mellerick and Fraser, 1987). Reactivation of latent HSV can be triggered by trauma, nerve damage or various stimuli to the neurone or dermatome (Stevens, 1975; Wildy et al., 1982; Hill, 1985).

Recently, it has been shown that the HSV-1 genome is not completely silent during latency. A region of the genome near the IE-1 gene is transcriptionally active during latent infections, giving rise to two RNA species, which result from splicing, called the latency associated transcripts or LAT (Stevens et al., 1987; Wechsler et al., 1988). The LAT is complimentary (antisense) to IE-1 mRNA and partially overlaps the 3'-end of the IE-1 gene (Deatly et al., 1987; Puga and Notkins, 1987; Rock et al., 1987; Spivak and Fraser, 1987; Stevens et al., 1987; Wagner et al., 1988; Wechsler et al., 1988). At present, the only evidence for the involvement of the LAT in latency is its presence in latently-infected ganglionic neurones, although the transcript is envisaged to play a role in the establishment and/or maintenance of HSV-1 latency.

In vitro latency systems have been developed in tissue culture cells harbouring non-replicating HSV in order to improve understanding of the molecular events that govern latent infections (O'Neill, 1977; Wigdahl et al., 1982; Youssoufian et al., 1982; Nilheden et al., 1985; Shiraki and Rapp, 1986; Russell and Preston, 1986). Using HSV mutants, Russell and Preston (1986) have demonstrated that only limited gene expression, probably of IE genes, is necessary for the latent state to be both established and reactivated in vitro. Indeed, Vmw110 (the product of IE gene 1) has been directly implicated in the reactivation event since on superinfection, dl1403 (a mutant containing a deletion in IE gene 1) fails to reactivate HSV-2 in vitro (Russell et al, 1987). Furthermore, adenovirus vectors that carry the IE-1 gene reactivate latent HSV-2 whereas HSV-1 mutants that contain mutations in IE-1, and specifically mutations that delete the "zinc finger" activating region of the polypeptide

(Everett, 1987), do not reactivate latent virus (R.Harris, personal communication). These results imply that Vmw110 modulates reactivation via transcription activation events.

1.3.2 Transformation

HSV-2 has been found associated with cervical cancer (Naib et al., 1966; Rawls et al., 1969; Nahmias et al., 1970), although the evidence for a causal link remains obscure. Although regions of the HSV genome have been detected in cervical intraepithelial neoplasia (pre-malignant cancer) and implicated in the transformation of rat cells to a malignant phenotype in tissue culture, it appears that retention of HSV-2 DNA is not required to maintain the transformed phenotype (Galloway and McDougall, 1983, Cameron et al., 1985). Using in vitro tissue culture systems, three distinct nonhomologous morphological transforming regions (mtr I, mtr II and mtr III) have been mapped in the genomes of HSV-1 and HSV-2 (Camacho and Spear, 1978; Reyes et al., 1979; Jariwalla et al, 1980; Galloway et al., 1984). The mtr III region in HSV-2, contained in the BglII c fragment, is composed of two independent transforming and immortalising regions, both of which are required for oncogenesis (Jariwalla et al., 1983; Jariwalla et al., 1986). However, these regions do not contain entire coding sequences for any one gene and no evidence exists to suggest that a viral protein is involved in the transformation process. Therefore it seems unlikely that the expression of stably inserted viral genes in cellular DNA is a function of transformation, particularly as HSV does not carry a viral oncogene analogous to those found in retroviruses or small DNA tumour viruses. Rather, a "hit and run" hypothesis for the oncogenic potential of HSV is favoured, whereby malignancy may develop from a transient or lasting disruption of normal cellular events to which HSV may have contributed (Galloway and McDougall, 1983; Macnab, 1987). Indeed, some cellular proteins, possibly including heat shock-related polypeptides, accumulate to high levels only in HSV-transformed cells (Macnab et al., 1985 and personal communication). However, their significance

regarding the transformed state and the factors responsible for their induction are unknown. These proteins are also induced in normal HSV infections (Macnab et al., 1985). Alternatively, insertion of HSV DNA sequences proximal to or within certain cellular gene loci may cause the inactivation, rearrangement or enhanced expression of critical cell-growth control genes. Indeed, the mtr regions of HSV all have the potential to adopt stem/loop structures, a feature reminiscent of insertion-like sequences which activate genes through enhancer-like activity (Galloway et al., 1984; Jones et al., 1986). In addition, HSV has been shown to act as a mutagen in infected cells (Pilon et al., 1986). The mutagenic activity of HSV could be an effect of virus-encoded enzymes involved in DNA biosynthesis that could cause a disruption in the pool of nucleotides necessary for error-free replication of cellular DNA. Therefore, the potential of HSV to cause mutations may be a significant property regarding an infected cell's predisposition to transformation.

Whatever the role of HSV in cervical cancer, transformation by mtrs or other HSV sequences occurs at low frequencies and certainly not by a one step mechanism (Macnab, 1987). It is more likely that HSV may act as a co-carcinogen in the development of tumours, in conjunction with other factors such as human papillomavirus (zur Hausen, 1982; Durst et al., 1983; Boshart et al., 1984).

1.3.3 Alteration of host macromolecular synthesis

Many host cell functions and gross changes in cellular protein synthesis accompany infection of permissive cells with HSV-1 or HSV-2. Cellular DNA and RNA synthesis is inhibited (Roizman and Roane, 1964), mitosis ceases (Wildy et al., 1961) and there is a rapid shut-off of most host polypeptide synthesis (Sydiskis and Roizman, 1966, 1967) accompanied by a general degradation of cellular mRNAs (Nishoika and Silverstein, 1977, 1978; Schek and Bachenheimer, 1985). HSV-2 is, in general, more efficient at host protein synthesis inhibition than HSV-1 (Powell and Courtney, 1975; Pereira et al., 1977; Fenwick et al., 1979, Schek and Bachenheimer, 1985) but the control of host

shut-off is poorly understood. Cells infected with UV-irradiated virus or infected in the presence of actinomycin D exhibit rapid shut-off of host protein synthesis, implying that the process is mediated by one or more virion components (Sydiskis and Roizman, 1967; Fenwick and Walker, 1979; Fenwick et al., 1979; Schek and Bachenheimer, 1985). Indeed, mutants of HSV-1 have been isolated that are defective for virion associated host shut-off (vhs mutants) and the gene responsible identified as UL41 (Read and Frenkel, 1983; Kwong et al., 1988). However, complete shut-off requires the expression of early and perhaps late genes, indicating that there is a secondary shut-off event in addition and distinct from the virion associated mechanism (Honess and Roizman, 1974; Marsden et al., 1976; Nishioka and Silverstein, 1978; Stenberg and Pizer, 1982). Secondary host shut-off may be mediated by a virus-encoded product or alternatively, might be the result of virus promoters competing with cellular promoters for host transcription factors.

In contrast to the overall reduction in cellular mRNA and protein synthesis levels during HSV infection, the synthesis of some cellular proteins, possibly including heat shock proteins and ubiquitin, are stimulated early in infection (Notarianni and Preston, 1982; La Thangue et al., 1984; Macnab et al., 1985 and personal communication; Patel et al., 1986; Latchman and Kemp, 1987). Kemp et al. (1986) have implicated the involvement of virion components, possibly including the HSV-1 IE gene transinducing factor Vmw65, in the stimulation of transcription of certain cellular genes. Such alterations in cellular gene expression during the initial stages of infection could ultimately determine the fate of an infection, whether it be lytic, latent or transforming.

1.4 THE STRUCTURE OF HERPESVIRUS GENOMES

1.4.1 Genome arrangement

Herpesvirus genomes consist of linear duplex DNA molecules (Becker et al., 1968) of molecular weights ranging from 80 to 150×10^6 depending on species (Roizman and

Furlong, 1974). The base composition of herpesvirus DNA varies considerably; VZV has a G+C content of 46%, Pseudorabies virus (PRV) 73% and HSV-1 68.3% (Ben-Porat and Kaplan, 1962; Kieff et al., 1971, Ludwig et al., 1972; McGeoch et al., 1988). Members of the herpesvirus family have been grouped according to the pattern of repeated sequences in their genomes (Roizman, 1982) although these structural criteria do not correlate with the biological classification. Figure 1 shows the genome arrangement of representative members of the herpesviridae.

1.4.2 Structure of the HSV-1 genome

Early studies of HSV-1 DNA, based on observations of self-annealing molecules by electron microscopy, revealed that the molecule was composed of two covalently linked segments, designated long (L) and short (S). Each segment contained unique sequences (U) flanked by a pair of distinct inverted repeat sequences (R), of which one of each was terminal (T) and one internal (I) (Sheldrick and Berthelot, 1974). The molecule also exhibits terminal redundancy due to the presence of a short (approximately 400bp) terminal direct repeat, called the "a" sequence. One or more additional copies of the "a" sequence are located internally at the "joint" between the L and S segments, but in the opposite orientation to the terminal repeats (Wadsworth et al., 1975; Wagner and Summers, 1978; figure 1). The "a" sequences are responsible for mediating inversion events between the L and S segments such that four sequence-orientation isomers of the molecule are possible (Delius and Clements, 1976; Wilkie, 1976). Isomerisation of the genome occurs by intramolecular recombination at the "a" sequences, probably during HSV DNA replication (Weber et al., 1988). One isomer, chosen arbitrarily, is designated as the prototype for purposes of genomic map representations (Roizman et al., 1979; figure 2). In addition to the "a" sequences and inverted repeats, HSV-1 contains a number of small tandem reiterated sequence elements that vary in copy number (McGeoch et al., 1988).

virus	no. of isomers	genome type
CCV	1	A
HVS	1	B
EBV	1	C
VZV	2	D
HSV	4	E
MCMV	1	F

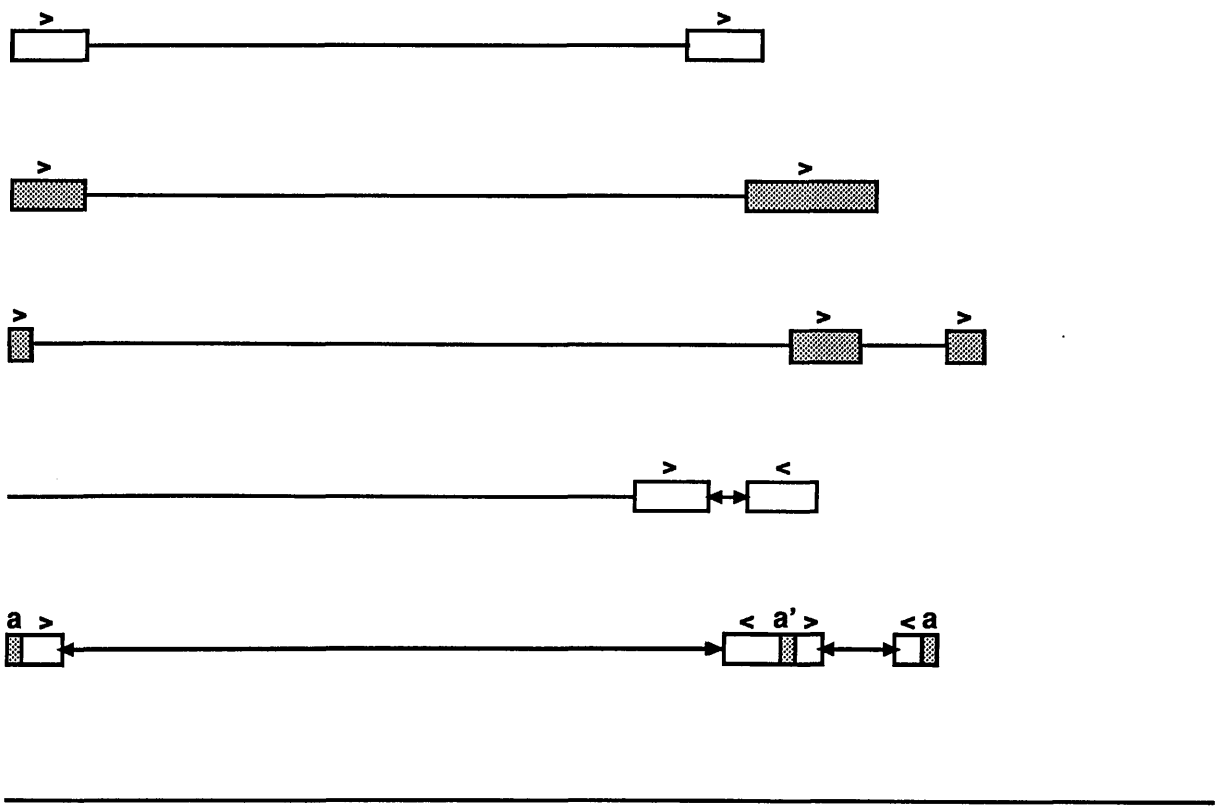


Fig. 1. Arrangement of sequences in herpesvirus genomes. Examples of genome types A to F are channel catfish virus (CCV), herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), varicella zoster virus (VZV), herpes simplex virus (HSV) and murine cytomegalovirus (MCMV) respectively. Lines represent unique sequences and arrowed lines show possible inversions. Open boxes are large (>1Kb) repeats and shaded boxes are repeats of small reiterated sequences; arrowheads above boxes denote whether repeats are direct or inverted. The small terminal direct and internal inverted repeats of HSV ("a" sequences) are indicated. The number of isomers of each molecule are also given.

1.4.3 The nucleotide sequence of HSV-1

The complete nucleotide sequence of the HSV-1 genome (strain 17) has now been determined (Davison and Wilkie, 1981; Murchie and McGeoch, 1982; McGeoch et al, 1985; McGeoch et al., 1986, McGeoch et al., 1988). Other herpesviruses whose complete sequences are known are VZV (Davison and Scott, 1986;) and EBV (Baer et al., 1984). The genome of HSV-1 comprises 152260 residues, although the exact lengh varies due to small reiterations and "a" sequences which differ in copy number. On the basis of open reading frame (ORF) predictions, the genome is estimated to encode 70 unique genes (2 are repeated); 56 in U_L (107.943 Kb), 12 in U_S (12.979 Kb), one in each of TR_L and IR_L (9.214 Kb) and one in each of TR_S and IR_S (6.677 Kb) (McGeoch et al., 1988; figure 2). The genes of HSV-1 are quite densely packed in the genome; 79% of U_S and 89% of U_L is occupied by ORFs which are considered to code for proteins (Rixon and McGeoch, 1985; McGeoch et al., 1988). It is clear from the close apposition or overlap of adjacent coding regions that in many cases transcriptional control elements must overlap with the polypeptide coding region of adjacent genes.

The overall G+C content of the genome is high (68.3%) and some regions of the genome are extremely G+C-rich, in particular the protein coding sequences of IE genes 1 (75.4%) and 3 (81.5%). The extreme ratio of G+C versus A+T in these genes is manifest in a large amount of G+C-rich codons, notably biased towards a G or C in the redundant third position. A preference for high G+C codon usage (resulting in restricted amino acid utilisation) is not understood, but the situation has probably arisen independently of protein functional demands (McGeoch et al., 1986; Perry et al., 1986). McGeoch et al. (1986) propose that much of the coding region DNA has evolved towards a potential for encoding common and innocuous residues such as alanine (whose codons contain in the first two positions only G and C), thereby introducing an element of "plasticity" in the event of mutation.

A knowledge of the complete nucleotide sequence of HSV-1 will revolutionise the identification of virus polypeptides and analysis of their function. For instance,

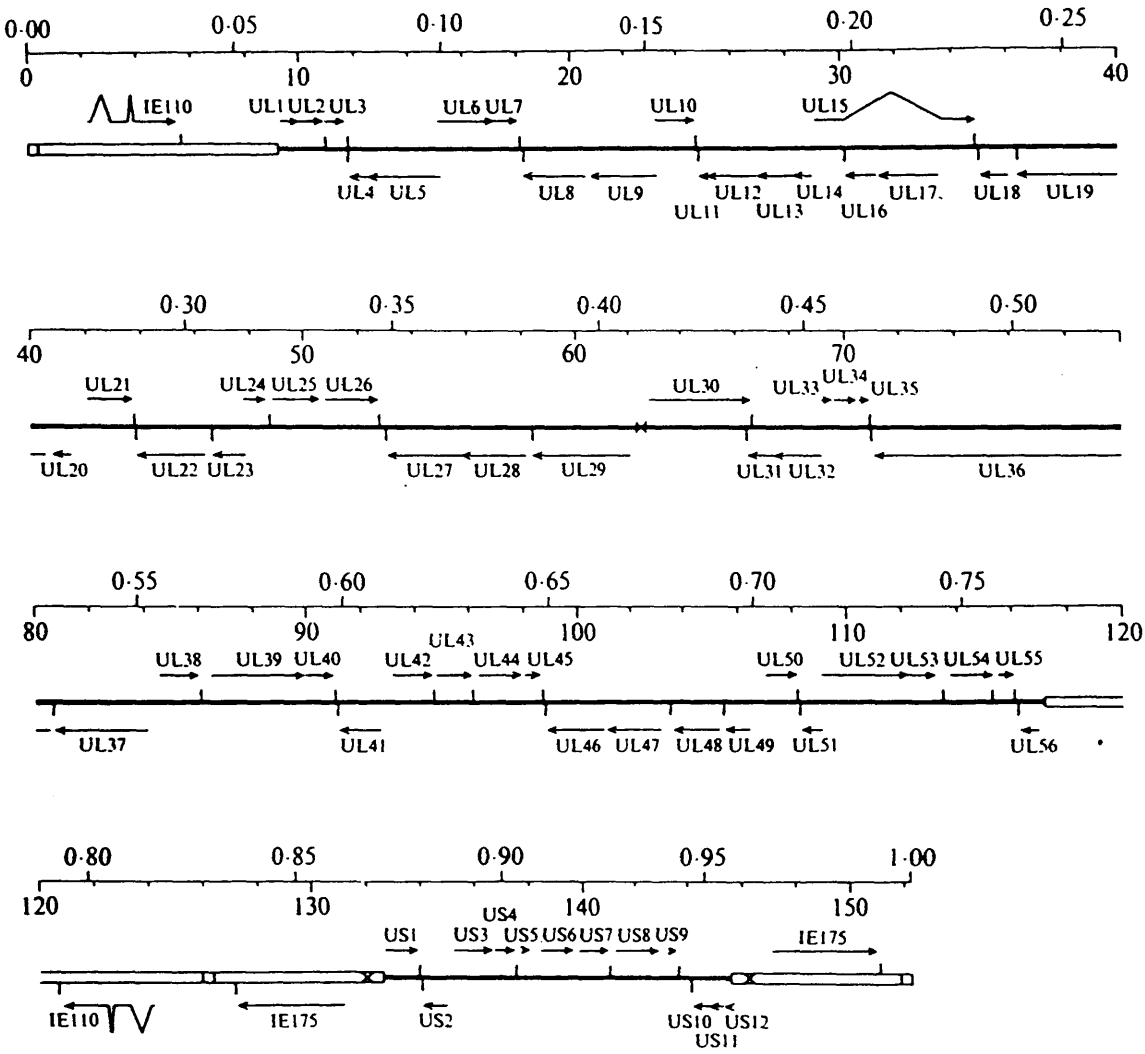


Fig. 2. Layout of genes in the genome of HSV-1. The HSV-1 genome is shown on four successive lines, with unique regions represented by solid lines and major repeat elements as open boxes as in Fig. 1. The lower scale represents kilobases, numbered from the left terminus, and the upper scale represents fractional map units. The sizes and orientations of proposed functional ORFs are shown by arrows. Overlaps of adjacent, similarly oriented ORFs are not shown explicitly. Locations of proposed transcription polyadenylation sites are indicated as short vertical bars. Locations of origins of DNA replication are shown as X. In the U_L region, on the first three lines, genes UL1 to UL56 are labelled. In the U_S region, on the bottom line, genes US1 to US12 are labelled. The locations of introns in the coding regions of gene UL15 and the two copies (TR_L and IR_L) of the IE110 gene are indicated.

Table 1.

Properties of HSV-1-encoded proteins (taken directly from McGeoch et al., 1988).

Gene	No. of residues	M_r*	Function or properties
IE110	(19) (222) (534) 775 Total	78452	IE transcriptional regulator (IE110)
UL1	224	24932	Hydrophobic N terminus
UL2	334	36326	–
UL3	235	25607	Hydrophobic N terminus
UL4	199		–
UL5	882	98710	DNA replication; ATP-utilizing?
UL6	676	74087	Virion protein
UL7	296	33057	–
UL8	750	79921	DNA replication
UL9	851	94246	DNA replication
UL10	473	51389	Multiply hydrophobic
UL11	96	10486	–
UL12	626	67503	Deoxyribonuclease
UL13	518	57193	–
UL14	215	23454	–
UL15	(343) (392) 735 Total	80918	–
UL16	373	40440	–
UL17	703	74577	–
UL18	318	34268	–
UL19	1374	149075	Major capsid protein
UL20	222	24229	Multiply hydrophobic
UL21	535	57638	–
UL22	838	90361	Virion glycoprotein H
UL23	376	40918	Thymidine kinase
UL24	269	29474	–
UL25	580	62666	Virion protein
UL26	635	62466	Capsid protein
UL27	904	100287	Virion glycoprotein B
UL28	785	85573	–
UL29	1196	128342	DNA replication; major DNA-binding protein
UL30	1235	136413	Replicative DNA polymerase
UL31	306	33951	–
UL32	596	63946	Locus of immune cytolysis resistance mutation
UL33	130	14436	–
UL34	275	29788	Virion protein; hydrophobic C terminus
UL35	112	12095	–
UL36	3164	335841	Virion protein
UL37	1123	120549	–
UL38	465	50260	Virion protein
UL39	1137	124043	Large subunit of ribonucleotide reductase

Table 1. (continued)

Gene	No. of residues	M_r*	Function or properties
UL40	340	38017	Small subunit of ribonucleotide reductase
UL41	489	54914	Virion protein causing host shut-off
UL42	488	51156	DNA replication; DNA-binding protein
UL43	434	44905	Multiply hydrophobic
UL44	511	54995	Virion glycoprotein C
UL45	172	18178	Hydrophobic N terminus
UL46	718	78239	-
UL47	693	73812	-
UL48	490	54342	Major tegument protein; activator of IE genes
UL49	301	32252	-
UL50	371	39125	Deoxyuridine triphosphatase
UL51	244	25468	-
UL52	1058	114416	DNA replication
UL53	338	37570	Multiply hydrophobic
UL54	512	55249	IE transcriptional regulator (IE63)
UL55	186	20491	-
UL56	197	21182	-
IE175	1298	132835	IE transcriptional regulator (IE175)
US1	420	46521	IE protein (IE68)
US2	291	32468	-
US3	481	52831	Protein kinase
US4	238	25236	Virion glycoprotein G
US5	92	9555	Putative glycoprotein
US6	394	43344	Virion glycoprotein D
US7	390	41366	Virion glycoprotein I
US8	550	59090	Virion glycoprotein E
US9	90	10026	Tegument phosphoprotein
US10	312	34053	Virion protein
US11	161	17756	-
US12	88	9792	IE protein (IE12)

References are cited in the original paper (McGeoch et al., 1988).

a powerful method for correlating gene and product relies on the ability to raise antisera against synthetic oligopeptides, predicted to occur in HSV-encoded proteins from sequence data. In addition, targeted mutagenesis of coding regions (based on sequence knowledge) will greatly assist the elucidation of polypeptide functions and their importance to virus viability (Longnecker and Roizman, 1987; Weber et al., 1987). Potential gene function can also be assigned by comparing the computer analysis of predicted protein sequences of an unknown gene with those of characterised genes in data banks.

Many viral proteins have already been identified and assigned to gene loci by the study of mutants (Stow et al., 1978; Preston et al., 1979b; Parris et al., 1980; Conley et al., 1981; Knipe et al., 1981) and analysis of intertypic recombinants (Marsden et al., 1978; Preston et al., 1978; Morse et al., 1978). HSV transcripts have been mapped by S1 mapping and Northern blotting analysis (e.g. Rixon and McGeoch, 1985) and correlated with gene products translated in vitro (Anderson et al., 1980; Preston and McGeoch, 1981; Rixon and McGeoch, 1984). The function of some proteins has also been determined by analysis of polypeptide activity in heterologous expression/assay systems using cloned genes (Preston and Cordingley, 1982; Everett, 1984b; Campbell et al., 1984; Preston and Fisher, 1984). However, the function and identity of of a large number of gene products have yet to be characterised. Figure 2 and table 1 summarise the map location of HSV-1 ORFs and genes encoding proteins with known functions (taken directly from McGeoch et al., 1988).

1.5 HSV REPLICATION

1.5.1 Overview

The replication of HSV is coordinated by temporal control of gene expression. The classification of three groups of genes, immediate-early (IE), early (E) and late (L) (Clements et al., 1977), or alpha, beta and gamma (Honess and Roizman, 1974) is based on their kinetics and

expression in the absence and presence of metabolic inhibitors of translation or DNA replication (Jones and Roizman, 1979; Kozak and Roizman, 1974). The IE genes are the first to be transcribed and their expression does not require de novo protein synthesis, whereas early and late gene expression is dependent on prior synthesis of IE polypeptides (Honess and Roizman, 1974; Clements et al., 1977). IE genes are expressed directly after release of DNA into the nucleus and, as discussed in section 2.2, their expression is stimulated by a component of the infecting virus particle (Post et al., 1981; Batterson and Roizman, 1983).

The IE proteins are potent regulators of HSV gene expression, able to activate E and L genes and repress IE genes (Everett, 1984b; O'Hare and Hayward, 1985a,b; Gelman and Silverstein, 1985; Quinlan and Knipe, 1985; DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986; Rice and Knipe, 1988; Roberts et al., 1988; Sekulovich et al., 1988). In particular the IE gene 3 product, Vmw175, is an essential control factor in the transition from IE to E and L stages of gene regulation (Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980). Early genes, which are involved in the synthesis of viral DNA, are abundantly expressed in the absence of DNA replication (Swanstrom et al., 1975). One early polypeptide, the major DNA-binding protein (MDBP), has been implicated in the general repression of HSV genes (Godowski and Knipe, 1983, 1985, 1986). Immediate early gene expression may also be subject to negative regulation by post transcriptional control mechanisms (Kozak and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985). Late genes are most abundantly expressed following the onset of DNA replication (Swanstron and Wagner, 1974; Powell et al., 1975). Two sub-classes of L genes, $gamma_1$ and $gamma_2$, exist. $Gamma_1$ genes are transcribed in the absence of viral DNA synthesis whereas $gamma_2$ genes require DNA replication before their full expression is attained (Jones and Roizman, 1979; Holland et al., 1980; Gibson and Spear, 1983; Johnson and Everett, 1986a). Many of the late proteins are structural components of the virion.

1.5.2 Cell penetration

HSV particles adsorb to cell surfaces and penetrate predominantly by fusion of the virion envelope with the cellular membrane. The virus encoded glycoprotein gB, present in the virus envelope, is required for fusion since mutants with defective gB are capable only of binding to cells (Sarmiento et al., 1979; Little et al., 1981). The viral capsids then migrate to nuclear pores where they disassemble and DNA is released into the nucleus. The HSV mutant, tsB7, is blocked at the stage of DNA release indicating that a viral-encoded protein is important for this process (Knipe et al., 1981; Batterson et al., 1983).

1.5.3 HSV transcript processing

The mechanism by which HSV DNA is transcribed and mRNAs processed is fundamentally similar to that for cellular RNA polymerase II genes. HSV mRNAs are transcribed in the nucleus by host RNA polymerase II (Ben-Zeev and Becker, 1977; Costanzo et al., 1977). The transcripts are processed at the 5'-terminus by capping, at the 3'-terminus by polyadenylation and are internally methylated (Bachenheimer and Roizman, 1972; Silverstein et al., 1973; Bartoski and Roizman, 1976; Moss et al., 1977). The 3'-flanking sequences of HSV genes contain the sequence YGTGTTYY, thought to be important for the termination of transcripts (McLaughlan et al., 1985).

Splicing of HSV mRNAs is rare (Frink et al., 1981, 1983; Watson et al., 1981, Costa et al., 1985). This is in contrast to many viruses, including CMV and EBV, which generate multiple individual mRNAs with common 5' and 3' termini, thereby providing an economic use of template DNA. HSV-1 IE mRNAs 4 and 5 are spliced within their common 5' untranslated regions located within TR_S and IR_S (Watson et al., 1981; Rixon and Clements, 1982), and IE gene 1 contains two introns within the coding sequence (Perry et al., 1986). One gene in the U_L segment (UL15) is also spliced and contains two ORFs in separate exons (Costa et al., 1985; McGeoch et al., 1988). The transcript found associated with latent infections (LAT) is also spliced but only a tentative

ORF has been designated (Wechsler et al., 1988). The purpose of these splicing events does not appear to be related to gene compression since the introns do not lie within overlapping reading frames. In fact, only a small degree of reading frame overlap exists in the genome. US10 and US11 are the only HSV genes in US whose products are actually known to be encoded by overlapping reading frames (Rixon and McGeoch, 1984; figure 3). In addition, there are 11 proposed overlaps of coding sequence within U_L (McGeoch et al., 1988). Many HSV transcripts, although not spliced, partially overlap and are arranged in nested groups with unique 5' ends and common 3' ends, as is the case for the mRNAs of genes US10, 11 and 12 (Hall et al., 1982; Costa et al., 1983; Rixon and McGeoch, 1984; figure 3). Families of genes with common 5' ends and different 3' ends are also found and are the result of readthrough of polyadenylation sites (Anderson et al., 1981; Hall et al, 1982).

1.5.4 IE genes

IE gene expression peaks about 2-3h after adsorption but IE mRNAs can still be detected in the cytoplasm at late times (Harris-Hamilton and Bachenheimer, 1985; Godowski and Knipe, 1986). During infection in the presence of cycloheximide, large quantities of IE mRNAs accumulate (Preston, 1979a). There is no transcription of E and L genes since protein synthesis is inhibited and IE polypeptides are required for the switch to E and L mRNA synthesis. Using these "immediate-early" conditions Clements et al. (1979) were able to map IE mRNA transcripts to the genome. There are 5 IE genes, the locations of which are shown in figures 2 and 15. IE genes 1 and 3 are located within the long and short inverted repeats respectively, and are therefore present in two copies each (Watson et al., 1979; Anderson et al., 1980; Mackem and Roizman, 1980; Rixon et al., 1982). The promoter and 5' untranslated leader sequences of IE genes 4 and 5 (encoding Vmw68 and Vmw12 respectively) are located within the short repeat and thus are common to both, but the coding sequences differ since they are located in the unique segments of the genome (Watson et al., 1981; Rixon and Clements, 1982; figure 3).

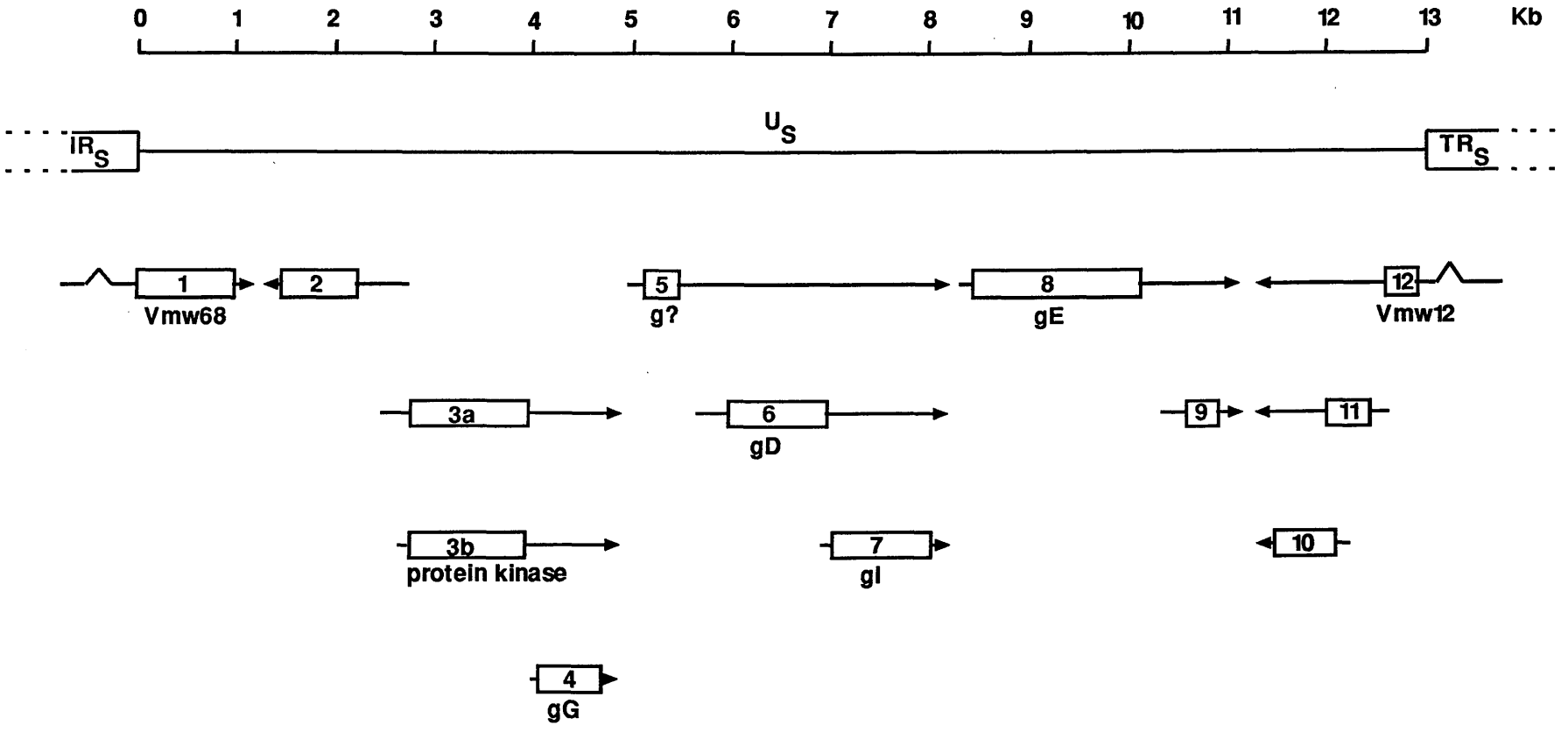

0 1 2 3 4 5 6 7 8 9 10 11 12 13 Kb
IRS
US
TRS
1
2
Vmw68
5
g?
8
gE
12
Vmw12
3a
6
gD
9
11
3b
protein kinase
7
gI
10
4
gG

Fig. 3. Transcripts in U_S of HSV-1. The 12 genes are encoded by 13 transcripts (McGeoch et al., 1985; Rixon and McGeoch, 1985). The probable coding regions (boxes) and non-coding regions (lines) are indicated, and the direction of transcription is shown by arrows. Known polypeptide functions are indicated below the genes. g? indicates a suspected glycoprotein (McGeoch, 1985) and a protein kinase has been assigned to US3 (McGeoch and Davison, 1986; Frame et al., 1987). 11 of the mRNA species (UL3 to UL12) belong to transcript families with 3' co-terminal ends. Part of the coding sequences of US10 and US11 overlap in different reading frames (Rixon and McGeoch, 1984).

IE gene 2 is located in the long unique segment of the genome.

IE transcription does not require de novo synthesis of viral or host proteins (Kozak and Roizman, 1974; Clements et al., 1977) but the transcription of IE genes is stimulated 5 to 10-fold by the major tegument protein of the infecting virion, Vmw65 (Post et al., 1981; Campbell et al., 1984; section 2.2). In these respects the regulation of IE genes is unique within the viral genome. The regulation of IE genes is discussed in detail in section 2.

1.5.5 IE polypeptides

The 5 IE polypeptides Vmw110 (ICP0), Vmw63 (ICP27), Vmw175 (ICP4), Vmw68 (ICP22) and Vmw12 (ICP47) are named according to their mobility by SDS-PAGE analysis and correspond to the products of IE genes 1 to 5 respectively. The IE proteins are all phosphorylated and located within the nucleus with the exception of Vmw12 (Pereira et al., 1977; Marsden et al., 1978, 1982; Preston 1979b; Fenwick and Walker, 1979; Ackermann et al., 1984). The functions of IE polypeptides have been elucidated by the study of virus mutants and, more recently, short-term transient transfection assays using cloned genes in tissue culture. Although the function of Vmw12 remains obscure, roles involved in the regulation of gene expression have been associated with the other IE proteins:

Vmw175 This polypeptide is critical for HSV replication due to its central role in the transcriptional programme. Various conditional HSV-1 mutants containing lesions within IE gene 3 (most notably tsK) fail to synthesise E and L proteins at the NPT and cannot replicate viral DNA (Marsden et al., 1976; Preston et al., 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1978, 1980). Temperature shift experiments using such mutants show that Vmw175 is continuously required for E and L gene expression and suppression of IE genes (Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980). The phenotype of ts Vmw175 mutants ranges from being non-permissive for E gene expression to partially or completely permissive

(DeLuca et al., 1984). However, some mutants which are permissive for E gene expression are nevertheless restricted in viral DNA synthesis and underproduce late gene products, suggesting that Vmw175 may have a direct role in DNA replication.

In transient expression assays using cloned IE-3 genes, E and L genes are activated by Vmw175 (Everett, 1984b; DeLuca and Schaffer, 1985; Gelman and Silverstein, 1985; O'Hare and Hayward 1985a,b; Quinlan and Knipe, 1985; Mavromara-Nazos et al., 1986). Vmw175 has been implicated in the autoregulation of IE gene expression due to the observation that ts Vmw175 mutants overproduce IE transcripts at the NPT (Dixon and Schaffer, 1980). Indeed plasmids that express Vmw175 can repress transcription from the IE-3 promoter (O'Hare and Hayward, 1985b). The other IE promoters can be repressed by Vmw175 under certain conditions in transfection assays (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1987a,b). During infection however, products of later genes may be involved in the turn-off of IE gene expression (Godowski and Knipe, 1986) and IE gene 3 seems to be the only one that is efficiently repressed at the level of transcription (Weinheimer and McKnight, 1987). Transactivation and repression of HSV promoters has also been demonstrated in vitro using partially purified Vmw175 (Beard et al., 1986; Pizer et al., 1986).

Vmw175 has been shown to bind specifically to the target consensus sequence 5'-ATGCTG-3' present at the transcription start site of its own gene, IE gene 3 (Beard et al., 1986; Faber and Wilcox, 1986; Kristie and Roizman, 1986a; Muller, 1987) and it is thought that this process mediates autoregulation (Gelman and Silverstein, 1987b; Muller, 1987; Roberts et al., 1988). However, it is unclear whether Vmw175 binds transactivation-specific promoter sequences present in other viral genes (Everett, 1984a; Eisenberg et al., 1985; Coen et al., 1986; Kristie and Roizman, 1986a,b; Michael et al., 1988).

In order to elucidate the mechanism of Vmw175, mutational analysis of the gene has been undertaken. An amino-terminal region of the polypeptide, which is highly

conserved in the VZV counterpart, was shown to be particularly important for both transactivation and repression functions in transfection studies (DeLuca and Schaffer, 1987, 1988; Paterson and Everett, 1988a,b). Both of these activities correlated with the ability of the protein expressed in transfected cells to bind the Vmw175-specific consensus sequence in gel retardation assays (Paterson and Everett, 1988b). Paterson and Everett (1988b) propose a model whereby Vmw175 mediates transactivation by association with cellular factors (e.g. TFIID) at promoter sequences (e.g. TATA boxes). Repression might then occur only if this complex were in the vicinity of a high affinity Vmw175 binding site, which may block the progression of the transcription machinery.

Vmw110 To date no ts mutants in IE gene 1 have been isolated. However, Vmw110 has been shown to be a potent activator of viral and cellular promoters in transient assay systems in tissue culture (Everett, 1984b; O'Hare and Hayward, 1985a,b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985; Mavromara-Nazos et al., 1986). An HSV-1 mutant, dl1403, was constructed that contained a large deletion in the IE-1 gene and this resulted in a protein that was inactive in transient assays (Stow and Stow, 1986; Perry et al., 1986). The deletion mutant grew inefficiently at low multiplicities of infection (moi), its growth yield being approximately 50 to 100-fold lower than wild-type (wt) virus. However, at high moi the mutant expressed normal amounts of viral proteins and replicated DNA as well as wt virus. The high particle/pfu ratio of dl1403 (approximately 700) resulted from a reduced ability to initiate productive infection at low moi and not from a defect in the virus particle (Stow and Stow, 1986, 1989). The conclusion is that Vmw110 is not absolutely essential in tissue culture, but at low moi viruses that lack the polypeptide exhibit a defect that can be overcome by increasing the number of infecting viruses. Vmw110 may therefore ensure that sufficient viral gene expression for lytic infection can occur at low moi; i.e. under conditions when Vmw175 alone is insufficient to commit the cell to the lytic cycle. Similar

conclusions have been reached more recently in studies using other insertion and deletion mutants constructed in the gene (Sacks and Schaffer, 1987; R.D. Everett, personal communication).

In transfection studies, Vmw110 has been shown to transactivate a wide variety of viral and cellular promoters either by itself or in combination, either additively or synergistically, with Vmw175 (Everett, 1984b, 1986; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986). However, the observation that an IE-3 deletion mutant, that expresses little if any Vmw175 coding sequences, is unable to grow in normal cells suggests that Vmw110 fails to activate E gene expression in the absence of Vmw175 during infection (DeLuca et al., 1985). This observation also argues against transdominant repression of Vmw110 by abnormal Vmw175 polypeptides in non-productive infections using ts Vmw175 mutants at the NPT.

Regions of the polypeptide that are involved in the intrinsic and synergistic activation effects have been defined by in vitro mutagenesis (Everett, 1987, 1988a). The synergism between Vmw175 and Vmw110 is largely dependent on the intregrity of the carboxy-terminal region of the polypeptide, whereas mutations in this region do not greatly affect the ability of the protein to activate by itself. Therefore, two different mechanisms of activation may work and synergy may require an association between Vmw110 and Vmw175. To date, no information regarding the DNA binding properties of the polypeptide is available, although a sequence of the coding region containing a consensus "metal finger binding domain" (Berg, 1986) was absolutely essential for the activity of Vmw110 in the absence of Vmw175.

Vmw63 This protein, the product of IE gene 2, is an essential regulatory polypeptide. A number of ts Vmw63 mutants have been isolated and have been shown to overproduce Vmw175 and Vmw110 at the NPT (Sacks et al., 1985). However, the mutants were able to synthesise normal levels of E proteins and DNA replication was not blocked, although they exhibited a severe reduction of late gene expression, notably of gB (Rice and Knipe, 1988).

In transfection assays, Everett, (1986), demonstrated that Vmw63 can further activate expression of the late gene promoter of VP5 above levels attained with Vmw110 and Vmw175, but only when all three proteins were present; Vmw63 had no effect by itself or in other combinations on other promoters tested including those of TK and gD. Recently, Vmw63 has been shown to both activate and repress a variety of HSV promoters in transfection assays when Vmw110 and Vmw175 are also present (Rice and Knipe, 1988; Sekulovich et al., 1988). In general, these results are consistent with the observations that ts Vmw63 mutants both overexpress and fail to express subsets of gene class products at the NPT (Sacks et al., 1985).

Vmw68 No ts mutants with lesions in the IE-4 gene have been isolated to date. A deletion mutant which removed the carboxy terminal third of Vmw68 was constructed (Post and Roizman,1981) which exhibits poor growth and reduced expression of at least one L gene in some cell lines (Sears et al., 1985). In addition, the mutant was not neurovirulent in mice.

Vmw12 There are no ts mutants in IE gene 5 and in fact viable deletion mutants have been constructed that lack the whole gene, indicating that the protein does not play an important role during infection in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

1.5.6 Regulation of early gene expression

Peak expression of the early genes occurs 4-6h post adsorption, although there is more variation in their kinetics of expression than for the IE genes. For example, although the large sub-unit of ribonucleotide reductase (RR_1) has predominantly early kinetics, it can still be detected in the presence of cycloheximide and in ts Vmw175 mutants that fail to express E genes at the NPT (Preston, 1979a; DeLuca et al., 1985). Thus RR_1 has been classed as a $beta_1$ gene product (Roizman and Batterson, 1985). Some genes, including gD, are detected at early times but they

are not fully expressed until after the onset of DNA replication (Gibson and Spear, 1983; Johnson and Everett 1986a). Genes whose expression is moderately dependent on DNA replication have been described as early-late (EL), leaky late (beta gamma) and $gamma_1$ (Roizman and Batterson, 1985; Wagner, 1985; Harris-Hamilton and Bachenheimer, 1985).

Early gene promoters have been studied in detail in an attempt to identify induction-specific sequences (Coen et al., 1986; Eisenberg et al, 1985; Everett, 1983, 1984a;) Early promoters contain upstream sequences necessary for full expression such as a CAAT box and GC-rich Spl sites for TK, and AG-rich regions for gD. The TATA box of these promoters is probably the main factor involved in the response to viral products, although other promoter elements are important (McKnight et al., 1981; McKnight and Kingsbury, 1982; Everett, 1983, 1984a; El Kareh et al., 1985; Coen et al, 1986;). Indeed the SV40 promoter, which is poorly responsive to HSV infection, can be transactivated when its TATA box is substituted with that of the gD promoter which suggests that HSV IE proteins can substitute for or directly modulate the TATA box transcription factor TFIID at HSV TATA box regions (Everett, 1988b). It should be noted however that although many HSV promoters contain homologies to TATA, CAAT and GC-rich elements, there is a great deal of diversity between promoters of the same class (Wagner, 1985; Mackem and Roizman, 1982c). These differences may account for the variation in the level of expression in equivalent groups of genes (Honess and Roizman, 1974; O'Hare and Hayward, 1985a; Harris-Hamilton and Bachenheimer, 1985).

An important observation made by Everett (1983, 1984b) was that a heterologous promoter, that of the rabbit beta globin gene, was also transactivated by HSV infection. When integrated into the viral genome, the beta globin gene was regulated with the kinetics of an E gene, suggesting that E gene promoter selectivity is not exclusively dependent on viral-specific sequences (Smiley et al., 1987). Interestingly, the endogenous cellular copy of the beta globin gene in rabbit kidney cells did not respond to HSV-1 infection, indicating that genes respond differently to

trans-acting factors depending on whether they are chromatin-associated or extrachromosomal.

1.5.7 Regulation of late gene expression

Late gene product accumulation peaks by 10-16h post adsorption, approximately 2h after the peak of viral DNA synthesis (Munk and Sauer, 1964; Roizman, 1969; Wilkie, 1973). Analysis of viral-induced polypeptides under conditions of virus DNA synthesis inhibition using chemical inhibitors or ts DNA$^-$ mutants reveals that late gene expression requires DNA replication to occur (Swanstrom and Wagner, 1974; Honess and Roizman, 1974; Powell et al., 1975; Marsden et al., 1976; Jones and Roizman, 1979; Holland et al., 1980; Conley et al., 1981; Pederson et al., 1981). Late genes have been classed as either "leaky late" (gamma$_1$), whose expression is reduced but still detectable in the absence of DNA replication, or "true late" (gamma$_2$), whose expression is very hard to detect under these conditions (Wagner, 1985; Roizman and Batterson, 1985). The major capsid protein, Vmw155, is an example of a leaky late protein while US11 exhibits true late characteristics, even to the extent that cloned US11 genes require plasmid DNA replication in cis for maximal expression in transient transfection assays (Johnson and Everett, 1986a,b; Johnson et al., 1986). These experiments used an HSV origin of DNA replication and the US11 promoter linked to an assayable gene, and it was found that only the TATA box region was required for fully efficient regulated expression upon HSV infection (Johnson and Everett, 1986b). This contrasts with the upstream requirement of E promoters which need regions upstream of the TATA box for full expression upon infection. In addition, the early promoter of gD is regulated as a true late promoter in the absence of its upstream region if linked to an origin of DNA replication (Johnson et al., 1986). The reasons how or why viral DNA replication leads to L gene induction is not understood. It is unlikely to be due simply to an increase in copy number of promoters, since E genes would then also be expected to be dependent on DNA synthesis. Replication could possibly cause a change in the DNA template and provide a switch for L gene expression, or

perhaps part of the replication machinery is needed *in situ* to initiate L gene transcription. However, analysis of mutants indicates that the IE gene products Vmw63 and Vmw68 are involved in L gene induction since these viruses underexpress certain late proteins (Sears *et al.*, 1985; Sacks *et al.*, 1985; section 1.5.5). In addition, L gene promoters, like those of E genes, are activated by IE proteins in transfection assays (Dennis and Smiley, 1984; DeLuca et al, 1985; Mavromara-Nazos *et al.*, 1986, Everett, 1986; section 1.5.5).

1.5.8 Enzymes

Many of the E proteins identified to date have enzymic activity and play roles in the metabolism or synthesis of viral DNA. The known virally encoded enzymes are thymidine kinase (TK) (Kit and Dubbs, 1963; Jamieson *et al.*, 1976), alkaline exonuclease (Morrison and Keir, 1968; Moss *et al.*, 1979), DNA polymerase (Keir *et al.*, 1966; Chartrand *et al.*, 1979, 1980), ribonucleotide reductase (RR) (Cohen, 1972; Dutia, 1983), dUTPase (Wohlrab and Francke, 1980; Preston and Fisher, 1984), uracil DNA glycosylase (Worrad and Caradonna, 1988; Mullaney *et al.*, 1989) and protein kinase (McGeoch and Davison, 1986; Purves *et al.*, 1986; Frame *et al.*, 1987).

Analysis of mutants has revealed that DNA polymerase is essential for growth in tissue culture (Chartrand *et al.*, 1980) whereas the TK, dUTPase, uracil DNA glycosylase, protein kinase and alkaline exonuclease proteins are not required for virus replication (Dubbs and Kit, 1964; Fisher and Preston, 1986; Mullaney *et al.*, 1989; Purves *et al.*, 1987; S. Weller , 13th International Herpesvirus Workshop abstracts, Irvine 1988). Mutants containing *ts* lesions in ribonucleotide reductase have been isolated, indicating that it is an essential polypeptide (Preston *et al.*, 1984, 1988; Dutia, 1983). However, Goldstein and Weller (1988) have shown that viruses in which the RR gene is deleted are viable in growing cells at 34^{o}C but not at 39.5^{o}C. They suggest that a cellular factor, capable of complementing viral RR, is present in growing cells at lower temperatures but inactive at 39.5^{o}C. Thus

the requirement of viral RR is largely dependent on the conditions in which infection occurs. RR consists of two subunits, one large and one small (Vmw136 or RR_1, and Vmw38 or RR_2) (Huszar et al., 1983; Bacchetti et al., 1984; Preston et al., 1984). Studies with ts1207 and ts1222, combined with immunological studies, show that the two subunits are inactive until associated with each other as a protein complex (Preston et al., 1984, 1988; Frame et al., 1985). A synthetic nonapeptide corresponding to the carboxyl 9 amino acids of the small subunit specifically inhibited the viral but not the cellular enzyme (Dutia et al., 1986; Cohen et al., 1986). It was proposed that inhibition of activity resulted from the ability of the nonapeptide to competitively inhibit Vmw38 from associating with the binding site on Vmw136. This finding suggests a novel approach to the development of antiviral agents that block protein:protein interactions between virus polypeptides.

The HSV DNA polymerase differs from most eukaryotic polymerases by virtue of its 3' to 5' exonuclease (proof reading) activity (Knopf, 1979). In addition, the HSV enzyme is sensitive to phosphonoacetic acid (PAA), a pyrophosphate analogue (Leinbach et al., 1976).

1.5.9 Structural polypeptides

HSV virions are estimated to contain between 15 and 33 polypeptides (Spear and Roizman, 1972; Heine et al., 1974) which are grouped into glycoproteins, tegument proteins and capsid proteins.

Glycoproteins HSV-1 encodes a minimum of seven glycoproteins (gB, gC, gD, gE, gG, gH and gI), identified by their ability to incorporate radiolabelled sugar molecules (Spear, 1976; Bauke and Spear, 1979; Buckmaster et al., 1984; Spear, 1985; Frame et al., 1986; Richman et al., 1986; Ackermann et al., 1986; Longnecker et al., 1987; Johnson et al., 1988). One additional open reading frame, whose protein is as yet unidentified, has characteristic glycoprotein amino acid sequences (McGeoch, 1985). The

glycoproteins are located within the membrane that composes the viral envelope and are probably exposed on the surface of the virion. Johnson et al. (1984) have implicated gB, gC and gD as having roles in cell surface adsorption. In addition, gB, gD and gH all play essential roles in the ability of HSV to fuse with plasma membranes and penetrate cells (Sarmiento et al., 1979; Little et al., 1981; Minson et al., 1986; Desai et al., 1988; Cai et al., 1988). Glycoprotein gD is thought to be an essential cell surface receptor-binding polypeptide (Johnson and Ligas, 1988). Glycoproteins gE and gI interact specifically with the Fc domain of immunoglobulin G (IgG) (Bauke and Spear, 1979; Johnson et al, 1988). Thus it appears likely that several glycoproteins, as well as structural proteins (Addison et al., 1984), participate in entry of HSV into cells in a multistep process.

Tegument proteins These proteins can be identified when released from virions in the presence of non-ionic detergents, although some associate more tightly to capsids than others (Lemaster and Roizman, 1980; Roizman and Furlong, 1974; Spear, 1980). Little is known about the orientation or the exact number of proteins that comprise this region, but polypeptides not classified as glycoproteins or capsid proteins are generally referred to as tegument proteins. The major tegument protein is Vmw65, the subject of this thesis.

Capsid proteins Capsids isolated from infected cells are either empty or contain HSV DNA. Empty capsids are made up of at least 5 proteins while mature nucleocapsids contain 2 additional polypeptides, one of which is VP22 (p40)(Gibson and Roizman, 1972, 1974; Heilman et al., 1979; Preston et al., 1983). Another polypeptide found in capsids is Vmw155, the major capsid protein.

1.5.10 DNA replication

Upon infection, linear herpesvirus DNA molecules circularise (Jean et al., 1977; Jacob and Roizman, 1977) by direct ligation of the termini (Jean and Ben-Porat, 1976; Davison and Wilkie, 1983; Poffenberger and Roizman, 1985). During the initial stages of DNA replication, loops and eyes

in the DNA molecule are visible by electron microscopy at multiple, widely separated loci (Friedman et al., 1977). Newly replicated DNA molecules are found in large concatemers, in head to tail configuration, which probably arise by a rolling circle replication process (Jacob and Roizman, 1977; Ben-Porat and Tokazewski, 1977). Electron microscopic studies and analysis of defective particles with incomplete genomes revealed that HSV DNA contains three origins of replication, two within the repeated R_S segment (ori_S) and one within U_L (ori_L) (Frenkel et al., 1976; Jean et al., 1977; Rixon and Ben-Porat, 1979; Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). Studies with mutant viruses have shown that deletion of ori_L or one copy of ori_S has little or no effect on virus replication in cultured cells (Longnecker and Roizman, 1986; Polvino-Bodnar et al., 1987). The cis-acting ori sequences were fine mapped by identifying DNA fragments capable of inducing replication of bacterial vectors in HSV infected cells. Ori_S consists of a 90bp fragment containing a 45bp palindromic sequence featuring 18 centrally located A or T residues flanked by G+C-rich tracts, located between the 5' ends of IE mRNAs 3 and 4/5 (Stow, 1982: Stow and McMonagle, 1983). Ori_L, located in the middle of U_L between the genes encoding DNA polymerase and the major DNA-binding protein (MDBP), contains a 72bp palindrome closely related (85% homologous) to ori_S (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller et al., 1985). The potential ability of the origins to form cruciform structures containing a stretch of low melting point (AT-rich) DNA probably facilitates strand separation in these regions of the genome (Stow, 1985). Ori_S and ori_L appear to be functionally identical in transient complementation assays (Wu et al., 1988).

1.5.11 Genes required for DNA replication

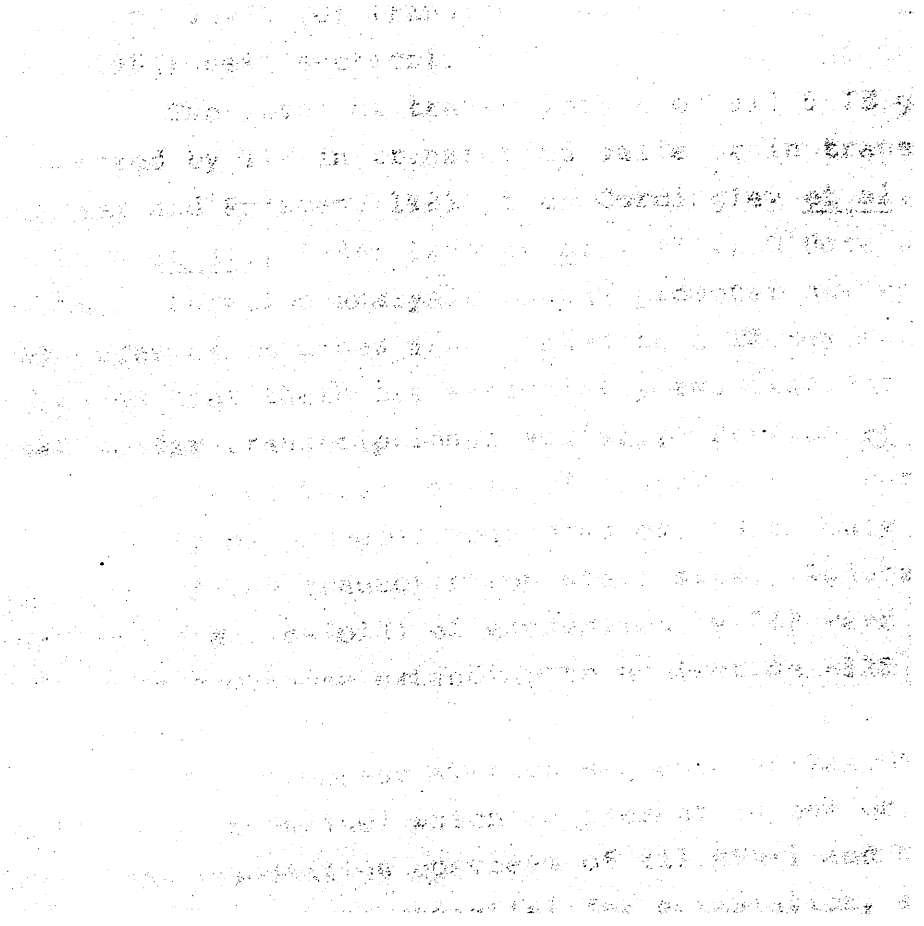
The major DNA binding protein (MDBP) is an early gene product essential for DNA replication (Conley et al., 1981), although the function of this protein is not well defined. The MDBP is a single-stranded DNA binding protein that may reduce the melting temperature at poly(dA).poly(dT) DNA tracts (e.g. at origins of DNA replication), thereby

facilitating strand separation (Powell et al.,1981). The MDBP also has a distict role in gene regulation, since ts MDBP mutants overexpress gC, and functional MDBP is also involved in repression of the IE-3 and Vmw155 genes (Godowski and Knipe, 1983, 1985, 1986). Genes required for viral DNA synthesis have also been identified by analysing the minimal fragments of the HSV genome necessary to support replication of plasmid vectors containing HSV origins in transient complementation assays (Challberg, 1986; Wu et al., 1988). Seven genes, based on predicted ORFs, were directly required for DNA replication. Two of these genes, encoding DNA polymerase and the MDBP, were already known to be essential for virus DNA replication by genetic analysis of mutants (Hay and Subak-Sharpe, 1976; Chartrand et al., 1979; Conley et al., 1981; Weller et al., 1983; Coen et al., 1984; Gibbs et al., 1985). The others were UL5, UL8, UL9, UL42 and UL52. The product of gene UL42 is a DNA-binding protein (Marsden et al., 1987) and the polypeptide encoded by UL9 binds specifically at ori DNA sequences (Olivo et al., 1988). In addition, there is evidence that UL5 encodes a helicase (L. Zhu and S. Weller, 13th International Herpesvirus Workshop abstracts, Irvine 1988). By analogy with prokaryotic systems, the other gene products might be expected to include a primase, a topoisomerase and accessory factors which may increase the processivity or efficiency of DNA polymerase (Wu et al., 1988).

1.5.12 DNA packaging and assembly of virions

Mature concatemeric DNA that has been replicated undergoes cleavage to produce unit length linear molecules which are then encapsidated into virus particles. Cleavage of DNA molecules occurs at the "a" sequence present at the termini of standard molecules (Davison and Wilkie, 1981; Mocarski and Roizman, 1982; Varmuza and Smiley, 1985; Nasseri and Mocarski, 1988). Stow et al. (1983) showed that the "a" sequence is also responsible for encapsidation of viral DNA into particles since addition of this sequence to plasmids containing HSV origins allowed plasmid DNA to be packaged in HSV-1 infected cells. Cleavage of concatemeric DNA into unit length genomes is either a prerequisite for, or

occurs concomitantly with, packaging into capsids (Vlazny et al., 1982). Analysis of an HSV mutant, ts1201, showed that packaging of DNA requires the processing of a structural polypeptide p40 (VP22a) to a form of lower electrophoretic mobility (VP22) (Preston et al., 1983). Unprocessed forms of p40 were associated with empty capsids, indicating that failure to package DNA was not due to a gross aberration in capsid structure (Preston et al., 1983). Another mutant, ts1204, which belongs to a different complementation group, was also unable to encapsidate DNA, indicating that other polypeptides are involved in the process (Addison et al., 1984).



2. REGULATION OF IE GENE EXPRESSION

2.1 CIS-ACTING REGULATORY SEQUENCES

Post et al. (1981) first demonstrated that cis-acting DNA sequences, present in the 5' flanking region of IE gene 3, determined the regulatory characteristics of IE gene expression. A hybrid gene was constructed that comprised the TK coding sequences linked to the promoter and upstream sequences of the HSV-1 IE-3 gene. This construct was introduced into viral DNA, and in the recombinant virus the gene exhibited IE kinetics and was expressed under conditions of a cycloheximide block/actinomycin release, a feature distinctive of IE genes. In addition, transcription from the plasmid construct when present in biochemically transformed cells was stimulated by superinfection with HSV-1 under "immediate early conditions", suggesting that IE gene expression was subject to "trans-induction" by a virus-encoded factor, later identified as the virion component Vmw65 (or transinducing factor, TIF) (Campbell et al., 1984; next section).

The level of transcription of all 5 IE genes is increased by TIF in transformed cells or in transient assays (Mackem and Roizman, 1982a,b,c; Cordingley et al., 1983; Preston et al., 1984; Lang et al., 1984; O'Hare and Hayward, 1985b). Deletion analysis of the promoter and upstream sequences of IE genes 4/5, linked to a TK reporter gene, revealed that there are essentially two distinct domains that confer transcriptional activity (Preston et al., 1984). Constitutive or basal levels of transcription were mediated efficiently by promoter sequences extending only 69bp upstream of the transcription start site. Induced levels (approximately 8-fold) of expression by TIF were achieved only when sequences extending to nucleotide -335 upstream were present.

The consensus AT-rich sequence 5'-TAATGARAT-3' (where R is a purine) which is present in one or more copies in either orientation upstream of all HSV-1 and HSV-2 IE genes appeared to be essential for stimulation, although flanking elememts modulated transcriptional activity (Preston et al., 1984; figure 4 and table 2). Additional

studies of other IE gene upstream sequences revealed a similar organisation of promoter and regulatory elements (Mackem and Roizman, 1982a; Cordingley et al., 1983; Kristie and Roizman, 1984; Lang et al., 1984; Bzik and Preston, 1986; O'Hare and Hayward, 1987; figure 4 and table 2). A synthetic 20-mer oligonucleotide containing the TAATGARAT target sequence was sufficient to confer inducibility by Vmw65 when linked to a IE promoter/CAT gene construct in either orientation and in the absence of other IE far-upstream sequences (Gaffney et al., 1985). This effect was increased when multiple copies of the 20-mer were present. Bzik and Preston (1986) also showed that a TAATGARAT element upstream of IE-3 essentially conferred inducibility in the absence of other sequences, and that flanking GA-rich tracts modulated activation by TIF. These GA-rich sequences (also present upstream of IE genes 2 and 4/5; figure 4 and table 2) were also capable of activating an otherwise non-functional homolgue of TAATGARAT. Triezenberg et al. (1988b) place more emphasis on the involvement of the GA-rich elements in the Vmw65 response, and suggest that induction can be mediated either by TAATGARAT or the GA-rich element. Gelman and Silverstein (1987b) observed that minimal IE promoters that lacked TAATGARAT elements were capable of TIF-mediated induction, but only when a high effector/target ratio was used, implying that an alternative, target-sequence independent mechanism of TIF augmented IE regulation might operate.

In addition to Vmw65 responsive regulatory signals, the upstream region of IE-3 and IE-4/5 contains a distinct enhancer-like cis-acting sequence (Lang et al., 1984; Preston and Tannahill, 1984). In the absence of Vmw65, the enhancer can stimulate expression from linked promoters in an orientation independent manner and at a considerable distance from heterologous promoters (Lang et al., 1984; Preston and Tannahill, 1984). However, unlike classical enhancer elements, the HSV enhancer does not function when present downstream of a gene (Preston and Tannahill, 1984). Deletion analysis of the enhancer suggests that it is composed of 3 separate elements, each of which impart a 2 to 3-fold effect (Bzik and Preston, 1986). Two of these

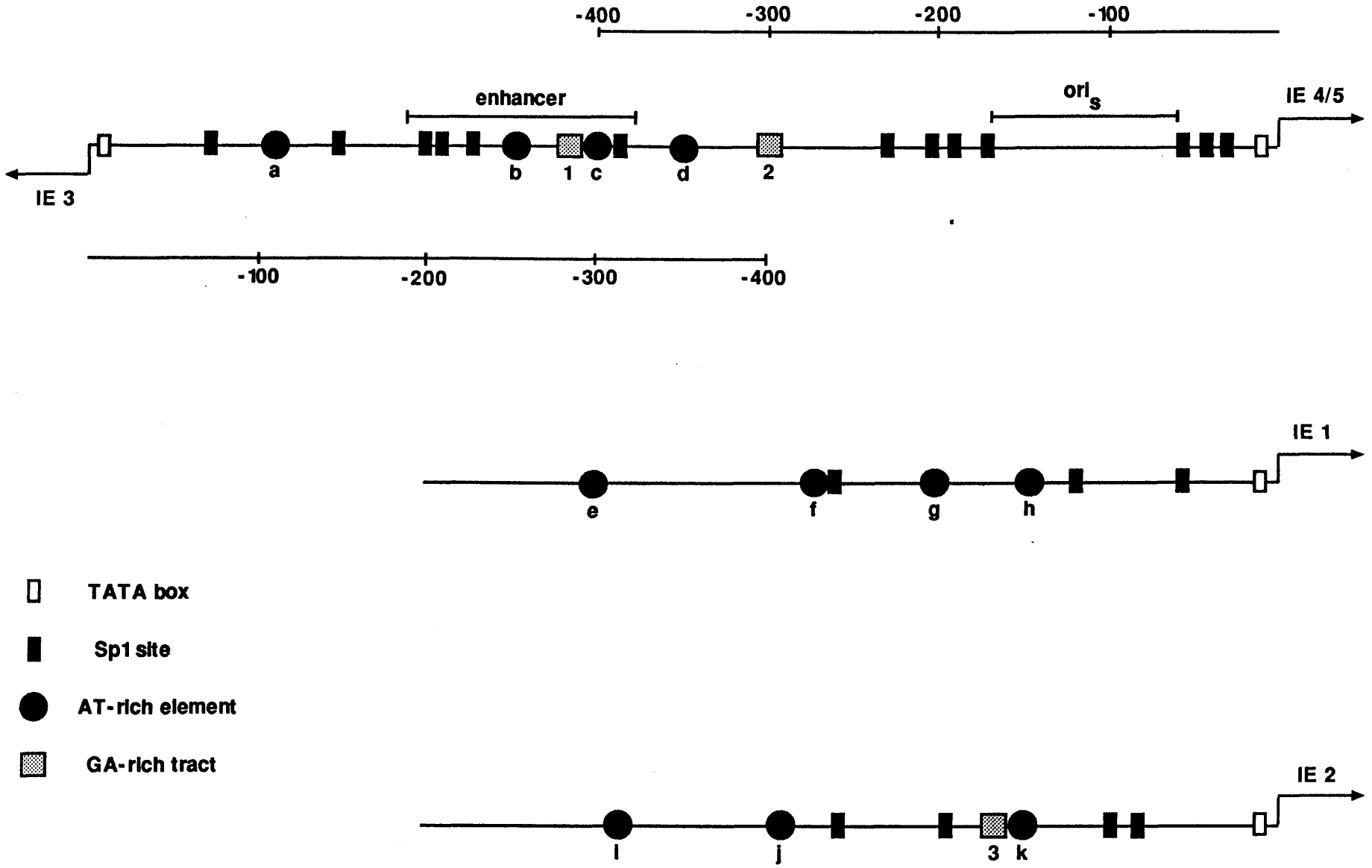

-400
-300
-200
-100
enhancer
ori S
IE 4/5
IE 3
a
b
1
c
d
2
-100
-200
-300
-400
IE 1
e
f
g
h
TATA box
Sp1 site
AT-rich element
GA-rich tract
IE 2
i
j
3
k

Fig. 4. Schematic representation of the distribution of promoter-regulatory elements in the upstream regions of IE genes. The location of four sequence motifs are displayed relative to the 5' end of the IE mRNAs (arrowed). The DNA sequence and orientation of the elements with respect to the IE genes is shown in table 2. The elements are the: TATA box, Spl binding site, AT-rich motifs (including "octamer" and TAATGARAT sequences) and GA-rich tracts. The nucleotide sequences of these promoters were taken from IE genes 1 (Mackem and Roizman, 1982b; Perry et al., 1986), 2 (Mackem and Roizman, 1982b; Whitton et al., 1983) 3 and 4/5 (Mackem and Roizman, 1982b; Murchie and McGeoch, 1982; McGeoch et al., 1986).

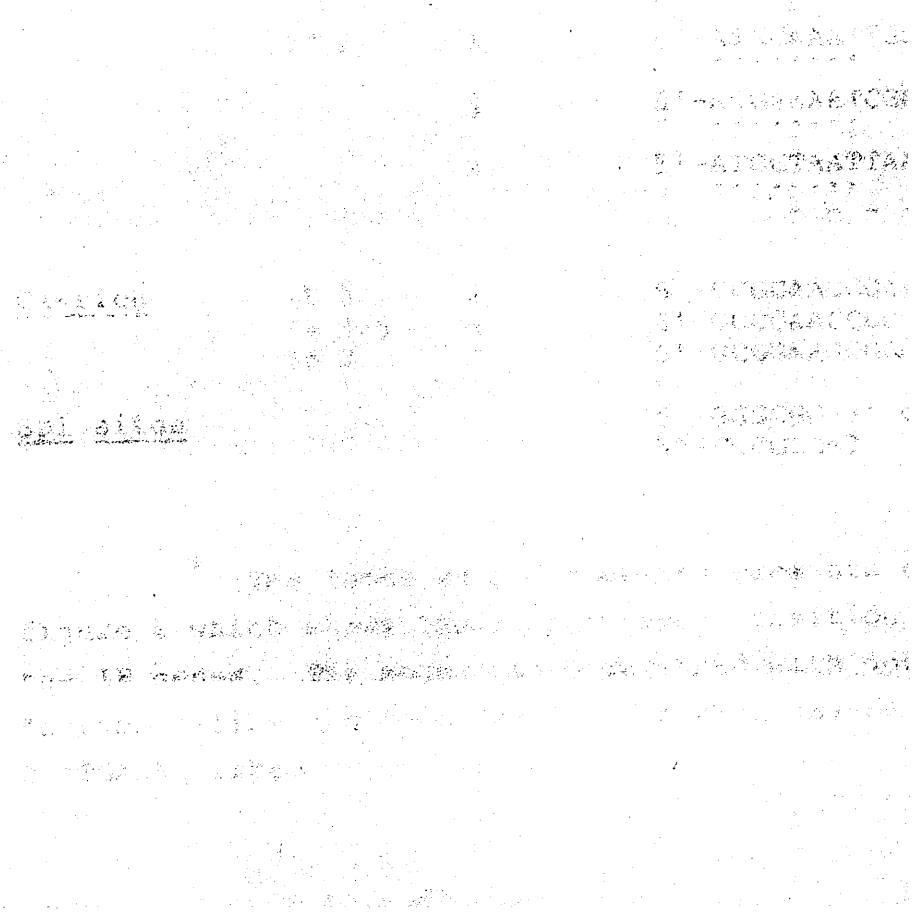


Table 2.

DNA sequence of regulatory elements upstream of IE genes

	gene	position*	sequence
AT-rich	IE 3	a	5'-TGCATAATGGAAT-3'
		b	5'-GCGGTAATGAGAT-3'
	IE 4/5	c	5'-ATGCTAACGAGGA-3'
		d	5'-GCGGTAATGAGAT-3'
	IE 1	e	5'-ATGCTAATTGCTT-3'
		f	5'-ATGCTAATGAGTT
		g	5'-ATGCTAATGGGGT-3'
		h	5'-ATGCTAATGATAT-3'
	IE 2	i	5'-ATGCAAATTGGAA-3'
		j	5'-ATGAAAATCGGTC-3'
		k	5'-ATGCTAATTAAAT
GA-rich	IE 3	1	5'-GCGGAACGGAAGCGGAAA-3'
	IE 4/5	2	3'-GCCGAACCGGGAAG-5'
	IE 2	3	5'-GCGGAAGCGGAAC-3'
Spl sites			5'-GGGCGG-3' or 5'-CCCGCC-3'

* The lettered and numbered elements correspond to figure 4 which shows their approximate position upstream of the IE genes. The sequences underlined with dots are "octamer"-like and those underlined with dashes are TAATGARAT-like.

elements correspond to the hexanucleotide 5'-GGGCGG-3' or its complement, which binds the cellular transcription factor Spl (Jones and Tjian, 1985). The third element contains the sequence 5'-GCGGAAAC-3', which shares homology with a domain in the SV40 enhancer that binds a factor GTIIA (Bzik and Preston, 1986; Xiao et al, 1987), and is similar to sequences in the adenovirus and polyoma virus enhancer motifs (Weiher et al., 1983; Hearing and Shenk, 1983; Herbommel et al., 1984).

Recently the octamer element 5'-ATGCAAAT-3', a regulatory signal common to many other eukaryotic promoters and enhancers (see sections 3.1 and 3.2), has been implicated in HSV IE gene regulation since a similarity exists between this consensus motif and sequences overlapping many TAATGARAT elements (Pruijn, 1986; O'Hare and Goding, 1988; Gerster and Roeder, 1988; see section 2.3). For example an element of the IE-1 gene has the sequence 5'-ATGCTAATGATAT-3', matching the octamer consensus in seven out of eight residues. Workers studying other systems have shown that the octamer DNA element is bound by at least two cellular protein factors, OTF-1 or NFIII, and OTF-2. OTF-1 and OTF-2 are implicated in the regulation of human histone H_2B and immunoglobulin heavy and light chains genes respectively (Harvey et al., 1982; Falkner et al., 1986; Sive and Roeder, 1986; Scheidereit et al., 1987; see section 3.2). The interaction of cellular factors with TAATGARAT elements is disscussed in section 2.2. The upstream regulatory regions of IE genes are thus complex, modular structures that probably interact with cellular factors and Vmw65 with the effect of increasing the efficiency of IE mRNA production.

2.2 A TRANS-INDUCING IE GENE REGULATORY FACTOR (TIF)

In transformed cells that harbour a gene construct composed of the IE-3 promoter and upstream sequences linked to the TK gene, TK expression could be induced on infection with TK^- HSV-1, suggesting a role for a virally encoded IE transinducing factor (TIF) (Post et al., 1981). Furthermore, TK mRNA synthesis was also induced by superinfection at the NPT with an HSV-1 mutant possesing a

ts defect in Vmw175 or with UV-inactivated virus, indicating that TIF was a protein component of the infecting virion (Post et al., 1981; Batterson and Roizman, 1983). The mutant tsB7, which is defective for capsid uncoating at the NPT, was also able to elicit the stimulatory response, showing that the TIF was located external to the nucleocapsid (Knipe et al., 1981; Batterson et al, 1983). The genomic identity of TIF was elucidated by cotransfection of cloned restriction fragments of HSV-1 DNA with an IE-TK reporter plasmid (Campbell et al., 1984). The gene was located and the polypeptide, Vmw65, identified by sub-cloning and immunoprecipitation studies (Campbell et al., 1984).

Subsequently, a number of viruses were tested for their ability to transinduce HSV IE promoters. While HSV-2 gave a positive response, PRV, HCMV and adenovirus type 2 were ineffective (Batterson and Roizman, 1983; Campbell and Preston, 1987). However, Vmw65 was able to stimulate expression from the PRV IE gene in cotransfection experiments, implying that PRV contains induction responsive sequences but no corresponding TIF (Campbell and Preston, 1987). DNA sequence analysis of the upstream region of the PRV IE gene revealed the presence of two TAATGARAT-like motifs, presumed to mediate the Vmw65 response. In addition, the region contained 6 repeats of a 15bp element 5'-GGCCAATGGGATTTY-3', which contains homology to the CCAAT box and also to the strong enhancer of HCMV (5'-C/ACTAACGGGACTTTCCAA-3') (Campbell and Preston, 1987; Boshart et al., 1985). A speculation made from these observations is that the PRV 15bp motifs may act as an enhancer (possibly including the interaction of CCAAT-binding factors) such that the requirement for TIF is overcome, and that the presence of TAATGARAT signals in HSV is predominantly to increase expression of IE genes that lack proximal enhancer elements (e.g. IE genes 1 and 2) (Campbell et al., 1984). Results presented in this thesis are consistent with this idea.

The DNA and predicted amino acid sequences of Vmw65 of HSV-1 have been determined (Dalrymple et al., 1985; Pellett et al., 1985). Sequence homology analysis with

Vmw65 has identified a putative 45K TIF homologue in VZV, but it is not known if this polypeptide has transinducing activity. A distinctive feature of the VZV homologue is that it lacks the 80 carboxy-terminal amino acids of Vmw65, a highly acidic domain of the protein (Dalrymple et al., 1985; Davison and Scott, 1986). Recently it has been shown that this acidic region of Vmw65, in common with acidic domains of other eukaryotic and prokaryotic activator proteins, is important for transinducing activity (Triezenberg et al., 1988a; Sadowski et al., 1988; see below). These observations, together with preliminary data from T.McKee (personal communication; section 7.1), suggests that the VZV homologue may not function as a TIF.

A study done concurrently with work presented in this thesis also investigated functional regions of Vmw65 by deletion mutagenesis (Triezenberg et al., 1988a). Two functionally distinguishable domains of the polypeptide were identified. A domain localised within the carboxy 78 codons of the Vmw65 ORF, which is unusually high in acidic residues (with a net charge of -18), is critical to the transcription activation function. This "acid tail" of Vmw65 appears to be analogous to the activating domains of the yeast gene activator proteins GCN4 (Hope and Struhl, 1986) and GAL4 (Ma and Ptashne, 1987a), which are also markedly acidic, since hybrid GAL4-Vmw65 proteins in which the GAL4 acidic region is replaced by the carboxy-terminal 78 amino acids of Vmw65 are potent activators of transcription (Sadowski et al., 1988; sections 3.2 and 3.3). A second domain of Vmw65, located upstream of the acid tail, was also important for transinduction. In the absence of the acid tail, the amino-terminal segment (approximately between amino acid positions 56 and 393) acts to dominantly and specifically interfere with the inducing activity of wt Vmw65 polypeptides. Thus the amino terminal "interfering" domain of Vmw65 may represent the region of the polypeptide responsible for tailoring the specificity of Vmw65 interaction at TAATGARAT elements by association with specific cell factors (see next section). Triezenberg et al. (1988a) propose that these truncated proteins retain a capacity to interact specifically with cellular DNA-binding

proteins that mediate interaction with IE cis-regulatory sequences, thereby competing with wt Vmw65.

2.3 VMW65 ASSOCIATES WITH CELLULAR FACTORS AT TAATGARAT ELEMENTS

Initial studies with Vmw65 demonstrated that it was not a DNA binding protein (Marsden et al., 1987). Thus it was speculated that TIF might mediate transinduction indirectly by interacting with cellular proteins, similar to mechanisms proposed for other transcription regulators that did not directly bind to DNA (e.g. adenovirus E1A). However, an important difference between Vmw65-mediated transinduction and gene regulation by other non-DNA binding proteins is the requirement for a specific cis-acting target sequence (TAATGARAT) located upstream of the basal promoter sequences.

Using gel retardation assays and DNAase I footprint protection analysis, it was demonstrated that unidentified cellular factors from uninfected cells specifically bound at and/or immediately surrounding the TAATGARAT responsive element (Kristie and Roizman, 1987, 1988; Triezenberg, 1988b). In addition, other workers noted the presence of a novel protein complex associating with TAATGARAT elements from infected cells or from uninfected cells that had in vitro synthesised Vmw65 or purified virion proteins added (McKnight et al., 1987; O'Hare and Goding, 1988; Preston et al., 1988). In band-shift assays using Vmw65-specific antibodies, Preston et al. (1988) showed that this virus induced protein:DNA complex, called the immediate early complex (IEC), contained Vmw65 and required cell factors for its formation. Analysis of mutant TAATGARAT templates revealed that the ability to form both the cellular protein:DNA complex and the virus induced protein:DNA complex was correlated with the induction response in transfection assays, suggesting that the two distinct complexes are both intermediates involved in transcription stimulation by Vmw65 (O'Hare and Goding, 1988; Preston et al., 1988). Results presented in this thesis confirm the importance of the virus induced complex (IEC) as an intermediate of transinduction and demonstrate directly that

formation of this complex is mediated by Vmw65 (section 6.3.6).

It was implied that the cellular factor which binds to IE regulatory sequences may recognise a sequence homologous to the common eukaryotic octamer element 5'-ATGCAAAT-3', located 5' to and overlapping the TAAT nucleotides of many HSV TAATGARAT sequences (table 2). In addition, the participation of the cellular factor was thought to be involved in transinduction since octamer elements conveyed inducibility when linked to reporter genes and competed with TAATGARAT sequences for formation of the cell protein:DNA complex (O'Hare and Goding, 1988). Recently the cellular factor required for the virus-induced complex was identified by Gerster and Roeder (1988) as the octamer binding transcription factor OTFI or OCTI, which is probably identical to nuclear factor III (NFIII) (O'Neill et al., 1988). These workers showed that affinity purified OTFI was required to form a Vmw65-induced complex called VIC (presumably the same as IEC) at IE-1 regulatory elements containing the sequence ATGCTAATGATAT. In addition, they demonstrated that both the ATGC and GARAT tracts of nucleotides were required for VIC formation, and that additional cellular factors as well as Vmw65 and OTFI were necessary for complex formation. The TAATGARAT elements of IE genes 3 and 4/5 show no obvious homology to the octamer element 5' to the TAAT nucleotides (table 2), but Preston et al. (1988) demonstrated that the IE4/5 TAATGARAT motif is still able to form the IEC efficiently. Thus, although many aspects of IE target-sequence binding remain unresolved, the available evidence suggests that Vmw65 mediates transinduction by associating with cellular factors including OTFI and at least one other protein to form a novel transcription complex which recognises octamer/TAATGARAT target elements.

3. REGULATION OF EUKARYOTIC GENES TRANSCRIBED BY RNA POLYMERASE II

3.1 PROMOTERS, UPSTREAM ELEMENTS AND ENHANCERS

The principal method of identifying DNA sequence elements that control transcription in eukaryotes has been to mutagenise DNA sequences near the start of transcription and then to test the altered templates either by reintroduction into the cell or in some other experimental substitute such as *in vitro* transcription systems. More recently, proteins that bind specifically to regulatory DNA sequences have been identified and in some cases purified or cloned. The current challenge is to understand how specific protein:DNA intreactions regulate gene expression.

The *cis*-acting transcriptional control elements identified in eukaryotic genes include core promoters, upstream promoter elements (UPEs) and distal enhancer elements. The basic components of UPEs and enhancers share many properties and may facilitate transcription by a common mechanism (Maniatis *et al.*, 1987). A typical promoter includes an AT-rich region designated the TATA box and one or more UPEs, such as the commonly found CCAAT or GGGCGG homolgies. The TATA box, regarded as the "core" of most promoters, is located between 25-30 bp upstream from the beginning of the transcribed sequence and functions primarily to ensure that transcripts are correctly and accurately initiated, and thus dictates the basal level of promoter activity (Nakajima *et al.*, 1988). Upstream promoter elements either contribute to basal promoter activity by increasing the rate of transcription or mediate the action of regulatory factors (Dynan and Tjian, 1985; Serfling *et al.*, 1985; Maniatis *et al.*, 1987). The strength of promoters is determined by the number and type of UPEs, which act independently of orientation but not distance, with respect to the TATA box. Examples of other UPEs include heat-shock and metal responsive elements. Thus it is speculated that specific factors associating with UPEs interact with proteins bound at the core promoter by stereospecific alignment to facilitate a stimulation in

transcription (Ptashne, 1988; Dynan and Tjian, 1985; Maniatis et al., 1987; see section 3.3).

Enhancers also increase the rate of transcription from promoters but they differ from UPEs in that they can act at great distances and even when present downstream of transcription units. Enhancers function in either orientation and generally contain multiple discrete DNA sequence elements, including motifs classed as UPEs in other situations, which interact specifically with protein factors (Serfling et al., 1985). These characteristics are best illustrated by the SV40 enhancer which consists of two domains, A and B, each composed of multiple sub-regions (Zenke et al., 1986; Fromental et al., 1988). The A and B domains alone have weak enhancer activity, but duplication of either region separately or in combination leads to a high level of activity (Zenke et al., 1986; Herr and Clarke, 1986). The synergistic effect of A and B is independent of their relative position or orientation. The sequence elements within the enhancer necessary for activity associate with specific and distinct DNA-binding protein factors which do not effect the binding of each other (Wildeman et al., 1986). These sequence motifs are recognised by different proteins in different cell-types, and such cell-line dependent interactions are critical for enhancer function in the respective cell-types (Davidson et al., 1986; Nomiyama et al., 1987). Thus different proteins that bind particular sites within a single enhancer stimulate transcription in a cell-type specific manner, and the complex organisation of viral enhancers may have evolved as a means of extending host range.

The organisation of enhancers and promoters that contain multiple UPEs is similar, and indeed some of the sequence elements of each are interchangeable. For instance, the octamer sequence element (5'-ATGCAAAT-3') contained within the immunoglobulin enhancer is also found in a number of other promoters (Bohmann et al., 1987; Parslow et al., 1987). The structural similarity between upstream elements and enhancers is reflected to a degree in functional relationship. For example, deletion of UPEs in the beta-globin promoter essentially abolishes transcription

when the enhancer is present far upstream. However, if the enhancer is substituted for the deleted UPEs, proximal to the TATA box, transcription is restored (Treisman and Maniatis, 1985). Therefore the distinction between enhancers and promoter elements is somewhat arbitrary, and the distance-independent nature of enhancers may simply be due to the number of transcription factor recognition elements within them, rather than to a fundamental difference in their mechanism of operation. This idea is consistent with the observation that a single heat-shock UPE cannot act at a distance, but duplication of this motif gives rise to an element with all the properties of an enhancer (Bienz and Pelham, 1986). However, since duplication of CCAAT boxes far upstream of a promoter does not have the same effect, this point cannot be generalised (Bienz and Pelham, 1986).

Generally, enhancers and UPEs function to regulate gene expression in a condition-dependent manner (Maniatis et al., 1987). For instance, inducible enhancers respond to environmental factors such as cell differentiation (Reichel et al., 1987; Velcich and Ziff, 1985; Hen et al., 1985), growth factors (Treisman, 1985), heat shock (Bienz and Pelham, 1986), viral infection (Goodbourn et al, 1985,1986), exposure to heavy metals (Serfling et al., 1985) and steroids (e.g. Renkawitz et al., 1984). In addition, such elements may only be active at specific times during development or the cell cycle, or only in specific tissues (Gerster et al., 1987; Maniatis et al., 1987).

3.2 PROPERTIES OF TRANSCRIPTION FACTORS

Purification studies have led to the identification of factors that interact and activate transcription through specific DNA sequence elements common to many eukaryotic promoters. These proteins include the GGGCGG motif-binding factor Sp1 (Kadonaga et al., 1986,1987), the CCAAT box-binding factor CBF (Chodosh et al., 1988a,b) and the TATA box-binding factor TFIID (Sawadoga and Roeder, 1985b). Additional factors that are necessary and sufficient for transcription from core promoter elements and that appear to be generally required for TATA box-containing promoters have

also been identified (Matsui et al., 1980; Sawadogo and Roeder; 1985a). TFIID has been shown to interact directly with the TATA and cap site region (Sawadogo and Roeder, 1985b; Nakajima et al., 1988) while others (TFIIB, TFIIE and RNA polymerase II) appear to operate at later steps (Fire et al., 1984; Reinberg and Roeder 1987; Reinberg et al., 1987). More recently, gene specific factors that operate through upstream elements have been purified and shown to function in vitro to stimulate transcription from the core promoter elements: these include factors that act constitutively, such as APl (or jun) and USF (or MTLF) (Lee et al., 1987; Mermod et al., 1988; Sawadogo et al., 1988; van Dyke et al., 1988), tissue specifically, such as OTFII (or OCT-2) and NF kappa B (Scheidereit et al., 1987; Kawakami et al., 1988) and inducibly, such as OTFI (or OCT-1) and Drosophila heat shock factor (Fletcher et al., 1987; Wu et al 1987; Wiederrecht et al., 1987).

Cloning of genes that encode transacting factors has allowed functional analysis of the proteins themselves. In the case of the inducible steroid receptor transcription factor family, studies have led to the identification of highly homologous domains of proteins involved in DNA-binding, hormone/ligand-binding and stimulation of transcription (Miesfeld et al., 1986; Giguere et al, 1986; Kumar et al., 1986; Godowski et al., 1987). The DNA binding domain has a cysteine-rich, zinc-binding "finger" region, a structural feature that has been found in many proteins with transcriptional regulatory function, most notably in the RNA polymerase III gene transcription factor TFIIIA (Miller et al., 1986; Berg, 1986; Harrison, 1986). The modular organisation of transcription factors is illustrated by experiments utilizing constructed hybrid polypeptides. For example, when the DNA-binding region of glucocorticoid receptor is introduced in place of the corresponding region in the progesterone receptor, the fusion protein still responds to progesterone but activates transcription from glucocorticoid DNA regulatory sequences (Green and Chambon, 1987). Thus the DNA-binding/transcriptional activation domain can be physically separated from the region responsible for steroid binding. However, chimaeric

proteins consisting of the ligand-binding domain of either the oestrogen receptor (OR) or glucocorticoid receptor (GR) linked to the DNA-binding region of the yeast transcription factor GAL4 stimulate transcription from promoters containing the GAL4-binding sequence in response to hormone (Webster et al., 1988b). Since the GAL4 DNA-binding region alone binds to DNA without activating transcription, there must also be a transactivating function located within or near the OR and GR ligand-binding domains. Similar studies showed that chimaeras consisting of the bacterial LexA repressor DNA-binding domain and the rat GR hormone-binding domain were unable to stimulate transcription from promoters containing the LexA-binding site in CV-1 cells but were active in HeLa cells, suggesting the involvement of cellular factors (Godowski et al., 1988). Thus the exact location of transactivating domains of steriod receptor molecules and their mechanism of action remains unresolved. However, hormone-binding appears to be involved in a number of steps which lead to "activation" of the DNA-binding domain of the steroid receptor (Godowski et al., 1987; Miesfeld et al., 1987; Hollenberg et al., 1987). When the hormone-binding domain is deleted, constitutive activation of transcription ensues, suggesting that this region normally masks the DNA binding/transactivating region of the receptor in the absence of hormone. The presence of bound hormone may induce a conformational change in the protein that frees the DNA-binding or nuclear transfer domain and this process might mediate transactivation (Picard and Yamamoto, 1987).

In contrast to the glucocorticoid receptor, DNA-binding and transcriptional activation are separable in the yeast transcriptional regulatory proteins GAL4 (Brent and Ptashne, 1985) and GCN4 (Hope and Struhl, 1986). Thus the DNA-binding region of GAL4 can be replaced by that of the LexA repressor and the hybrid protein activates transcription from yeast genes bearing an upstream LexA operator binding-site (Brent and Ptashne, 1985), whereas DNA-binding domains on their own are capable of binding target sites but not of activating transcription (Keegan et al., 1986). Further studies led to the finding that the activating domains of yeast proteins GAL4 and GCN4 and the

human jun (AP1) protein were functionally interchangeable (Struhl, 1987) and that the only similarity between these domains was a stretch of amino acids bearing a significant net negative charge. Mutational analysis revealed that simply the presence of acidic regions, which were not conserved at the amino acid sequence level, was required to provide activating function (Ma and Ptashne, 1987b; Hope and Struhl, 1986; Hope et al, 1988). It was also apparent that the strength of the activating regions corresponds to the number of acidic residues present, as exemplified by the extremely acidic and highly efficient activating region of the HSV-1 transactivator, Vmw65 (Sadowski et al., 1988). In summary, the work on yeast transcription factors (reviewed by Ptashne, 1988) suggests the following generalisations: (i) Activators are modular proteins whose activating regions are distinct and readily interchangeable. (ii) Although the DNA-binding regions are specific structures which interact with distinct regulatory sequences, the activating regions are less precisely defined domains characterised by an excess of acidic residues and perhaps an amphipathic alpha helix structure. (iii) Activators that work well in yeast also work in mammalian, plant and insect cells indicating a conserved mechanism of transcriptional activation (Fischer et al., 1988; Ma et al., 1988; Kakidani and Ptashne, 1988; Webster et al., 1988a). (iv) Activating regions can act synergistically and in either orientation to stimulate transcription (Kakidani and Ptashne, 1988; Webster et al., 1988b; Lin et al., 1988). (v) Stronger activators work at greater distances from the promoter (Sadowski et al., 1988).

Recently, the factor that recognises the common eukaryotic regulatory sequence 5'-ATGCAAAT-3', the "octamer" element, has been purified and cloned (Scheidereit et al., 1987; Ko et al., 1988). The octamer element is found upstream of a variety of genes including the Ig heavy and light chains (Parslow et al., 1984; Falkner and Zachau, 1984; Mason et al., 1985; Ballard and Bothwell, 1986), human histone 2B (Sive and Roeder, 1986) and Xenopus snRNAs (Mattaj et al., 1985). This element is also present in the SV40 enhancer (Davidson et al., 1986; Rosales et al., 1987). Two species of transcription factor bind to the octamer

element in vitro, OCT1 and OCT2. OCT2 is present only in B cells and is probably responsible for tissue specific activation of Ig genes, whereas OCT1 is produced in all cell-types and is likely to play a more ubiquitous role in gene activation (Singh et al., 1986; Augereau and Chambon, 1986; Mocikat et al., 1986; Staudt et al., 1986; Schlokat et al., 1986; Sive and Roeder, 1986; Bohmann et al., 1987; Fletcher et al., 1987; Pruijn et al., 1987). Indeed, it has recently been shown that OCT1, but not OCT2, mediates induction of HSV-1 IE genes by associating with the virus factor Vmw65 (Gerster and Roeder, 1988; section 2.3). Thus although OCT1 and OCT2 bind the same sequence, their protein functions do not correlate.

A situation similar to the interaction of OCT1 and Vmw65 in HSV infection has been observed between the products of two nuclear proto-oncogenes c-fos and c-jun. The polypeptide fos is capable of activating transcription in yeast cells (Lech et al., 1988), and can induce transcription from jun (also called API) responsive genes in mammalian cells (Chiu et al., 1988). The jun protein, which mediates induction of transcription by phorbol esters such as TPA, binds a specific upstream element (TRE) in responsive promoters and activates transcription (Lee et al., 1987; Angel et al., 1987, 1988), while fos induces this level of expression by associating with jun at the regulatory sequence (Franza et al., 1988; Chiu et al., 1988; Sassone-Corsi et al., 1988a). However, fos (like Vmw65) does not bind DNA directly itself but rather mediates its activity via protein:protein interaction (Chiu et al., 1988). The fos-jun protein complex is thought to be stabilised by the interaction of a heptad repeat of leucine residues (the "leucine zipper") present in both polypeptides, and also in an array of other proteins (Landschulz et al., 1988; Kouzarides and Ziff, 1988; Sassone-Corsi et al., 1988b). Although this mechanism of transcriptional induction resembles that seen in HSV IE gene expression, jun does not function in a manner completely analogous to OCT1 since, unlike TRE elements in jun responsive genes, the absence of octamer elements in IE promoters does not decrease the basal level of expression in

transfection assays (Bzik and Preston, 1986).

The octamer-binding proteins contain conserved domains (called POU domains) that include regions which are homologous to the proposed DNA-binding homoeobox domains of Drosophila developmental regulatory transcription factors (Ko et al., 1988; Muller et al., 1988; Scheidereit et al., 1988). In addition, POU domains are found in a cell-type specific pituitary transcription factor (Pit-1) that also binds sequences with homology to the octamer element, and in the regulatory factor, unc-86, of the roundworm C. elegans (Bodner et al., 1988; Ingraham et al., 1988; Finney et al., 1988). Therefore it is speculated that common conserved sequence elements upstream of many genes may differentially regulate expression by binding a family of different factors with common DNA-binding domains, whose availability is dependent on the cellular environment, cell-type or stage of cell cycle or development.

3.3 MECHANISM OF TRANSCRIPTION ACTIVATION

The activating regions of transcription factors such as the yeast regulatory proteins GAL4 and GCN4 presumably interact with transcription components that are common to many organisms (Hope and Struhl, 1986; Ma and Ptashne, 1987a,b; Fischer et al., 1988; Ma et al., 1988; Kakidani and Ptashne, 1988; Webster et al., 1988a,b). Obvious candidates for this interaction include RNA polymerase II (Sigler, 1988) and/or various accessory factors required for the formation of transcription initiation complexes. One of these factors, the TATA box-binding protein TFIID, is required for transcription in vitro (Sawadogo and Roeder, 1985a,b) and studies using partially purified proteins indicate that mammalian upstream element-binding transcription factors directly interact with TFIID. For example, when the factor ATF, which is involved in the regulation of multiple cellular cyclic-AMP induced and adenovirus EIA induced genes (Lin and Green, 1988), binds its recognition site upstream of a core promoter, it induces a conformational change of bound TFIID facilitating promoter recognition by RNA polymerase II and additional factors TFIIE and TFIIB (Horikoshi et al., 1988a). This

ATF-induced preinitiation complex has an extended DNA-binding domain over the transcription start site and is correlated with stimulation of transcription (Horikoshi et al., 1988a; Hai et al., 1988). Thus TFIID is a possible common target for the action of eukaryotic activators. Evidence for this view comes from experiments in which a GAL4 derivative activating protein was shown to alter the conformation of DNA-bound TFIID in a manner identical to mammalian transcription factors (Horikoshi et al., 1988b). The TATA-binding factor TFIID is interchangeable between yeast and mammals (Buratowski et al., 1988; Cavallini et al., 1988) and this observation could account for the ability of yeast activators to stimulate transcription of mammalian genes and vice-versa. Therefore it is speculated that the acidic activating regions of transcription factors bound upstream could interact with TFIID to promote the formation of a preinitiation complex at the core promoter with looping out of intervening DNA (Ptashne, 1986, 1988).

During studies on yeast (GAL4) and viral (Vmw65 of HSV-1) transcription factors, it was noticed that overproduction or high concentrations of activator protein caused repression of genes that lacked the activator recognition site while genes containing the relevant upstream elements were activated (Gill and Ptashne, 1988; Sadowski et al., 1988; Triezenberg et al., 1988a). This activity was not dependent on the presence of the DNA-binding domain (Gill and Ptashne, 1988). Ptashne (1988) suggests that this phenomenon, called "squelching", occurs when activator regions of transcription factors associate with the target protein (e.g. TFIID) off the DNA, thus limiting the pool of general transcription factors but favouring preinitiation complexes at promoters containing specific activator DNA-binding sites. Indeed, the strongest activators tended to be the most efficient squelchers (Gill and Ptashne, 1988; Triezenberg et al., 1988a). The activating region of Vmw65 of HSV-1 is unusually potent when attached to a GAL4 DNA-binding domain (Sadowski et al., 1988) and in addition is very efficient at squelching (Gill and Ptashne, 1988). It is envisaged that potent activators such as Vmw65 may only be used profitably if expressed

transiently as in a viral infection, since continuous expression of powerful cellular activators, and thus presumably powerful squelchers, would be harmful to cells (Sadowski et al., 1988). Ptashne (1988) speculates that cellular systems overcome the risk of squelching by harbouring multiple weak activators which, when bound to upstream DNA elements, efficiently cooperate to interact with bound core promoter transcription factors, but that none of them on their own are strong enough to squelch. This idea is consistent with observations that upstream regions and enhancers contain multiple and distinct protein-binding elements (Schaffner et al.,1988; Ondek et al., 1988; Herr and Clarke, 1986; Davidson et al., 1988; Fromental et al., 1988).

4. MATERIALS

4.1 CHEMICALS

Chemicals were purchased from BDH Chemicals UK, Pharmacia Fine Chemicals, Koch-Light Laboratories and Sigma (London) Ltd. Solvents were obtained from James Burroughs UK Ltd and Koch-Light Laboratories.

4.2 RADIOCHEMICALS

Radiochemicals were obtained from NEN Dupont and Amersham International plc.

4.3 OLIGONUCLEOTIDES

Synthetic restriction enzyme linker oligonucleotides were obtained from New England Biolabs.

4.4 ENZYMES

Restriction endonucleases, DNA modifying enzymes and enzyme buffers were obtained from Bethesda Research Laboratories, New England Biolabs., Nbl Enzymes Ltd, Boehringer Mannheim GmbH. and Promega Biotech.

4.5 CELLS

BHK-21 clone 13 cells (Macpherson and Stoker, 1962) were used throughout, unless specified otherwise. HFL, Vero and HeLa cells (Flow Laboratories) were used for virus titrations. HeLa cells were used for nuclear extracts.

4.6 TISSUE CULTURE MEDIUM

BHK cells were grown in Glasgow modified Eagle's medium (GMEM [Bus⌣by et al., 1964]) supplemented with 10% tryptose phosphate broth, 10% calf serum, 100 units/ml penicillin and 100ug/ml streptomycin (ETC_{10}).

HFL cells were grown in GMEM supplemented with 10% foetal calf serum, 100 units/ml penicillin and 100ug/ml

streptomycin (EF_{10}).

Vero cells were grown in EF_{10}.

HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco Ltd) supplemented as for EF_{10}.

The following modified tissue culture media were also used:

EHU_5	Eagle's medium containing 5% human serum.
PBSA	170mM NaCl, 3.4mM KCl, 2mM KH_2PO_4 (pH7.2).
PBS	PBSA plus $CaCl_2.H_2O$ and $MgCl_26.H_2O$ both at 1g/l.
Versene	0.6mM EDTA dissolved in PBSA containing 0.0002% w/v phenol red.
Trypsin	0.25% w/v trypsin dissolved in tris-saline.

4.7 VIRUSES

HSV-1 viruses and HSV-1 mutants used or constructed in this study were all derived from Glasgow strain 17(syn^+) (Brown et al., 1973).

The HSV-2 mutant ts2203 was obtained from Dr V.G. Preston and was derived from strain HG52 (Timbury, 1971).

4.8 PLASMIDS

Plasmid pMC1 (Campbell et al., 1984) was obtained from E.M. Campbell. Plasmids pFTK, pTK1, pTKN7 and pS20TK were obtained from C.M.Preston. Plasmid pGEM2 (Promega Biotech) was obtained from J.McLauchlan. Plasmids p111 (Everett, 1987) and pIE3CAT (Stow et al., 1986) were obtained from R.Everett.

4.9 BACTERIAL STRAINS

The bacterial strain used was DH-1 from E.coli K12 (Hanahan, 1983).

4.10 BACTERIAL CULTURE MEDIUM

L.Broth 10g/l NaCl, 10g/l Bactopeptone and 5g/l

yeast extract.

L.Broth agar — L.Broth plus 1.5% w/v agar.

Ampicillin, where appropriate, was added to L.broth or L.broth agar at 50ug/ml.

4.11 COMMONLY USED BUFFERS

TBE	90mM Tris; 90mM Boric acid, 1mM EDTA (pH8.3).
E	36mM Tris, 30mM NaH_2PO_4, 1mM EDTA (pH8.3).
EEB	40mM Tris HCl (pH8.5), 0.5mM sodium acetate, 1mM EDTA.
SSC	150mM NaCl, 15mM trisodium citrate (pH7.0).

5. METHODS

5.1 CONSTRUCTION AND PREPARATION OF PLASMIDS

5.1.1 Restriction enzyme digests

DNA was digested in a final volume of between 10 and 100ul in buffer reaction conditions as specified by the manufacturers. The number of units of enzyme added was dependent on the activity of the enzyme and the amount of DNA present. Reaction mixtures were generally incubated at 37°C for 1h.

For the production of linear partial molecules, DNA (2ug) was digested with 2 units of enzyme in the presence of 10 to 500ug/ml ethidium bromide to identify conditions that produced a maximum of singly cut molecules.

5.1.2 Separation of DNA fragments by non-denaturing gel electrophoresis

Agarose gels: 200ml horizontal slab gels (260mmx160mmx5mm) containing 0.5 to 1.5% (w/v) agarose were electrophoresed in E buffer plus 0.5ug/ml ethidium bromide, for approximately 16h at 50V. Samples were loaded in 2% Ficoll, 20mM EDTA (pH7.5), E buffer and 20ug/ml bromophenol blue.

50ml "mini" agarose gels (100mmx70mmx7mm) were also used. These gels were electrophoresed in TBE at 40V for 1h. Samples were loaded in 10% glycerol, 1xTBE and 20ug/ml bromophenol blue. 1ug/ml ethidium bromide was added prior to visualisation of DNA by UV transillumination.

Polyacrylamide gels: Vertical non-denaturing polyacrylamide gels were used for resolving DNA fragment smaller than 0.5kbp.

A solution of 50ml containing 5 to 10% acrylamide (diluted from a stock of 29% acrylamide and 1% N,N'-methylene-bisacrylamide) and 0.55xTBE was prepared and 10% APS and 50ul TEMED added immediately prior to pouring into a prepared gel sandwich (260mmx160mmx1mm). Acrylamide was allowed to polymerise and then samples were applied in 5% glycerol, 0.5xTBE and 20ug/ml bromophenol blue.

Electrophoresis was carried out in 0.55xTBE at 100-200V for 3-4h. Bands were visualised by soaking the gel in lug/ml ethidium bromide prior to UV illumination, or when DNA bands were radiolabelled, by autoradiography of a dried gel.

5.1.3 Purification of DNA fragments from gels

Agarose gels: a slice of agarose containing the required DNA fragment was removed from the gel. The DNA was isolated from the agarose by electroelution in 1xEEB at 20mA/sample for 1-2h using wells designed for this purpose. The DNA was removed in 200ul EEB and was purified by phenol/chloroform extraction and precipitation with an equal volume of isopropanol at room temperature in the presence of carrier E.coli rRNA. Carrier was not added if the DNA was subsequently to be used in phosphatase, kinase or primer extention reactions.

Polyacrylamide gels: gel slices containing DNA fragments were cut into small pieces prior to incubation overnight in 600ul of gel electroelution buffer (GEB)(0.5M sodium acetate, 0.1% w/v SDS, 2mM EDTA, 20mM Tris HCl (pH7.5)) at 45°C. The sample was then centrifuged for 2min at 13,000rpm in a benchtop microfuge and the supernatant retained. The acrylamide pellet was incubated for a further 2h in GEB then centrifuged as before. Supernatants were pooled and filtered through Whatman GF/C paper in a 2ml syringe. The DNA was then precipitated by addition of 2 volumes of ethanol at -20°C for 1-2h.

5.1.4 Ligation of DNA fragments

For ligation of vector sequences to DNA fragments, 50ng of linearised vector and 200-500ng of purified fragment were incubated with 1 unit of T4 ligase in 20mM Tris HCl (pH7.6), 10mM $MgCl_2$, 10mM DTT and 0.6mM ATP in a 4ul reaction at 15°c for 16h.

To prevent reannealing, the vector was treated with calf intestinal phosphatase in the same conditions as restriction enzyme digestions. The sample was then phenol/chloroform extracted and precipitated prior to the ligation reaction.

Staggered cut termini of DNA fragments were

converted to blunt ends by treatment with T4 polymerase. Reactions contained 0.5 to 1.0ug DNA, 33mM Tris HCl (pH7.8), 66mM potassium acetate, 10mM magnesium acetate, 100ug/ml BSA, 200uM dCTP, dGTP, dATP and dTTP plus 4 units of T4 DNA polymerase and were incubated at 15°C for 3h.

Phosphorylated oligonucleotide linkers were inserted into plasmids by ligation with linear molecules using the same procedure as for vector/fragment ligations. A 50-fold molar excess of linker over plasmid DNA was used. DNA ligase was inactivated by heating for 5 min at 60°C and the DNA was digested with linker restriction enzyme. Linear molecules were resolved by electrophoresis on a 1% agarose gel, eluted from the gel and religated.

5.1.5 Transformation of E.coli

10ml of L.Broth was inoculated with 5ul of glycerol stock of DH-1 and incubated at 37°C overnight. 1ml of this culture was added to 90ml of L.Broth and the mixture shaken at 37°C until the OD_{600} was 0.2 (normally for 2.5h). After cooling on ice for 10 min, the culture was centrifuged at 3,000rpm for 15min at 4°C in a Sorvall SS34 rotor. Bacterial pellets were resuspended in a total of 25ml of ice cold 100mM $CaCl_2$ and incubated on ice for 1h. This mixture was centrifuged as before and E.coli suspended in 1ml ice cold $CaCl_2$. A portion, usually 100ul, of $CaCl_2$ shocked cells was added to 2ul ligation mix (section 5.1.4) and incubated on ice for 1h. The mixture was then heated at 42°C for 2min and added to 2ml of L.Broth. Cultures were agitated at 37°C for 90min and 200ul samples were spread on L.Broth agar plates containing ampicillin. Plates were incubated at 37°C overnight.

5.1.6 Analysis of transformed E. coli colonies

Colonies were picked from agar plates into 2ml of L.Broth and shaken at 37°C for 6h. Chloramphenicol was added to a final concentration of 25ug/ml and incubation continued overnight at 37°C. The following day the E.coli cells were pelleted at 5,000rpm for 5min in a benchtop microfuge and the pellet was resuspended in 100ul STET (8% w/v sucrose, 5% v/v Triton X100, 50mM EDTA, 50mM Tris HCl

[pH8.0]) containing 1mg/ml lysozyme. Following incubation at 100°C for 1min the mixture was centrifuged at 13,000rpm in a benchtop microfuge for 10min and the pellets discarded. The supernatant was precipitated with 100ul of isopropanol at -20°C for 30min. After centrifugation at 13,000rpm for 5min in a benchtop microfuge the pellets were washed in ethanol, dried and resuspended in 30ul H_2O.

The "mini-prep" DNA samples were generally assayed for the insertion of a fragment by screening for an increase in size compared to vector DNA. Further analysis was carried out by restriction endonuclease digestion of samples.

5.1.7 Large scale preparation of plasmid DNA

Large scale DNA preparations were carried out by the "hard lysis" procedure, an adaptation of the method of Guerry et al. (1973).

5ul of glycerol stock of bacteria containing the appropriate plasmid was added to 10ml of L.Broth plus antibiotic and incubated at 37°C overnight. 0.5ml of this stock was used to inoculate 200ml of L.Broth containing ampicillin and the culture was shaken at 37°C for 8h. Chloramphenicol was then added to a final concentration of 25ug/ml and incubation continued overnight. The culture was centrifuged at 8,000rpm for 10min in a Sorvall GSA rotor. After decanting the supernatant the pellet was suspended in 5ml of 25% w/v sucrose, 50mM Tris (pH8.0), and 2.5ml of 10mg/ml freshly prepared lysozyme. The samples were mixed and retained on ice for 30min. 2ml of 250mM EDTA (pH7.5) was added and after a further 5min 1.5ml of 5M NaCl and 1.5ml of 20% w/v SDS was mixed with the extract. Incubation on ice was continued for 2h and the preparations were centrifuged at 20,000rpm for 1h at 4°C in a Sorvall SS34 rotor. The supernatant containing the plasmid DNA was further purified by two extractions with phenol/chloroform, one with chloroform followed by precipitation in two volumes of ethanol at -20°C. DNA was pelleted by centrifugation at 2,500rpm for 15min in a MSE coolspin centrifuge and dissolved in 10ml of TNE (20mM Tris (pH7.5), 100mM NaCl and 1mM EDTA) plus 10ug/ml RNAase. After incubation at 37°C for

4h, approximately 2mg of proteinase K was added and samples maintained at 37°C overnight. The proteinase K was removed by two phenol/chloroform extractions plus a chloroform extraction. Sodium acetate (pH7.0) was added to a concentration of 0.3M and DNA was precipitated by addition of 0.5 volumes of isopropanol at room temperature for 2h. After centrifugation at 5,000rpm for 15min at 20°C in a MSE coolspin centrifuge the pellet was washed with ethanol, dried and dissolved in 300ul H_2O.

5.1.8 Estimation of DNA concentration

A series of dilutions of plasmid DNA, linearised with appropriate restriction enzyme, was prepared. The samples were electrophoresed beside DNA of known concentration and photographed on Polaroid 665 film under UV illumination. Concentration of DNA was estimated from densitometric traces on a negative film.

5.2 FUNCTIONAL ANALYSIS OF PLASMIDS

5.2.1 Transfection of plasmid DNA into cells

Plasmid DNA was transfected into BHK cells using the calcium phosphate precipitation technique, a modification of the method of Shen et al., (1982).

Cocktails were prepared containing plasmid DNAs plus carrier to a total of 3ug, 66ul of 2xHeBS (260mM NaCl, 9.8mM KCl, 1.6mM Na_2HPO_4, 11mM D-glucose, 42mM HEPES (pH7.05) and deionised water to 132ul. 9ul of 2M $CaCl_2$ was added and samples immediately vortexed for 0.5min. The tubes were allowed to stand at room temperature for 10min. The medium was then removed from 80% confluent cell monolayers grown in 35mm Petri dishes and they were overlaid with the calcium phosphate precipitate and incubated at 37°C for 30min, with intermittant rocking.

2ml of ETC_{10} medium was added to each monolayer and the incubation continued for a further 1-3h at 37°C. The medium was then removed from the cells and 25% v/v dimethyl sulphoxide in 1x HeBS was applied for 4min at room temperature (Stow and Wilkie, 1976). The dimethyl sulphoxide was removed and cells were washed twice with

ETC_{10}, a further 2ml of ETC_{10} was added and monolayers were incubated at either 31°C, 37°C or 38.5°C as appropriate. Infection with virus, when required, was performed after 1h. Incubation was then continued for 3 to 40h as appropriate.

5.2.2 Preparation of cell extracts

TK assays: medium was removed from the cell monolayers which were then washed with ice cold PBS. 1ml of fresh PBS was added and cells were scraped into this medium. Cell suspensions were transferred to 1.5ml reaction tubes and centrifuged at 5,000rpm for 1min in a benchtop microfuge. The supernatant was carefully removed and cells were resuspended in 100ul ice cold TK lysis buffer (20mM Tris HCl (pH7.5), 2mM $MgCl_2$, 10mM NaCl, 6.5mM beta mercaptoethanol, 0.5% v/v NP40) by vortexing and the tubes placed on ice for 5min. Samples were centrifuged at 13,000rpm for 2min in a benchtop microfuge and then the supernatant transferred to fresh tubes and maintained on ice or stored at -70°C.
For CAT assays: medium was removed and the monolayers were washed in PBS as above. The cells were scraped in 1ml of ice cold TEN (100mM NaCl, 50mM Tris HCl (pH7.5), 10mM EDTA) and transferred to 15ml Falcon tubes. The cells were centrifuged at 2,000rpm for 1min in a MSE coolspin centrifuge and resuspended in 75ul 0.2M Tris HCl (pH7.5). The cells were lysed by sonication, transferred to 1.5ml vials and centrifuged at 13,000rpm for 2min in a benchtop microfuge to remove debris. The cell extract supernatants were stored at -20°C.

5.2.3 Estimation of protein concentration

Estimation of protein concentration was performed using the method of Bradford (1976). 10ul of protein sample/cell extract was diluted in 90ul of H_2O and mixed with 1ml Bradford reagent (0.01% Coomassie brilliant blue G, 0.0003% SDS, 4.75% v/v ethanol, 8.5% v/v phosphoric acid) and left at RT for 10min. The absorbance of the solution was measured at 595nm and converted to milligrams of protein by comparison to a standard curve produced by using known quantities of BSA made up in H_2O or 0.2M Tris HCl (pH7.5).

5.2.4 TK assays of transfected cells

Samples of cell extract (10ul) were assayed for TK activity in a reaction mix containing 100mM sodium phosphate (pH6.0), 10mM $MgCl_2$, 100uM dTTP, 5mM ATP and 100uCi/ml [^{3}H]-thymidine (47 Ci/mmol) in a 50ul volume at 30°C for 2h. The reaction was terminated by the addition of thymidine to 33uM and tubes were heated at 90°C for 4min. They were cooled on ice for 5min and centrifuged at 13,000rpm for 2min in a benchtop microfuge. 50ul of the supernatant was spotted onto a DE81 filter paper disc. The discs were washed three times in 4mM ammonium formate (pH4.0), 10mM thymidine at 37°C and twice in absolute alcohol. Discs were then dried under a heat lamp and placed in vials containing 5ml of scintillation fluid. Conversion of [^{3}H]-thymidine to nucleotides was determined by scintillation counting.

5.2.5 CAT assays of transfected cells

CAT assays were performed using the method of Gorman (1982). Assay mixtures containing 25ul of cell extract (section 5.2.2), 1ul 50mM acetyl co-enzyme A, 14ul H_2O and 0.5ul [^{14}C]-chloramphenicol (45uCi/mmol), were incubated at 37° for 1h. The reaction was stopped by extracting chloramphenicol and acetylated products in 200ul ethyl acetate. The ethyl acetate was evaporated under vacuum, and the pellets were then dissolved in a smaller volume (20ul) of ethyl acetate before spotting onto thin layer chromotography (TLC) plates. After running in 95% v/v chloroform / 5% v/v methanol, plates were air dried and autoradiographed. The amount of radioactivity in the chloramphenicol and its 3'-monoacetylated product was determined by scintillation counting, enabling the percentage conversion of substrate to product to be calculated. The amount of radioactivity in 1'-acetylated chloramphenicol was not significant.

5.2.6 Marker rescue analysis

Marker rescue was carried out as described by Stow et al., (1978). Plasmid and virus DNA were co-transfected into cells and the cells incubated as described in section 5.3.2. Infected cells were scraped into growth medium,

disrupted by sonication, and virus yield at both the PT and the NPT determined by titration.

5.2.7 In vitro transcription and translation of plasmids

Plasmids were digested with an endonuclease restriction enzyme to produce linear molecules that were cleaved 3' to the coding sequence. In vitro transcription was performed using the Riboprobe system (Promega Biotech) following the manufacturer's protocol. 1ug of linear DNA was mixed at RT with 5ul SP6 buffer (200mM Tris HCl (pH7.5), 30mM $MgCl_2$, 10mM spermidine), 0.01% BSA, 10mM DTT, 0.4mM ATP, UTP, CTP, GTP, 0.5mM G(5')ppp(3'), 40 units RNAasein (Promega Biotech) and 10 units T7 RNA polymerase in a total volume of 25ul. The reaction mixtures were incubated at 37°C for 1h.

In vitro translation was carried out by addition of 2.5ul of transcription reaction mixture to 20ul of rabbit reticulocyte lysate (Amersham). Duplicate samples were incubated for 90min at 30°C either in the presence or in the absence of 25 to 50uCi [^{35}S]-methionine (>800Ci/mol) in a 25ul reaction mixture. Non-radioactive samples were stored at -70°C for use in gel retardation assays. The radiolabelled translation mixture was treated with 200ug/ml RNAase for 15min at 30°C and then processed for SDS-PAGE as described in section 5.2.10.

5.2.8 3'-end labelling of probes

10ug of plasmid DNA was digested with restriction enzyme. Following phenol/chloroform extraction and ethanol precipitation, the DNA fragments were labelled in T4 polymerase buffer (33mM Tris acetate (pH7.9), 66mM sodium acetate, 10mM magnesium acetate) containing 10mM DTT, 0.1% BSA, 0.5mCi/ml alpha-[^{32}P]-dATP, 0.5mCi/ml alpha-[^{32}P]-dTTP, 100uM dGTP, 100uM dCTP and 1 unit of T4 polymerase in a total volume of 20ul at 37°C for 1h. The radiolabelled fragments were separated in a non-denaturing polyacrylamide gel (section 5.1.2) and the appropriate band was identified by autoradiography, isolated and purified (section 5.1.3).

5.2.9 Gel retardation assay with proteins synthesised in vitro

Formation and analysis of protein-DNA complexes was performed as described by Preston et al (1988). DNA fragments were end-labelled with ^{32}P (section 5.2.8) and purified from a polyacrylamide gel (section 5.1.3). Reaction mixtures contained the following components: 10mM HEPES (pH7.9), 0.6mM dithiothreitol, 2.3mM $MgCl_2$, 85mM NaCl, 0.1mg/ml BSA, 4ug poly dI. poly dC., approximately 0.2ng ^{32}P-3' end labelled fragment, 5.0ug mock infected HeLa cell nuclear extract (section 5.2.11) and 5ul reticulocyte lysate which had been incubated with in vitro synthesised Vmw65 mRNA (section 5.2.7) or 0.3-1.0ug of virion extract that contained Vmw65 (section 5.4.5). Incubation was at 25°C for 30 min, and reaction mixtures were analysed by non-denaturing PAGE on a 3.5% polyacrylamide gel (section 5.1.2). After electrophoresis for 3.5h at 160V, the gel was dried and exposed for autoradiography.

5.2.10 SDS polyacrylamide gel electrophoresis

40ml of gel mix was prepared containing the required amount of 30% acrylamide / 3% DATD to give a final concentration of between 8% and 10% acrylamide in 1xGB (4xGB = 1.5M Tris HCl (pH8.9), 0.4% SDS). Polymerisation was initiated by the addition of 0.5ml 10% w/v APS and 40ul TEMED, and the mixture was poured into a vertical gel mould (170mmx130mmx1.5mm). While still liquid the acrylamide was overlaid with 2ml 0.25xGB and allowed to polymerise for 30min.

24ml stacking gel solution was prepared, containing 5% acrylamide , 0.5% DATD, 1xSGB (4xSGB = 0.5% Tris HCl (pH6.7), 0.4% SDS), 0.5ml of 10% APS and 35ul of TEMED. The buffer was removed from the surface of the running gel and after washing, the mould was filled with the stacking gel solution and a suitable teflon comb was inserted.

Samples were prepared by boiling for 5min in a denaturing buffer of final concentration 50mM Tris HCl (pH6.7), 2% SDS, 10% glycerol, 5% beta mercaptoethanol. Gels were electrophoresed for 3-4h in a buffer containing 50mM Tris, 65mM glycine and 0.1% SDS and fixed by soaking

overnight in 25% methanol, 6% acetic acid. Gels containing low levels of radioactivity were shaken for 1h in EN^{3}HANCE, followed by 1h in H_2O prior to being dried. Autoradiographs were made using Xomat-S film (Kodak).

5.2.11 Nuclear extracts

HeLa cell nuclear extracts were prepared from monolayer cultures using a modification of the method of Dignam et al. (1981). Cells were scraped from 90mm diameter Petri-dishes and centrifuged at 2000rpm in a Sorvall SS34 rotor for 5min at 4°C. Cell lysis was performed by resuspending the cell pellet in 2 packed volumes of Buffer A (10mM HEPES pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT and 0.5mM phenylmethylsulphonyl fluoride [PMSF]) containing 0.5% NP40, with intermittant mixing at 4°C for 10min. Nuclei were pelleted by centrifugation at 15000rpm in a Sorvall SS34 rotor for 15min at 2°C. The supernatant was carefully discarded and the nuclei lysed by resuspending the pellet in 3ml per 10^9 cells of Buffer C (20mM HEPES pH7.9, 25% v/v glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF and 0.5mM DTT), with intermittant mixing at 4°C for 30min. The suspension was centrifuged at 15000rpm in a Sorvall SS34 rotor for 15min at 2°C and the supernatant (nuclear extract) collected and stored in small samples at -70°C.

5.3 CONSTRUCTION AND PREPARATION OF VIRUSES

5.3.1 Tissue culture

BHK cells were grown in 800ml plastic culture flasks from a seed stock of approximately $1x10^7$ cells at 37°C in 50ml of ETC_{10} and an atmosphere of 5% CO_2 and 95% air. Two days later confluent monolayers were harvested in 2ml trypsin/versene (1:1 v/v), 5ml of fresh ETC_{10} was added and cells were resuspended by pipetting. This suspension was used to seed subsequent monolayers.

5.3.2 Co-transfection of virus DNA and plasmid DNA into cells

0.5ug of virus DNA and 0.5ug of linearised plasmid DNA (cleaved within the vector sequence) was transfected

into cells as described in section 5.2.1, except that 2.0ug of calf thymus DNA was used as carrier and precipitates were gently mixed upon addition of $CaCl_2$ and not vortexed. Transfected monolayers were incubated in ETC_{10} at 31°C for 3 days and virus progeny were harvested by scraping the infected cells into the growth medium. The medium was transferred to a 5ml glass vial, cells disrupted by sonication and the sonicate stored at -70°C.

5.3.3 Isolation of virus plaques

Virus was titrated on cell monolayers at dilutions calculated to give rise to approximately two plaques per Petri dish. The monolayers were incubated for 3 days in EHU_5 at 31°C. The medium was removed, the cells washed twice with 2ml PBS and then 0.5ml of PBS was applied to the monolayers. Single plaques were scraped up in approximately 20ul PBS using a micropipette, transferred to 500ul ETC_{10}, sonicated and stored at -70°C.

5.3.4 Small-scale preparation of virus-infected cell DNA

200ul of virus plaque stocks was used to infect cells in multi-well plates containing 15mm diameter wells. The cells were incubated in ETC_{10} at 37°C for 2-3 days, or until a cytopathic effect was observed. The medium was then removed and stored at -70°C as a viral stock. 0.2ml of CLB (0.5% SDS, 20mM Tris HCl (pH7.5), 2mM EDTA) containing 0.25mg/ml proteinase K was added to the cells and incubation was continued for 3h at 37°C. The glutinous cell extract was then transferred to a 1.5ml vial and NaCl was added to a final concentration of 0.3M. DNA was extracted once with phenol pH8.0 (saturated with 0.5M Tris HCl (pH7.5) and 50mM EDTA) and once with chloroform, precipitated in two volumes of ethanol, dried and dissolved in 50ul H_2O.

5.3.5 Southern blot analysis of virus DNA

5.3.5.1 Internal labelling of probes by nick translation

DNA was labelled with ^{32}P as described by Rigby *et al.*, (1977). 1.0ug of plasmid DNA was incubated in a reaction mix containing 40mM dATP, 40mM dTTP, 2.5ul of

10xNTB (5M Tris HCl (pH7.5), 1M $MgCl_2$, 1M DTT and 5mg/ml BSA) and $2x10^{-4}$ug DNAase at RT for 3min. The mix was placed on ice and diluted to 25ul by the addition of 30uCi of both alpha-[^{32}P]-dCTP and alpha-[^{32}P]-dGTP, 3 units of E.coli DNA polymerase I plus distilled water. The reaction was incubated at 15^{o}C for 1h. ^{32}P labelled DNA was then separated from unincorporated triphosphates by centrifugation (2000rpm in a MSE coolspin centrifuge, 4min) through a 1ml Sephadex (fine) G50 column that had been tightly packed by centrifugation (2000rpm in a MSE coolspin centrifuge, 4min) in a 1ml plastic syringe.

5.3.5.2 Transfer of DNA to nitrocellulose

The method used was essentially that of Southern (1975). The agarose gel to be blotted was shaken for 45min in Gel Soak I (200mM NaOH, 600mM NaCl), then for a further 45min in Gel Soak II (1M Tris HCl (pH8.0), 0.6M NaCl) at room temperature. The gel was then transferred to two sheets of Whatman 3MM filter paper, supported by a glass plate. The edges of the filter paper were dipping into a tray containing 6xSSC. A sheet of nitrocellulose, the same dimensions as the gel, was placed on top covered by two sheets of 3MM paper and a stack of paper towels, also cut to size. The towels were weighted and the blot left overnight in order for the DNA to transfer to the nitrocellulose. The following day the nitrocellulose was removed and allowed to dry in air and baked at 80^{o}C for 2h.

5.3.5.3 DNA/DNA hybridisation

Hybridisations were carried out under conditions of high stringency by the method of Southern (1975). The nitrocellulose containing separated DNA fragments was prehybridised in 100ml 6xSSC, 5xDenhardts (0.5% w/v Ficoll, 0.5% w/v polyvinylpyrollidone, 0.1% w/v SDS, 0.5% w/v BSA) and 20ug/ml denatured calf thymus DNA at 65^{o}C for 2h in a sealed polythene bag. Meanwhile the probe was denatured by incubation in 0.2M NaOH for 10min at RT, followed by neutralisation by the addition of 0.2M HCl.

The pre-hybridisation mixture was removed and replaced with the denatured probe in a mix of same final

composition as the pre-hybridisation solution. The nitrocellulose filter was shaken overnight at 65°C then washed in 10xDenhardts, 4xSSC for 30min at RT, followed by three washes in 0.3xSSC, 0.1% SDS at 65°C. It was air dried and autoradiographed.

5.3.6 Large-scale preparation of virus stocks

Confluent BHK monolayers in 850cm^2 roller bottles were infected with 0.003 pfu virus per cell in 50ml ETC10. After incubation at 31°C for 3-4 days, when extensive cpe had developed, cells were shaked into the medium and the cell debris pelleted out by low speed centrifugation (1,500rpm in a Sorvall GSA rotor, 15min). Cell-released virus in the supernatant was concentrated by centrifugation at 12,000 rpm for 2h at 4°C in a Sorvall GSA rotor. The virus pellet was resuspended in 10ml ETC_{10}, sonicated and stored at -70°C in small samples.

5.3.7 Large-scale preparation of virus DNA

Virus infected cells showing extensive cpe (from 5 850cm^2 roller bottles), were harvested into the medium, pelleted by low speed centrifugation and the supernatant retained. Cell released virions were pelleted by centrifugation (12,000rpm for 2h in a Sorvall GSA rotor), resuspended in 20mM Tris HCl (pH7.5), 100mM NaCl, 2mM EDTA, 0.2 %SDS containing 0.25mg/ml proteinase K and incubated with gentle agitation at 37°C overnight. DNA was extracted three times with phenol/chloroform and once with chloroform, precipitated in 2 volumes of ethanol, dried and dissolved in 0.4ml H20.

5.4 ANALYSIS OF VIRUSES

5.4.1 Titration of virus stocks

Serial tenfold dilutions of stock virus were made in ETC_{10}. The medium was removed from confluent cell monolayers, and 200ul of diluted virus was added to to each dish. Following virus adsorption for 60min at 37°C, cells were overlaid with EHU_5 to prevent the spread of released virus. After incubation at either 38.5°C 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.

5.4.2 Virus particle counts

Particle counts were determined by electron microscopy, kindly performed by Mr. J. Aitken.

5.4.3 Virus adsorption assay

5.4.3.1 Preparation of [^{3}H]-thymidine-labelled virus

Cell monolayers in 90mm Petri dishes were infected with 3 pfu/cell and incubated in ETC_{10} at 37°C for 5h. [^{3}H]-thymidine was added to the dishes to a final concentration of 20uCi/ml and incubation was continued overnight or until extensive cpe was observed. The medium, containing cell-released virus, was collected and cell debris was pelleted out by low speed centrifugation. The virus was pelleted from the supernatant by centrifugation through a 10% sucrose / 10% PBS cushion at 12,000rpm for 2h in a Sorvall SS34 rotor. The supernatant was discarded, the pellet washed twice in ETC_{10} and resuspended in 0.5ml ETC_{10} by sonication. The number of cpm/ml was determined by TCA washing and scintillation counting (section 5.4.10) and the number of virus particles/ml was determined by electron microscopy.

5.4.3.2 Estimation of virion adsorption rate

[^{3}H]-thymidine-labelled virus was adsorbed to cell monolayers in 30mm plates using a moi of 100 particles/cell, for 0, 2 and 4h at 37°C. Cells were washed twice with H_2O and harvested at the appropriate time by scraping into 0.5ml of H_2O. The cells were transferred to conical tubes and incubated on ice for 30min in 5% TCA. The samples were filtered through 25mm diameter GF/C discs, the discs were TCA washed (section 5.4.10), the amount of radioactivity retained on the discs determined by scintillation counting and the percentage adsorption calculated.

5.4.4 Quantitation of viral DNA in nuclei

Cell monolayers in 90mm Petri dishes were infected with 100 particles of virus/cell in ETC_{10} containing 200ug/ml cycloheximide. The dishes were incubated at 38.5°C for 3h before cells were harvested. Cell extracts were made as described in section 5.2.2 for TK extracts, except that the cytoplasm (supernatant) was discarded and the nuclei (pellet) was retained. The nuclei were centrifuged through a 10% w/v sucrose / 10% PBS cushion and then lysed in 50mM Tris HCl (pH7.8), 10mM EDTA, 100mM NaCl and 0.4% SDS containing 200ug/ml proteinase K. DNA was extracted twice with phenol/chloroform and once with chloroform, precipitated in 2 volumes of ethanol, dried and dissolved in 100ul H_2O. The amount of virus DNA present was determined by endonuclease restriction enzyme digestion, agarose gel electrophoresis (section 5.1.2) followed by Southern blot analysis (section 5.3.5).

5.4.5 Extraction of virion proteins for gel retardation assays

$1x10^{10}$ cell released virus particles (prepared as described in section 5.3.7) were concentrated by centrifugation at 25,000xg for 2h, resuspended in PBS/0.5% calf serum, and centrifuged through a cushion of 10% w/v sucrose in 10% PBS at 25,000xg for 2h. The pellet was resuspended in 10mM Tris HCl, 50mM NaCl, 1mM EDTA (pH7.5), NP40 added to a final concentration of 0.03%, and the samples kept on ice for 1h. The extract was centrifuged at 50,000xg for 1h and the supernatant stored at -70°C.

Before the samples were analysed in gel retardation assays (section 5.2.9) protein level comparisons were made by SDS-PAGE and coomassie brilliant blue staining (section 5.2.10).

5.4.6 Virus transinduction assay

Cell monolayers in 35mm Petri dishes were transfected with 3ug of plasmid as described in section 5.2.1, except that the DMSO boost was performed 1h after the medium overlay. The cells were incubated at 38.5°C for a further 1h and then superinfected with 1000 particles/cell.

After adsorption for 1h, ETC_{10} was added and incubation was continued for 3h. Cytoplasmic extracts were made (section 5.2.2) and CAT assays performed (section 5.2.5).

5.4.7 Dot blot analysis of virus mRNA

5.4.7.1 Preparation of cytoplasmic RNA

Cells in 35mm Petri dishes were infected with 1000 particles of virus/cell in the presence of 200ug/ml cycloheximide. After adsorption for 1h at 38.5°C, ETC_{10} containing 200ug/ml cycloheximide was added and the incubation continued for 4h. Cytoplasmic RNA was extracted using the method of White and Bancroft, (1982). Medium was removed from the dishes, the cells washed with PBS and scraped into 1ml PBS. The cells were transferred to a 1ml vial and pelleted by low speed centrifugation. The cells were resuspended in ice cold TE (10mM Tris HCl (pH7.0), 1mM EDTA) containing 0.5% v/v NP40 and incubated on ice for 5min. The samples were centrifuged at 12,000rpm for 2min in a benchtop microfuge and the supernatant retained. The extracts were denatured by incubation at 60°C for 15min in 6xSSC and 20% v/v formaldehyde and stored at -70°C.

5.4.7.2 Transfer of RNA to nitrocellulose

A nitrocellulose filter was washed in H_2O and then 20xSSC and placed on a Hybridot multi-well vacuum aparatus. RNA dilutions were made in 20xSSC and applied to nitrocellulose under suction. The filter was air dried and then baked at 80°C for 2h.

5.4.7.3 DNA/RNA hybridisation

Hybridisations were carried out using the method of White and Bancroft (1982). The nitrocellulose containing bound, denatured RNA was prehybridised in 50% formamide, 5xSSPE (100mM NaCl, 50mM NaH_2PO_4 (pH7.4), 20mM EDTA (pH7.4)), 5xDenhardts solution (0.5% w/v Ficoll, 0.5% w/v polyvinylpyrollidone, 0.1% SDS, 0.5% w/v BSA) and 100ug/ml denatured calf thymus DNA at 42°C for 2h in a sealed polythene bag. Meanwhile the probe was denatured by incubation in 0.2M NaOH for 10min at RT, followed by

neutralisation by the addition of 0.2M HCl.

The prehybridisation mixture was removed and replaced with the denatured probe in a mix of the same final composition as the pre-hybridisation solution (except using 1xDenhardts). The nitrocellulose was shaken overnight at 42°C and then washed twice in 2xSSC/0.1% SDS and twice in 0.1xSSC/0.1% SDS at RT for 15min each time. It was then air dried and autoradiographed.

5.4.7.4 Internal labelling of probes by primer-extension

Primer extension was carried out using the method of Feinberg and Vogelstein (1983). 20ng of purified DNA fragment was incubated at 90°C for 2min in a total volume of 20ul, and cooled on ice. The DNA was then incubated in a reaction mix containing 0.4mg/ml BSA, 4mM Tris HCl (pH8.0), 0.4mM $MgCl_2$, 0.4% beta mercaptoethanol, 200mM HEPES (pH6.6), 2 OD units hexa-deoxynucleotides (Pharmacia), 20uM dATP, 20uM dTTP, 30uCi alpha-[^{32}P]-dGTP, 30uCi alpha-[^{32}P]-dCTP, and 6 units of Klenow fragment polymerase (LFP) at RT overnight. The DNA was then separated from unincorporated triphosphates in a 1ml Sephadex G50 column as described in section 5.3.5.1.

5.4.8 S1 nuclease analysis of mRNA 5' terminus

5.4.8.1 5'-end labelling of probes

10ug of plasmid containing the appropriate insert was digested with restriction enzyme. Following phenol/chloroform extraction and ethanol precipitation the DNA pellet was dried and dissolved in kinase buffer (70mM Tris HCl (pH7.6), 10mM $MgCl_2$, 5mM DTT). 50uCi of gamma-[^{32}P]-ATP plus 2 units of T4 polynucleotide kinase were added and the mixture incubated at 37°C for 3h. The labelled sample was loaded on a non-denaturing acrylamide gel, as described in section 5.1.2. Bands were detected by autoradiography using Xomat-S film. The appropriate band was cut out and DNA extracted according to the method in section 5.1.3.

5.4.8.2 DNA/RNA hybridisation and S1 nuclease digestion

Cytoplasmic extracts from infected 35mm Petri dishes were prepared as described in section 5.2.2 for TK extracts and RNA was extracted twice with phenol/chloroform and once with chloroform, precipitated in ethanol, dried and dissolved in water.

Approximately 0.5ug of 5'-end labelled DNA was precipitated with 10ug of cytoplasmic RNA. The resultant pellet was resuspended in 20ul of 90% v/v deionised formamide, 0.4M NaCl, 40mM PIPES (pH6.8), 1mM EDTA and heated at 90°C for 5min. The samples were then transferred rapidly to a water bath at 58°C and incubated for 16h. They were then placed on ice. 4,000 units of S1 nuclease was added in S1 buffer to give a final concentration of 25mM NaCl, 3mM sodium acetate (pH4.5), 0.1mM $ZnSO_4$ in a volume of 200ul.

The samples were incubated at 37°C for 90min. EDTA (pH7.5) was then added to a concentration of 20mM and sodium acetate (pH7.0) to 0.3M. The samples were phenol/chloroform extracted, ethanol precipitated and electrophoresed on a denaturing polyacrylamide gel.

5.4.8.3 Denaturing polyacrylamide gels

50ml of acrylamide solution was prepared, which contained 8-10% acrylamide (diluted from a stock of 29% acrylamide, 1% N,N'-methylene-bisacrylamide, 7M urea), 21ml of 10M urea, 0.55xTBE, 300ul of 10% APS and 50ul TEMED. This solution was poured into a mould (230mmx450mmx0.35mm). After polymerisation the gel was prerun at 40W for 1h prior to loading samples. The samples were dissolved in 80% de-ionised formamide, 0.55xTBE, 1mM EDTA, 0.1% w/v xylene cyanol and 0.1% bromophenol blue and denatured by heating at 100°C for 3min before loading. Electrophoresis was performed in 0.55xTBE at 40W for approximately 2h.

5.4.9 Analysis of viral proteins

5.4.9.1 Radiolabelling of viral polypeptides

IE polypeptides: an incubation temperature of 38.5°C was used throughout. Cell monolayers in 35mm Petri dishes were infected with a moi of 1000 particles/cell and the virus was adsorbed in the presence of 200ug/ml cycloheximide for 1h. The cells were then overlaid with 2ml ETC_{10} containing 200ug/ml cycloheximide and incubated for a further 4h. The medium was removed from the dishes and the cells washed three times with ETC_{10} containing 1ug/ml actinomycin D and once with PBS containing 1ug/ml actinomycin D. 0.3ml of PBS containing 1ug/ml actinomycin D and 100uCi/ml [^{35}S]-methionine was added and the monolayers incubated for a further 1h before being harvested.

Early and late polypeptides: cell monolayers in 35mm dishes were infected with 1000 particles/cell, adsorbed for 1h at 38.5°C and incubated with 2ml ETC_{10} for 8h. [^{35}S]-methionine was added to a final concentration of 100ug/ml and the cells were harvested after a further incubation period of 1h.

5.4.9.2 Preparation of protein samples for SDS-PAGE

The medium was removed from each dish and the monolayers washed with ice cold PBS. The cells were then rapidly washed in ice cold TE (10mM Tris HCl (pH7.5), 1mM EDTA), 0.3ml TE was added and the monolayers incubated at 4°C for 10min. The cells were transferred to a 1.5ml vial and denatured by boiling for 20min in "boiling mix" (50mM Tris HCl (pH6.7), 2% SDS and 10% glycerol, 5% beta mercaptoethanol). The amount of radioactivity in the samples was determined by TCA washing and scintillation counting (section 5.4.10). Samples of 25ul were analysed by SDS-PAGE (section 5.2.10).

5.4.10 Determination of incorporation of radiochemicals in labelled substrates

10ul samples were spotted on 25mm Whatman no.1 filter paper discs. The discs were washed once in 10% w/v

TCA and twice in 5% TCA, rinsed with ethanol and dried. The discs were placed in vials containing 5ml scintillation fluid and the radioactivity was measured by scintillation counting.

5.4.11 TK assays of infected cells

Cells in 35mm Petri dishes were infected with the appropriate moi in the presence of 200ug of phosphononacetic acid (PAA) per ml. After incubation for 15h at 38.5°C cytoplasmic extracts were made (section 5.2.2) and TK assays were performed (section 5.2.4).

5.4.12 Virus complementation assay

Cells in 35mm Petri dishes were transfected with 3ug of plasmid DNA (section 5.2.1) and treated with DMSO 1h later. After a further 1h at 37°C, virus was titrated on the monolayers and plaques counted 3 days later.

5.4.13 Virulence assay

This study was performed by J.M.Ryan and J.M.Cameron of Glaxo Group Research Ltd. Female Charles River mice, each weighing approximately 15g, were inoculated either intracranially (ic) or intraperitoneally (ip) with 200ul of 10-fold dilutions of virus stocks. Ten mice were inoculated for each virus dilution, and the number of survivors after 21 days was recorded. The mean LD_{50} values from two experiments were calculated.

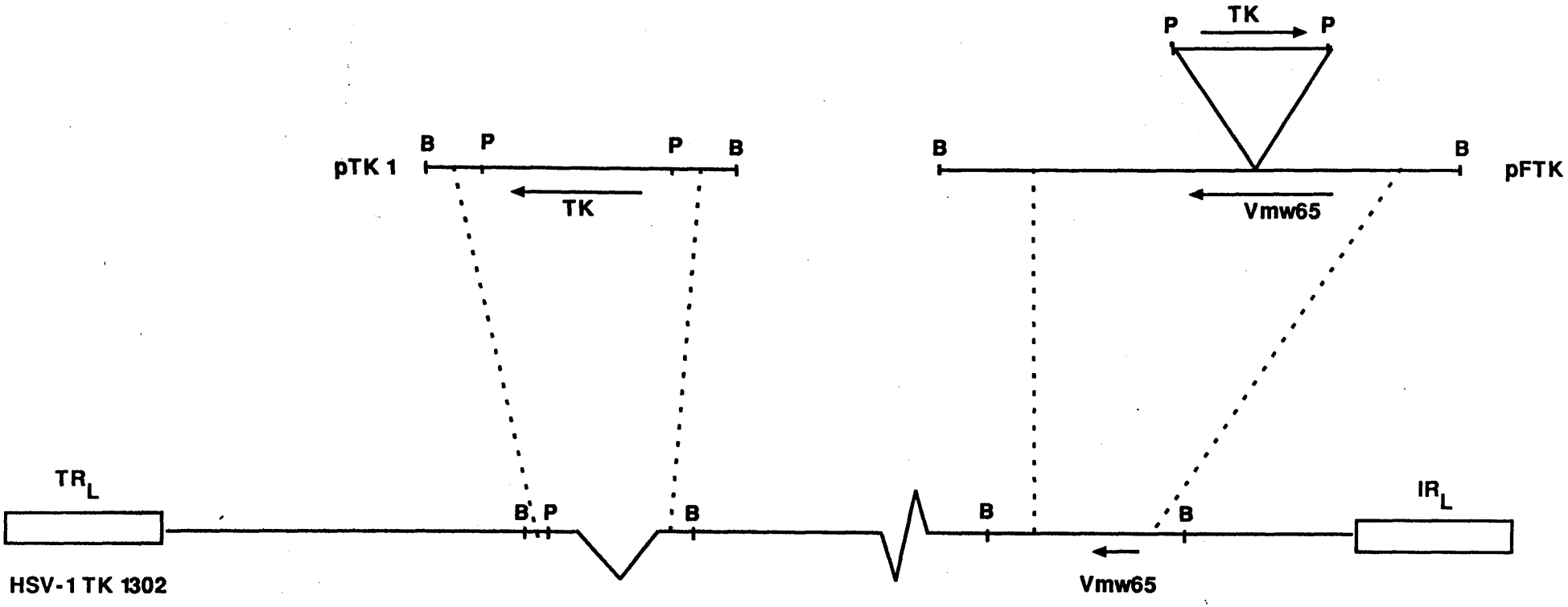
6. RESULTS

6.1 OBJECTIVE

The aim of the work presented in this thesis was to produce and study an HSV-1 mutant virus that lacked the ability to transinduce IE genes, with the aim of understanding the biological importance of this phenomenon. The approach taken was to construct and characterise mutant Vmw65 polypeptides encoded on plasmid DNA and then attempt to introduce mutations that abolished the transinducing activity of the protein into viral DNA. Preliminary experiments indicated that Vmw65 was required for viral replication (section 6.2), and studies on the HSV-2 mutant ts2203 revealed that the polypeptide has an essential role in virus assembly, probably at the level of encapsidation of viral DNA (F.Ramsay, thesis). Therefore mutations that disrupted this function would not be tolerated in virus. For this reason the initial aim of the project was to map functional domains of the polypeptide by linker insertion mutagenesis. It was hoped that mutations could be targetted to regions of Vmw65 that were important for transinduction but not involved in the essential role of DNA packaging.

6.2 VMW65 IS ESSENTIAL FOR VIRUS VIABILITY

A preliminary experiment was performed to determine whether Vmw65 is essential for virus growth by attempting to construct virus mutants that lacked an intact gene encoding the polypeptide. Plasmid pFTK contains a disrupted copy of the Vmw65 gene in which the entire promoter and coding sequence of the HSV-1 TK gene has been inserted at the PvuII site (amino acid position 189 of Vmw65; figure 5). This plasmid was cotransfected with DNA of the TK^- deletion mutant, HSV-1(17)TK1302 (Sanders et al., 1982) and the progeny harvested after 3 days. The progeny were then used to infect BHK cell monolayers, and TK levels assayed after 16h. An increase in TK level above that of TK1302-infected cells would indicate thr presence of TK recombinants. Homologous recombination between the plasmid encoded TK gene in pFTK and its natural locus in the virus (and therefore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HSV-1 TK 1302
Vmw65

Fig. 5. Rescue of HSV-1(TK)1302 with DNA fragments containing a TK gene. Plasmid pTK1 contains the BamHI p fragment in which the TK gene is located. Plasmid pFTK contains the BamHI f fragment in which a disrupted copy of the Vmw65 gene (with an inserted TK gene) is located. HSV-1(TK)1302 DNA contains a deletion in the TK coding sequence which extends past the 5' PvuII site. BamHI and PvuII sites are represented by B and P respectively. Dotted lines indicate regions of DNA where homologous recombination is possible.

Table 3.

Marker rescue of HSV-1(17)TK1302

Virus	TK activity (cts/min. /min. assay/ug protein)
Mock infected	2.9
TK1302 + pUC9	2.6
TK1302 + pTK1	30.0
TK1302 + pFTK	3.9

BHK cells were infected with progeny virus from cotransfections of HSV-1(17)TK1302 and pUC9, pTK or pFTK.

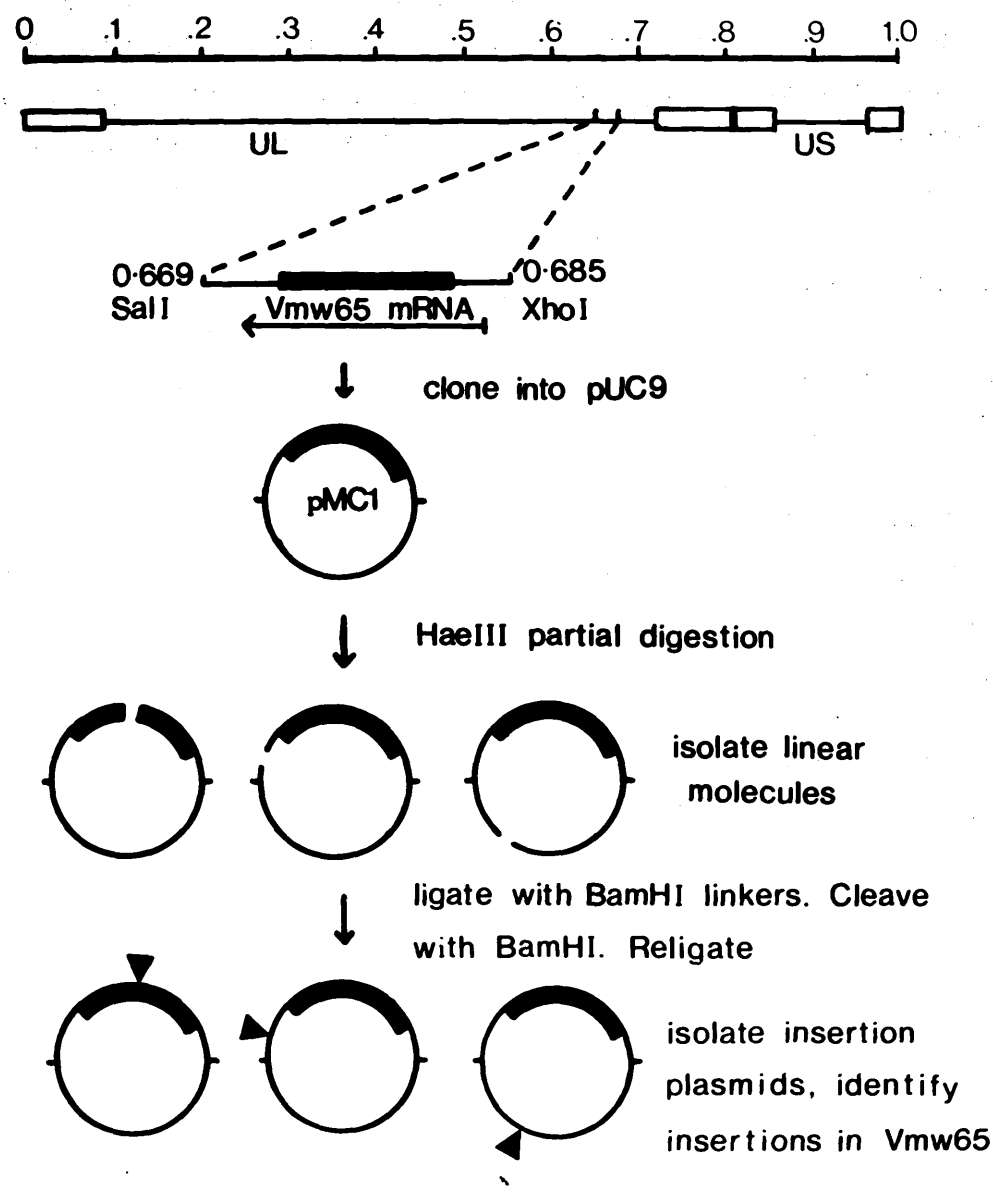
not within the Vmw65 gene) could not have readily occurred since the deletion in TK1302 extended past the 5' limit of the insertion (a PvuII site) in pFTK (figure 5). Table 3 shows that TK^+ recombinants could not be detected using pFTK, whereas TK^+ recombinants were readily identified when pTK1 (a plasmid which contains the TK gene in its normal locus) was used in the cotransfection. This result indicates that viruses which contain a large insertion (the TK gene) within the Vmw65 coding sequence are not viable, and therefore that the protein is essential for viral replication. Subsequent analysis of the HSV-2 mutant ts2203, which was found to contain a mutation in the coding sequences for Vmw65, confirmed this conclusion (F. Ramsay, thesis and section 6.3.7).

6.3 ANALYSIS OF IN-FRAME INSERTION MUTATIONS IN PLASMID ENCODED VMW65

6.3.1 Construction of insertion mutants

The gene encoding Vmw65 lies between map coordinates 0.669 and 0.685 in the U_L region of the prototype HSV-1 genome, and is contained within plasmid pMC1 (figure 6; Campbell et al., 1984; Dalrymple et al., 1985; Pellett et al., 1985). The approach used to mutagenise Vmw65 was to construct a series of mutants each with a 12 bp BamHI oligonucleotide inserted into pMC1 at a different location within the Vmw65 coding region. To achieve this, oligonucleotides (5'-CGCGGATCCGCG-3') were ligated with partially restricted, linear pMC1 molecules which had been cleaved at only one of a number of HaeIII sites within the plasmid (figure 6). The ligation products were cleaved with BamHI and religated to ensure that the insertions did not consist of multimers of linker; pMC1 contains no BamHI sites, and so the introduced site is unique. Plasmids with insertions at ten of the seventeen HaeIII sites within the gene were identified by restriction enzyme analysis and isolated (figures 7 and 8). The mutant plasmids were named pMC1.in(x). The resultant amino acid changes introduced by each mutation is shown in figure 28.

The rationale for employing small insertions for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ligate with BamHI linkers. Cleave with BamHI. Religate
isolate insertion plasmids, identify insertions in Vmw65

Fig. 6. The upper section shows a map of the HSV-1 genome in the prototype orientation, and position of Vmw65. U_L and U_S are the long and short unique regions bounded by repeated sequences represented by open boxes. pMC1 contains the HSV-1 sequence between SalI and XhoI sites cloned into pUC9. The lower section shows the construction of insertion mutants within the gene encoding Vmw65. Plasmid pMC1 was cleaved with HaeIII in the presence of ethidium bromide using conditions designed empirically to produce a maximum of singly cut linear molecules. Plasmid DNA (2ug) was digested with 2 units of HaeIII for 30 min at 37°C with concentrations of ethidium bromide varying from 10ug/ml to 500ug/ml, in a total volume of 20ul. Samples containing a high proportion of singly cut molecules were identified, and 0.2ug of DNA was ligated with a 50-fold molar excess of phosphorylated 12 bp BamHI linker oligonucleotide (CGCGGATCCGCG). DNA ligase was inactivated by heating for 5 min at 60°C and DNA was digested with BamHI. Linear molecules were resolved by electrophoresis on a 1% agarose gel, eluted from the gel and religated. Plasmids containing linker insertions were identified by analysis of small scale plasmid preparations. The locations of linker insertion sites were determined by restriction endonuclease mapping.

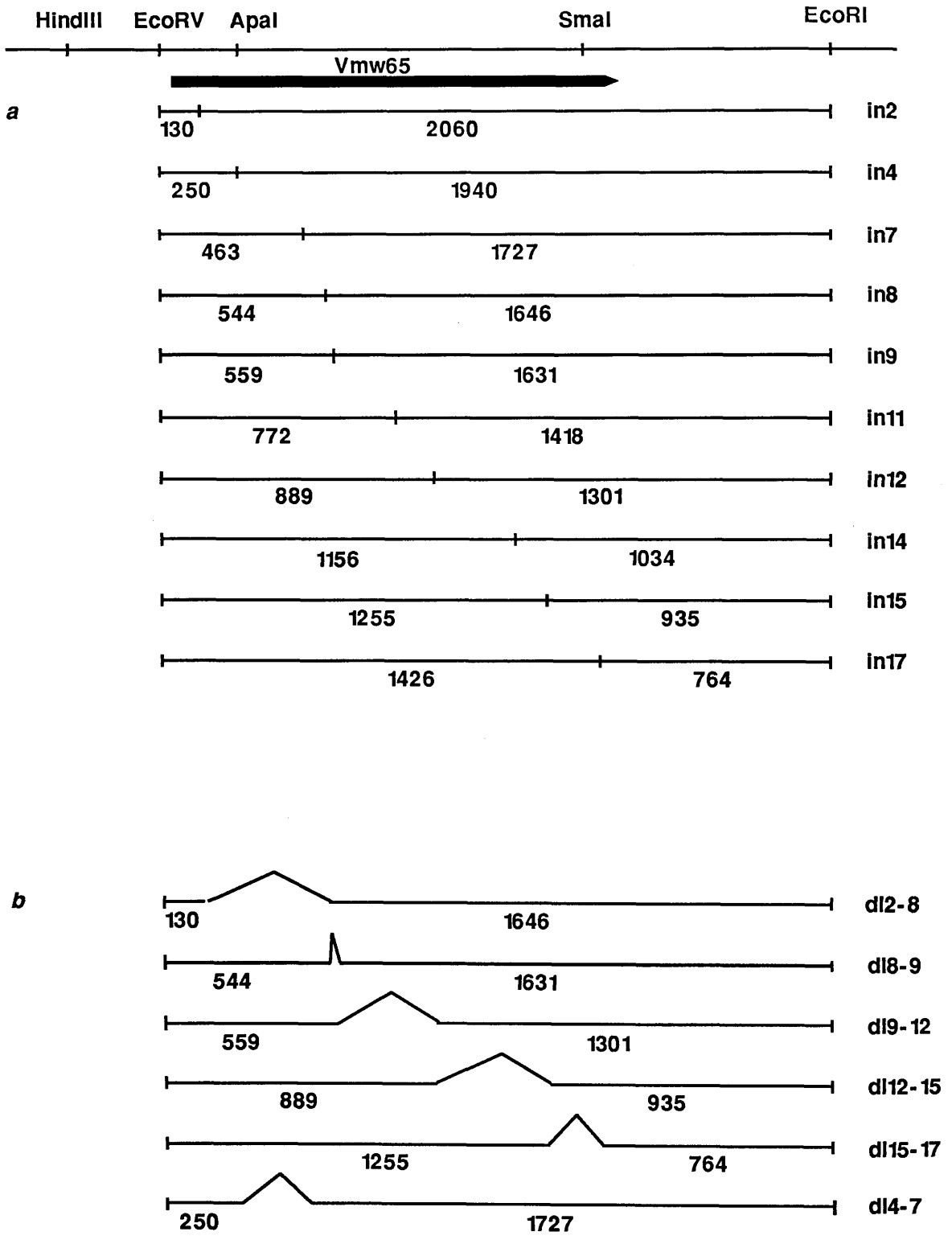
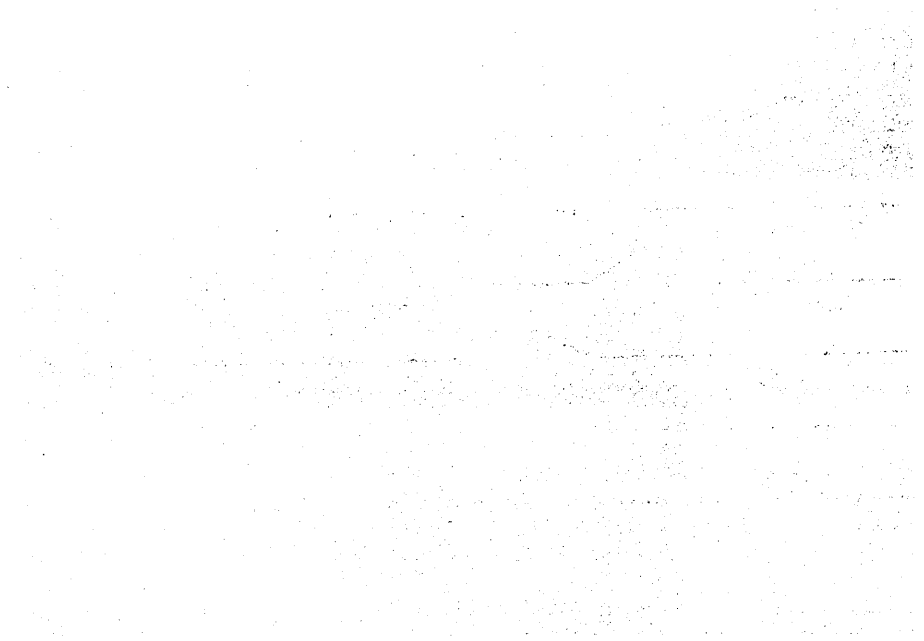
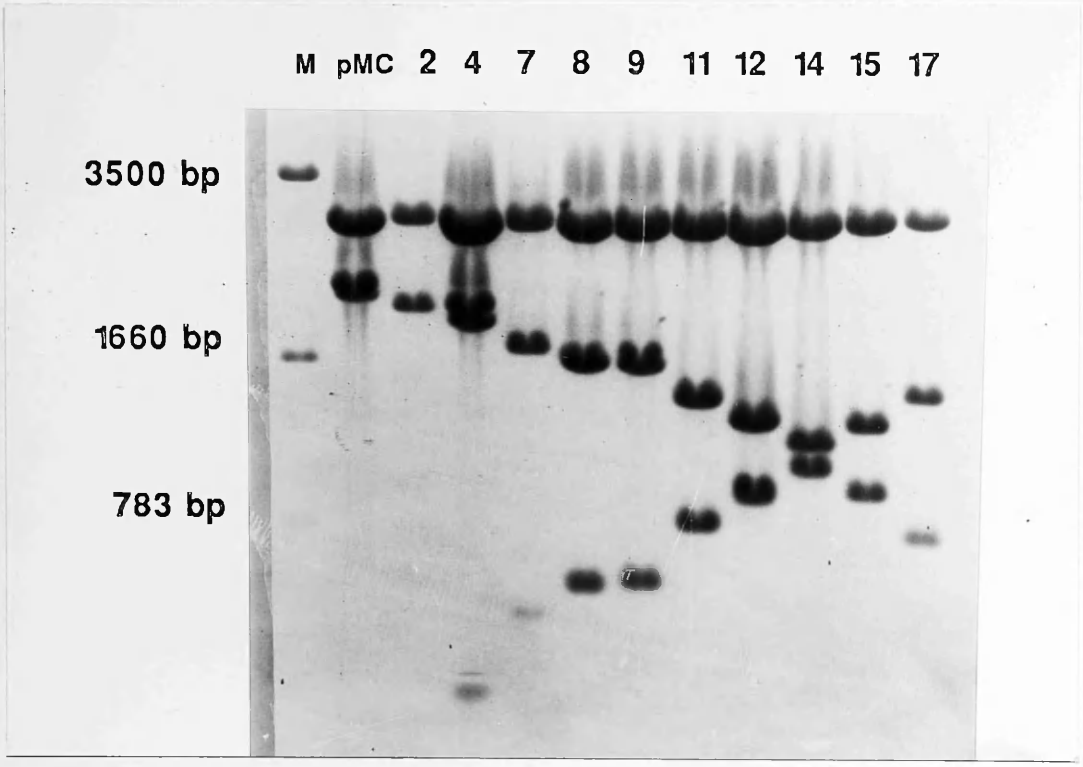

HindIII
EcoRV
ApaI
SmaI
EcoRI
Vmw65
a
130
2060
in2
250
1940
in4
463
1727
in7
544
1646
in8
559
1631
in9
772
1418
in11
889
1301
in12
1156
1034
in14
1255
935
in15
1426
764
in17
b
130
1646
dl2-8
544
1631
dl8-9
559
1301
dl9-12
889
935
dl12-15
1255
764
dl15-17
250
1727
dl4-7

Fig. 7. Position of insertion linkers (a) and deletions (b) introduced into Vmw65. Fine mapping of the location of linkers was achieved by restriction enzyme analysis using BamHI and the enzymes shown in the figure. In-frame deletions were created by excising the coding region represented between two seperate insertion mutant plasmids whose BamHI linker sites were in the same reading frame. The location of insertions and deletions represent BamHI sites which dissect the wt EcoRV/EcoRI fragment into two fragments of the length indicated (in base pairs).

A separate sheet showing the DNA sequence of the Vmw65 gene and position of linker insertions is included at the back of this thesis.




M pMC 2 4 7 8 9 11 12 14 15 17
3500 bp
1660 bp
783 bp

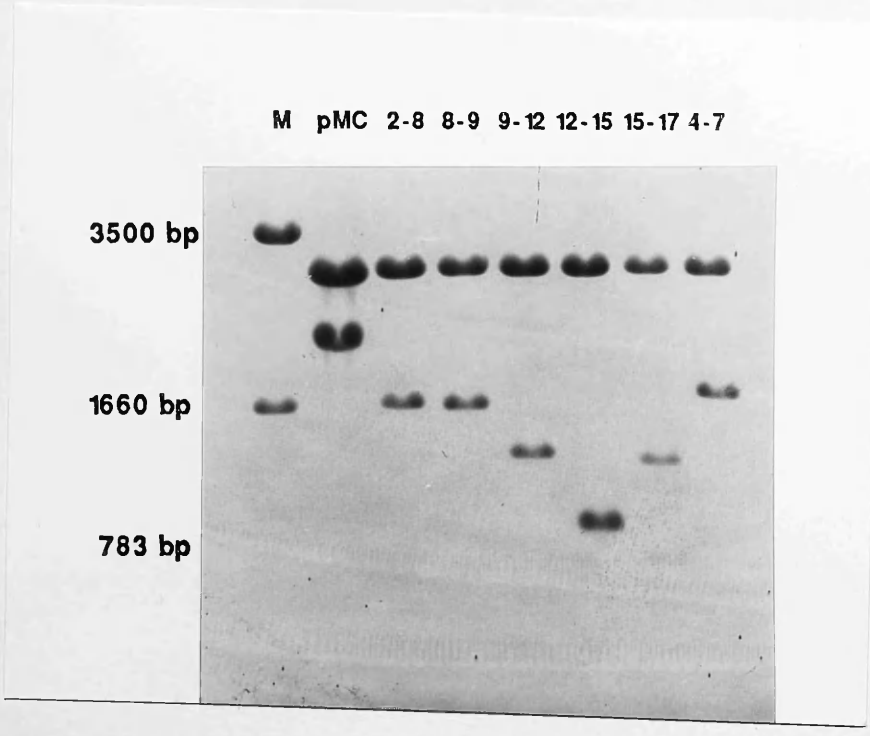
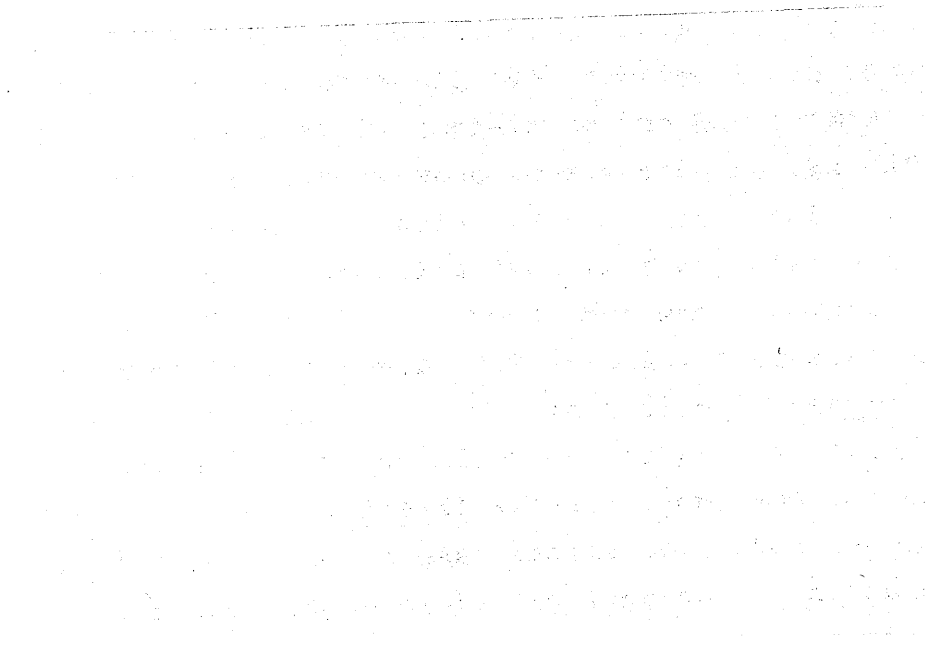

M pMC 2-8 8-9 9-12 12-15 15-17 4-7
3500 bp
1660 bp
783 bp

Fig. 8. Restriction enzyme digestion pattern of mutant plasmids containing insertions and deletions. The plasmids were cleaved with EcoRV, EcoRI and BamHI, the fragments separated on a 1% agarose gel and stained with ethidium bromide. The resultant fragments of the pMCl.in (top) and pMCl.dl (bottom) series correspond to those depicted in figure 7. The lengths of marker fragments (first lane) are given in base pairs.



mutagenesis was that their effect would be expected to be local within the polypeptide, and therefore separable domains of Vmw65 could be mapped. Furthermore, mutations in the same reading frame could be used to construct in-frame deletions in the Vmw65 coding sequences (section 6.3.4).

6.3.2 Construction of plasmid pIE4/5CAT

A plasmid that contained the IE4/5 promoter linked to the CAT coding sequences was constructed to act as an assayable reporter of transinduction in co-transfection experiments. Plasmid pIE4/5CAT was constructed by replacing the HSV-2 promoter in pLW2 (Gaffney et al., 1985) with the promoter and upstream regulatory fragments of the HSV type 1 (HSV-1) IE gene 4/5 (figure 9).

6.3.3 Transinducing activity of insertion mutants

Previous studies have shown that cloned HSV DNA fragments which encode Vmw65 can stimulate transcription from IE promoters in short-term co-transfection assays (Campbell et al., 1984). This approach was used to analyse the transinducing phenotype of insertion mutants, and the results of a representative experiment are shown in figure 10. When a plasmid containing the HSV-1 IE4/5 promoter and upstream regulatory sequences linked to the CAT gene (pIE4/5CAT) was transfected into BHK cells a basal level of CAT expression from this promoter was detected (figure 10, track 5). When pMC1 was included in the transfection, however, CAT activity increased by 6-8 fold (figure 10, track 1). The insertion in HaeIII site 4 (in4) did not affect transinduction (figure 10, track 2) whereas the insertion in HaeIII sites 8 (in8) and 14 (in14) (figure 10, tracks 3 and 4) essentially abolished the effect. Since transfection experiments are inherently variable at a quantitative level, this experiment was repeated to ensure reproducibility of the effects. In some cases (for in2, in7 and in12), independent isolates of the same mutant were tested. A summary of the results from three to six experiments using the complete series of plasmids, together with calculated standard errors, is shown in table 4.

Six of the mutant plasmids (in2, in4, in7, in11,

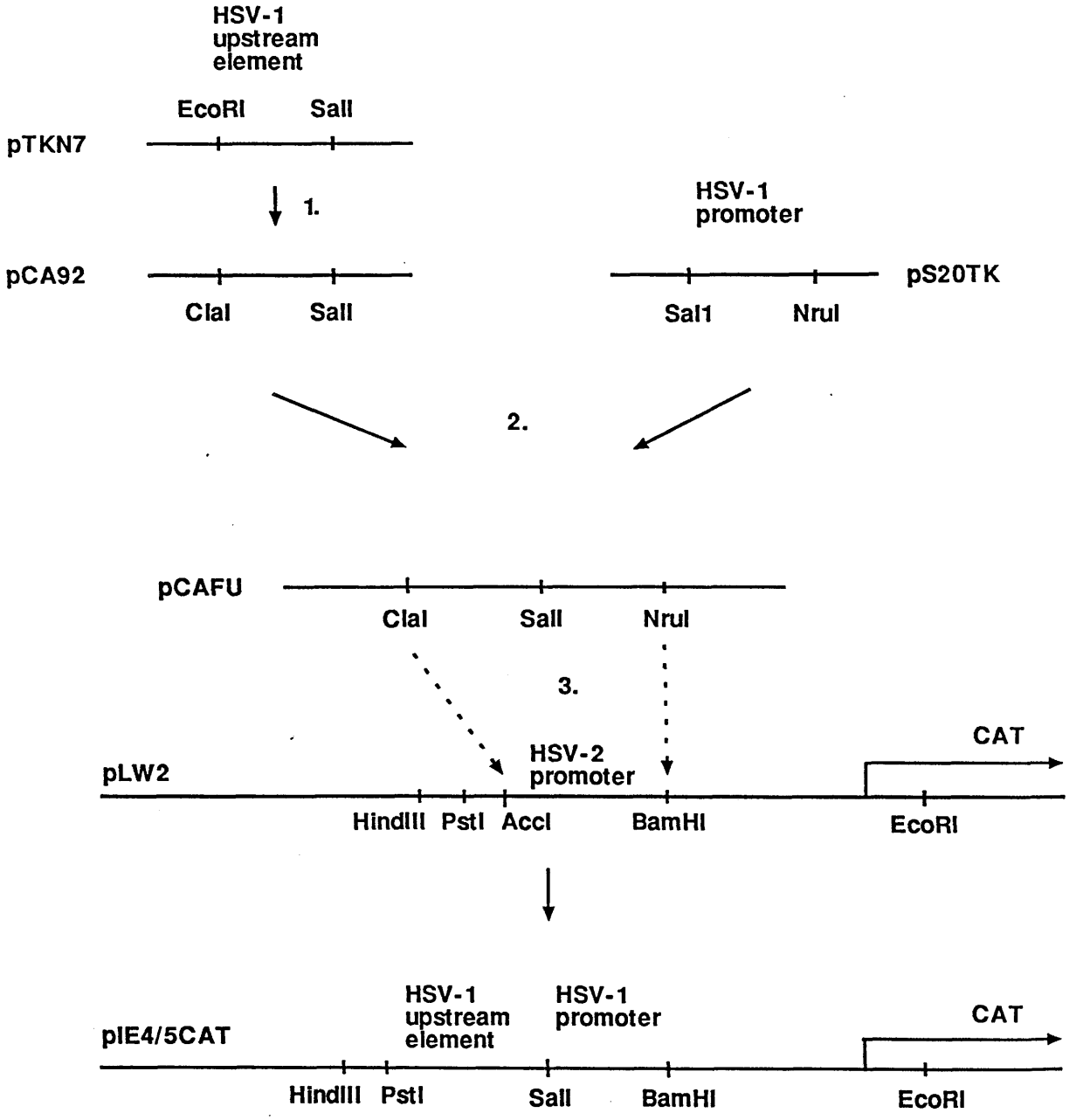

HSV-1
upstream
element
EcoRI
SalI
pTKN7
1.
HSV-1
promoter
pCA92
pS20TK
ClaI
SalI
Sal1
NruI
2.
pCAFU
ClaI
SalI
NruI
3.
CAT
HSV-2
promoter
pLW2
HindIII
PstI
AccI
BamHI
EcoRI
HSV-1
upstream
element
HSV-1
promoter
CAT
pIE4/5CAT
HindIII
PstI
SalI
BamHI
EcoRI

Fig. 9. Construction of pIE4/5CAT. the IE 4/5 upstream fragment used was defined by EcoRI and SalI sites in plasmid pTKN7 (Preston et al., 1984) which represent nucleotides -402 and -240 respectively.* (1) The EcoRI site was converted to ClaI by insertion of an oligonucleotide linker (CATCGATG). The IE4/5 promoter was contained in a SalI (converted from SmaI)/NruI fragment spanning nucleotides -69 to +99 in plasmid pS20TK (Murchie and McGeoch, 1982; Preston et al., 1984). (2) The upstream and promoter fragments were ligated together at the SalI site. (3) The resultant 330 base pair (bp) ClaI/NruI product was inserted into pLW2 between the AccI site and a filled-in BamHI site. Thus the HSV-2 IE4/5 promoter in pLW2 was replaced by the upstream and promoter sequences of the HSV-1 IE4/5 gene, without the HSV-1 ori_s sequences (Stow and McMonagle, 1983; Preston et al., 1984).

* This fragment contains two TAATGARAT elements, referred to as 'c' and 'd' in figure 4 and table 2.

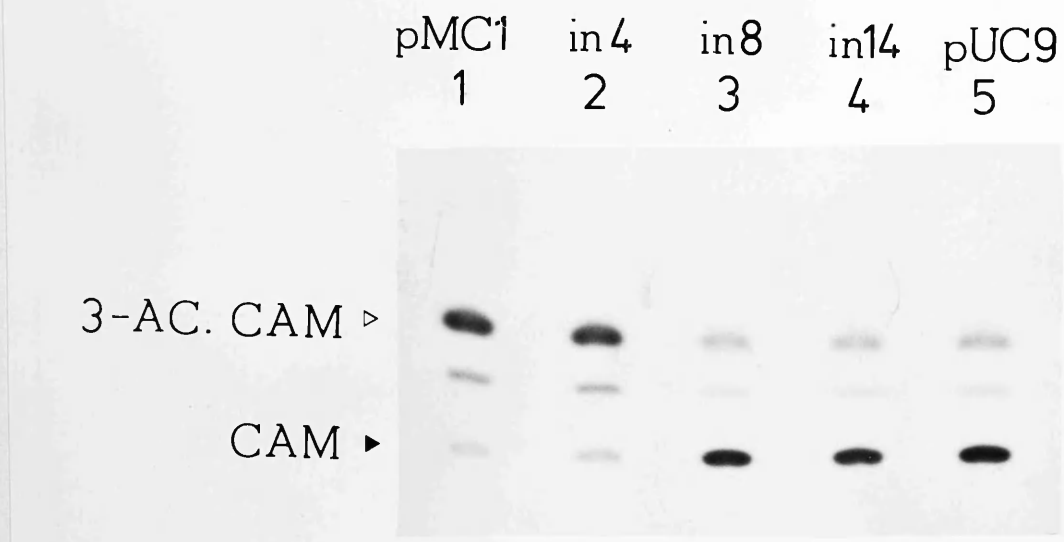

pMC1
1
in 4
2
in 8
3
in14
4
pUC9
5
3-AC. CAM ▷
CAM ▶

Fig. 10. Transinduction of IE transcription by mutant plasmids. CAT assays were carried out on extracts of BHK cells co-transfected with 1.0ug pIE4/5CAT plus 1.0ug pMC1 (lane 1), mutant plasmids in4 (lane 2), in8 (lane 3) or in14 (lane 4), or pUC9 (lane 5) after 16h of incubation at 37°C. The positions of chloramphenicol (CAM) and 3'-acetyl chloramphenicol (3'-AC. CAM) are shown.

Table 4.

Transinducing activity of Vmw65 mutants on IE4/5:CAT

Plasmid	Transinducing activity (%)*	
pMC1	100	
in2	88	(9)
in4	104	(4)
in7	78	(6)
in8	4	(4)
in9	10	(3)
in11	112	(3)
in12	46	(6)
in14	3	(4)
in15	111	(3)
in17	105	(3)
no pMC1	0	

* The stimulation of expression from the IE4/5 promoter is given as a percentage of that obtained in parallel experiments with pMC1.

The mean of a number of independent determinations (presented in brackets) is shown.

in15 and in17) were indistinguishable from pMC1 in their ability to stimulate IE transcription, showing that insertions of four amino acids can be tolerated in certain regions of Vmw65. Three mutants, in8, in9 and in14, were reduced by 90% or more in their transinducing ability and thus represent plasmids in which the insertion caused a strong impairment of activity, within the limits of sensitivity of the assay. Any residual activity of in8 and in14 amounted to not more than 5% of the pMC1 level. One plasmid, in12, gave an activity which was reduced by about 50% but not abolished. Thus the ten insertion mutants exhibited transinducing phenotypes ranging from no alteration to abolition of activity.

6.3.4 Construction and analysis of deletion mutant plasmids

A series of deletions in Vmw65 was constructed by joining together the coding regions upstream and downstream of in-frame BamHI sites from separate linker insertion plasmids. Figure 7 shows a summary of the deletions constructed in Vmw65 and figure 8 shows a restriction enzyme profile of the plasmids. The plasmids were named pMC1.dl(x). The deletion mutants were analysed for transinduction at 31^{o}C and 37^{o}C in the same manner as the insertion plasmids (figure 11). Although the mutants (lanes 2 to 7) appeared to have residual activity above levels of pUC9 (lane 8), all of the polypeptides are believed to lack transinducing function since further analysis of dl15-17 revealed that it exhibited no detectable activity (T.McKee, personal communication). Rather, it was thought that the negative control sample in this experiment was at fault. Thus, allowing for the problem with the negative control and the fact that only a single experiment was performed, it was concluded that all of the deletions constructed abolished transinduction by Vmw65.

6.3.5 Expression of mutant Vmw65 polypeptides in vitro

A high level expression system was required to synthesise insertion mutant Vmw65 polypeptides such that they could be assayed for their ability to bind cell

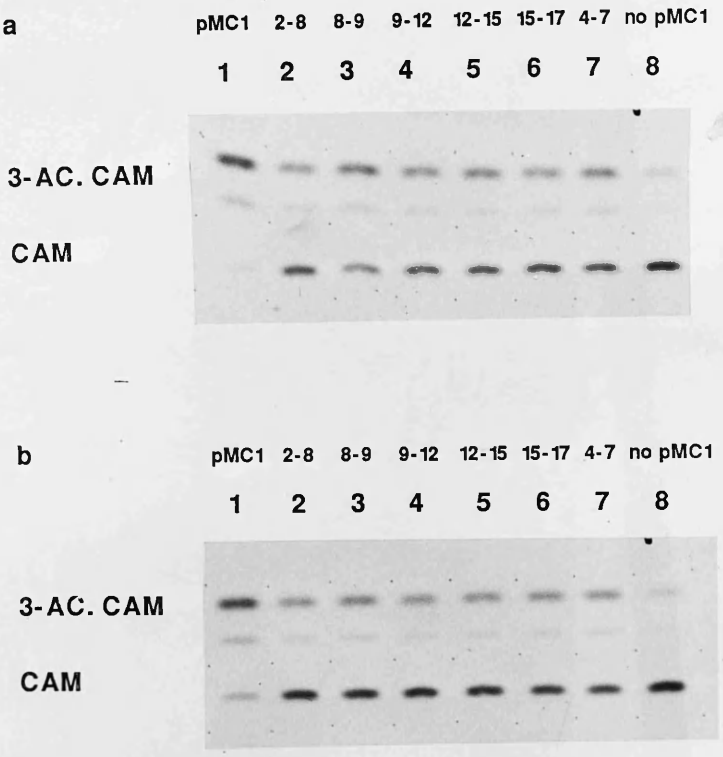

a
pMC1 2-8 8-9 9-12 12-15 15-17 4-7 no pMC1
1 2 3 4 5 6 7 8
3-AC. CAM
CAM
b
pMC1 2-8 8-9 9-12 12-15 15-17 4-7 no pMC1
1 2 3 4 5 6 7 8
3-AC. CAM
CAM

Fig. 11. Transinducing activity of deletion mutant plasmids. CAT assays were performed on extracts of BHK cells co-transfected with 1.0ug pIE4/5CAT plus 1.0ug pMC1, mutant plasmids d12-8, d18-9, d19-12, d112-15, d115-17 or d14-7, or pUC9 (lanes 1 to 8 respectively) after incubation for 16h at either 37°C (panel a) or 31°C (panel b).

proteins and form the IE complex (section 6.3.6). Essentially, the insertion mutant genes were cloned downstream of a T7 promoter in the pGEM2 expression vector (Promega Biotech), as described below.

A 1521 bp EcoRV/FokI fragment of pMC1, containing the entire coding sequence for Vmw65, was cloned into the HincII site of pUC9 to give pMC17 (figure 12.1). The HindIII/EcoRI fragment from pMC17 was inserted into the corresponding sites of the multilinker in pGEM2 (Promega Biotech) to give pGEMTIF (figure 12.2). Plasmid pGEMTIF therefore contained the entire coding sequence of Vmw65 under the control of the bacteriophage T7 promoter. Insertion mutations of Vmw65 were introduced into pGEMTIF by exchanging DNA fragments of pGEMTIF containing coding sequence with corresponding fragments of the pMC1.in series of plasmids that contained the insertion (figure 12.3). Due to the distribution of convenient sites, only those mutations between the unique ApaI and SstI restriction sites (in7, in8, in9, in11, in12, in14 and in15) were introduced into pGEMTIF, and the resulting constructions were designated pGEMTIF.in(X).

The pGEMTIF.in(X) expression templates were transcribed and translated in vitro. Figure 13 shows that Vmw65 is the mRNA-dependent protein sythesised in vitro using the wt and mutant plasmid templates. This result rules out the possibility that frameshift mutations, resulting from deletion of a terminal nucleotide during cloning, occurred during construction of the insertion mutants. In all cases, Vmw65 appeared to run as a multiple band. This phenomenon has been observed before (Campbell et al., 1984) and may be due to initiation of protein synthesis at a downstream AUG cod on (Dalrymple et al., 1985).

6.3.6 Formation of a protein-DNA complex directed by Vmw65 synthesised in vitro

Vmw65 associates with cellular factors to form a complex, IEC, which binds specifically to TAATGARAT elements (McKnight et al., 1987; Preston et al., 1988). The supposition inherent in these findings, that IEC is important for IE gene induction, was investigated by

correlating the ability of mutated forms of Vmw65 to produce the complex with their transinducing phenotypes. Wt and mutant Vmw65 were synthesised by coupled *in vitro* transcription and translation, using the pGEM system described above. The products were incubated with HeLa cell nuclear extracts and IEC was detected by analysis of retardation of the electrophoretic mobility of a 74 bp DNA fragment containing the regulatory region, including TAATGARAT, of HSV-1 IE gene 4/5.

When Vmw65 extracted from virus particles was added to reaction mixtures containing HeLa cell nuclear extract and ^{32}P-labelled 74 bp DNA fragment, the slow migrating complex IEC was formed (figure 14, track 1). Addition of reticulocyte lysate containing Vmw65 synthesised *in vitro*, in place of HSV virion extract, also resulted in the production of IEC (track 2), whereas this complex was not formed when reticulocyte lysate incubated without mRNA was added (track 3). The complex labelled HC3, present in all tracks, is the result of HeLa cell nuclear proteins binding to TAATGARAT (Kristie and Roizman, 1987; O'Hare and Goding, 1988; Preston *et al.*, 1988), and the band in track 3 which migrated more rapidly than IEC probably represents the previously described complex HC4, which results from nonspecific binding of Hela cell proteins to DNA fragments (Preston *et al.*, 1988). HC4 was observed only when reticulocyte lysate without transcription mix was used in the gel retardation assay, presumably because pGEM DNA from the transcription mix acts as an additional nonspecific competitor. In control experiments, it was found that HeLa cell nuclear extract was neccessary for the formation of IEC, thus the reticulocyte lysate did not contain significant amounts of the cell polypeptide which interacts with Vmw65 (C.M.Preston, unpublished results) in agreement with the observations of Gerster and Roeder (1988). When mutant Vmw65 synthesised *in vitro* was used in the gel retardation assay, a strong correlation between complex formation and transinducing activity was observed (figure 14, tracks 4 to 10). Mutant polypeptides in7, in11 and in15 all formed IEC and were also capable of stimulating transcription from IE promoters (section 6.3.3). Similarly

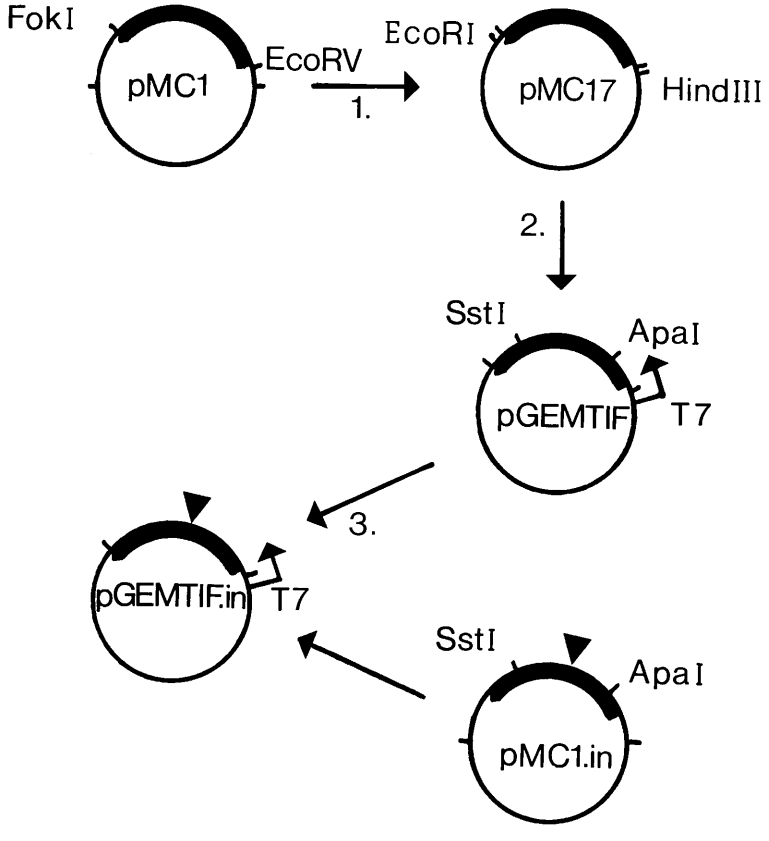

FokI
pMC1
EcoRV
1.
EcoRI
pMC17
HindIII
2.
SstI
ApaI
pGEMTIF
T7
3.
pGEMTIF.in
T7
SstI
ApaI
pMC1.in

Fig. 12. Construction of templates for in vitro transcription. (1) pMC17 was constructed by insertion of a 1521 bp EcoRV/FokI fragment from pMC1 into the HincII site of pUC9. (2) The 1521 bp fragment was excised from pMC17 with HindIII and EcoRI and cloned between the HindIII and EcoRI sites of the pGEM2 transcription vector, to give pGEMTIF. (3) Construction of insertion mutants. A 1048 bp ApaI/SstI fragment from the pMC1.in plasmids was excised and exchanged with the corresponding fragment in pGEMTIF to give the pGEMTIF.in series.

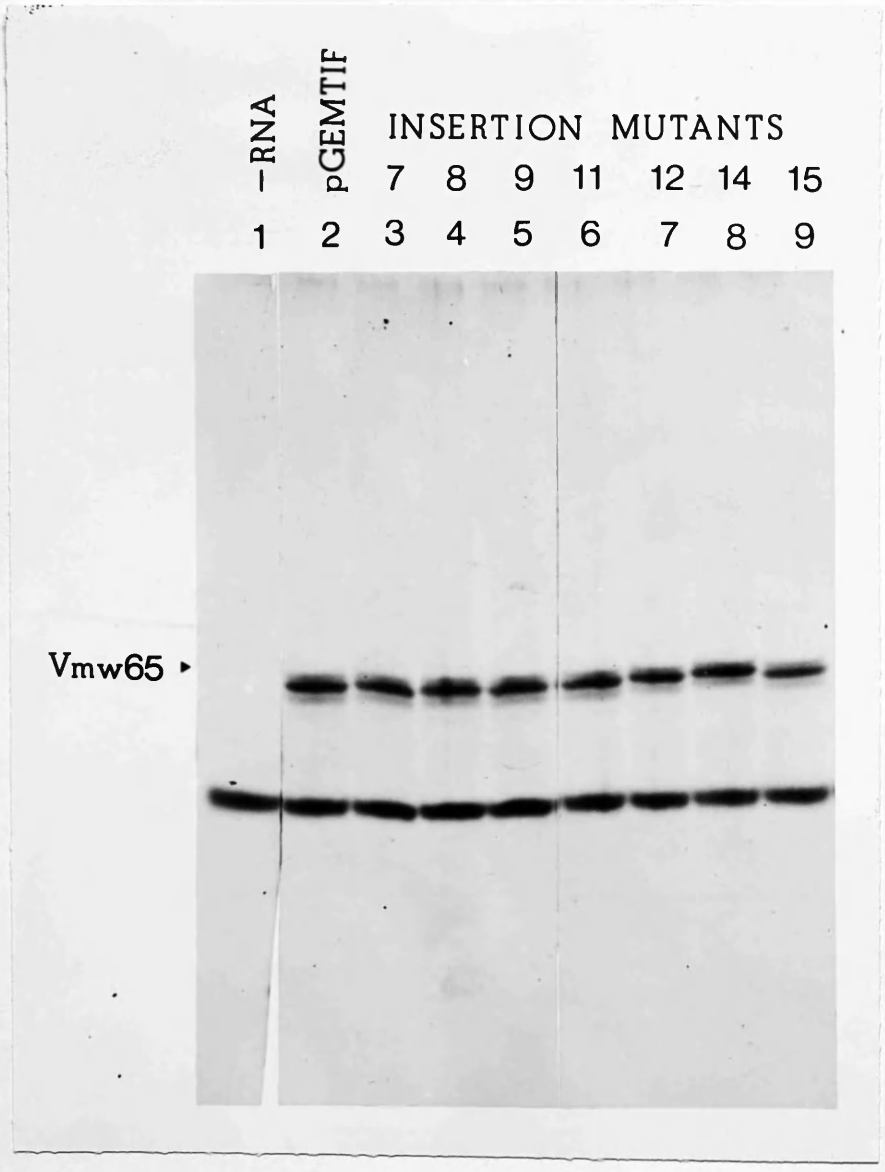

−RNA
pGEMTIF
INSERTION MUTANTS
7 8 9 11 12 14 15
1 2 3 4 5 6 7 8 9
Vmw65

Fig. 13. *In vitro* translation of Vmw65 polypeptides. RNA transcribed from the pGEMTIF.in plasmid series was translated *in vitro* radiolabelled with [^{35}S]-methionine. Lane 1 represents lysate without added RNA. Lane 2 shows the translated products of RNA transcribed from pGEMTIF, and lanes 3 to 9 show the translated products from pGEMTIF.in7 to pGEMTIF.in15.

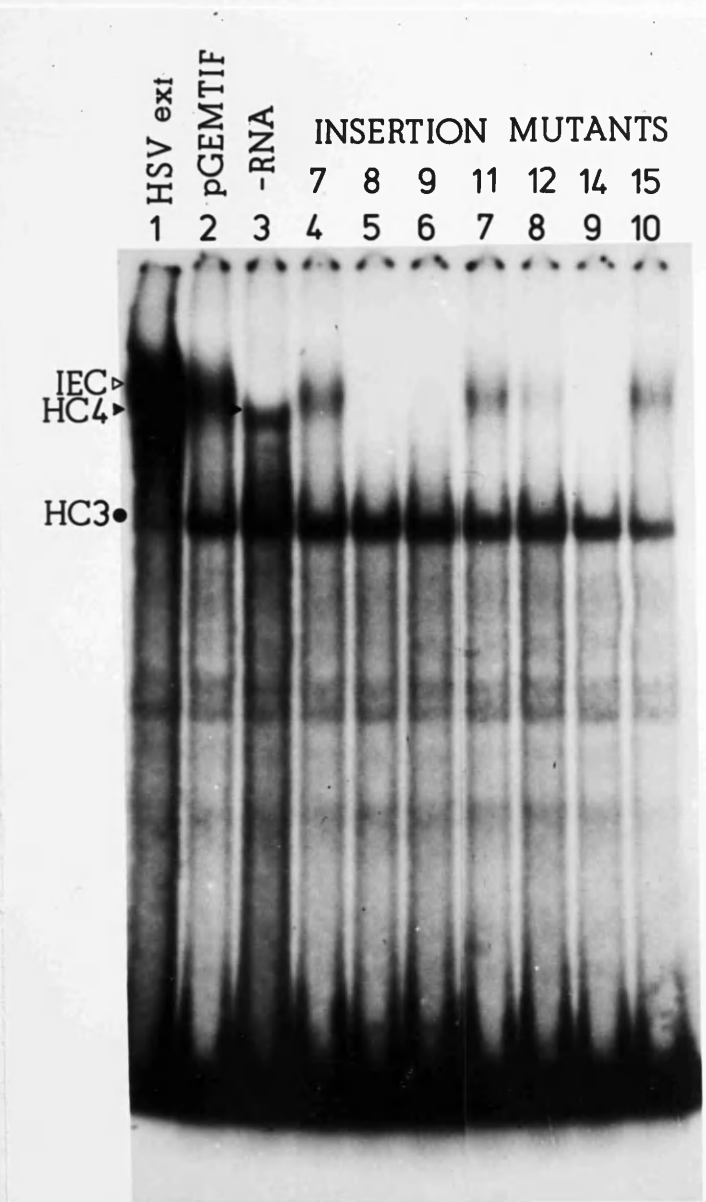

HSV ext
pGEMTIF
-RNA
INSERTION MUTANTS
7 8 9 11 12 14 15
1 2 3 4 5 6 7 8 9 10
IEC
HC4
HC3

Fig. 14. Gel retardation assay using in vitro synthesised Vmw65 products. A 74 bp DNA fragment containing IE gene 4/5 specific TAATGARAT sequences located between the SmaI sites at -362 and -297 relative to the transcription start site plus extra nucleotides due to the presence of BamHI and BglII linkers (Preston et al., 1988) was incubated with HeLa cell nuclear extract and virion extract (lane 1), rabbit reticulocyte lysate (lane 3), lysate containing Vmw65 synthesised in vitro (lane 2), or lysate containing mutant proteins synthesised in vitro (lanes 4 to 10).

mutants in8, in9 and in14 did not form IEC and did not transinduce IE transcription. The correlation is highlighted by in12 which had intermediate transinducing activity and formed the IEC but in lesser amount. These results strongly suggest that the production of IEC is an important stage in the stimulation of transcription from IE promoters.

6.3.7 Marker rescue of ts2203 by insertion mutants

To investigate domains of Vmw65 important for the correct assembly of virions, mutant plasmids were analysed for the ability to rescue the ts mutation in the HSV-2 mutant ts2203. Mutant ts2203 was produced by F.H. Ramsay and V.G. Preston from the double mutant ts13 (Halliburton and Timbury, 1976), which was known to contain a ts mutation in the alkaline exonuclease and a second ts mutation located between genome coordinates 0.64 and 0.70 which affects the virus particle (Moss et al., 1979). The BglII i fragment (genome location 0.62-0.72) of ts13 was subcloned, recombined with wt HSV-2 DNA, and a ts mutant, ts2203, was isolated and purified. The location of the mutation in ts2203 was established by marker rescue experiments using cloned HSV-2 DNA fragments. The mutation was mapped to a DNA fragment which overlaps the 5' end of the gene encoding Vmw65, as deduced from comparison with the known gene arrangement of HSV-1 (F.H.Ramsay, thesis).

The mutation in ts2203 did not affect the production of IE polypeptides at 38.5°C over a range of input moi, and cloned BglII i from ts13 was as effective as BglII i from wt HSV-2 in transinducing IE promoters at 31° or 38.5° in transient expression assays (F.H.Ramsay, thesis). The mutation in ts2203 therefore appears to have no effect on IE transcription, and further studies have revealed a block in DNA packaging and virus assembly at 38.5°C (F.H. Ramsay and V.G. Preston, personal communication).

Intertypic marker rescue was carried out using pMC1 and the insertion mutants, on the assumption that (i) the HSV-2 equivalent is functionally homologous to Vmw65, and (ii) that plasmids which fail to rescue ts2203 have defects incompatible with the proper assembly of virions, provided

the ts2203 mutation is in the HSV-2 counterpart of Vmw65. It was found that pMC1 rescued ts2203 although, as expected from DNA sequence differences, the efficiency was lower than with HSV-2 fragments (table 5). Five mutants (in2, in7, in14, in15 and in17) rescued ts2203, whereas five (in4, in8, in9, in11 and in12) did not. One conclusion from this experiment is that the ts2203 mutation must lie within the coding sequences of the HSV-2 homologue of Vmw65, since this is the only gene affected by the oligonucleotide insertions in4, in8, in9, in11 and in12. Furthermore, the failure of these plasmids to rescue ts2203 indicates that the insertions affect regions essential for virus assembly. The interpretation of positive rescue is less clear, since two mechanisms might operate. Such a result may be obtained because the insertion genuinely does not affect virion assembly, but it is also possible that the insertion lies sufficiently far from the ts2203 mutation that intragenic recombination, without incorporation of the insertion mutation into viral DNA, could account for the observations. The latter argument does not apply to in7, since the flanking mutations in4 and in8 are unable to rescue, nor to in2 if the ts2203 mutation is truly at the 5' end of the coding sequences. Attempts were made to identify the presence of the BamHI linker in rescued, recombinant viruses by restriction enzyme analysis and Southern blot hybridisation. However, after considerable effort it was apparent that ready interpretations using this approach were hampered by an incomplete knowledge of restriction sites and their location in the corresponding region of HSV-2. Although preliminary evidence for the incorporation of in2, in7, in14, in15 and in17 into viral DNA was obtained, attempts to purify recombinant plaques were unsuccessful.

Nevertheless, in situations where mutant plasmids failed to rescue ts2203, recombinant virus DNA is presumed to contain the linker insertion (which is lethal) since its presence is the only difference from the wt DNA fragment of pMC1. Thus, of the five sites at which insertions prevent rescue, only two (in8 and in9) are loci of major importance for both virus assembly and transinduction of IE transcription. The features of Vmw65 required for virion

Table 5.

Marker rescue of ts2203 by mutant plasmids

Mutation	Marker rescue (NPT/PT x 100)*
BglIIi	22.0
BglIIi (ts13)	<0.001
pMC1	2.6
in2	2.1
in4	<0.001
in7	0.2
in8	<0.001
in9	<0.001
in11	<0.05
in12	<0.001
in14	0.77
in15	1.2
in17	1.8
no pMC1	<0.02

* The ability of mutant plasmids to rescue the ts lesion in HSV-2 ts2203 is expressed as the percentage of resulting non-ts recombinants in the total virus progeny. The results are a mean of two independent determinations.

assembly are not invariably the same as those involved in transcription activation and thus there are at least short regions of the polypeptide, if not domains, that are functionally separated. Mutant in14 is unable to transinduce but can rescue ts2203, suggesting that it is defective only for transinduction. In turn, this interpretation implies that viable viruses containing the in14 mutation could be constructed, and that transinduction is not essential for viral growth.

6.4 ANALYSIS OF A VIRUS CONTAINING A MUTATION IN VMW65

6.4.1 Construction of a mutant virus containing an insertion within the Vmw65 gene

As described in section 6.3.3, a 4 amino acid insertion at codon 397 in Vmw65, encoded on plasmid pMC1.in14 (figure 15), abolished the transinducing activity of the polypeptide. The mutation disabled the binding of Vmw65 to the host cell factor(s) and thus defined a region of the polypeptide involved in this interaction (section 6.3.6). The mutation, however, did not appear to affect the polypeptide's essential role during virion assembly as inferred from its ability to rescue a HSV-2 mutant with a ts mutation in Vmw65 (section 6.3.7), suggesting that a viable virus could be constructed that contained the transinducing mutation specified by pMC1.in14. Since attempts to isolate an intertypic recombinant by marker rescue of ts2203 were unsuccessful, it was decided to recombine the in14 insertion directly into wt HSV-1 DNA. To construct a mutant virus, pMC1.in14 was cotransfected with intact wt HSV-1 DNA into BHK cells, and the structure of progeny virus DNA was examined by restriction enzyme analysis and Southern blot hybridisation. One plaque from a total of 84 screened was identified as a recombinant that contained the BamHI linker insertion (figure 16.a). This mutant isolate was titrated on BHK cells and progeny plaques were screened. It was apparent that the original plaque was contaminated with wt HSV-1 (figure 16.b) and so a secondary plaque was titrated and screened in an attempt to purify the mutant DNA (figure 16.c). A further round of plaque purification was necessary

to obtain progeny that displayed only the mutant DNA profile (figure 16.d), and a large scale stock of virus was prepared from one of these plaque stocks. The mutant virus was called in1814

6.4.2 Marker rescue of in1814

To rule out the possibility of a second site mutation in in1814 that might affect the phenotype of the mutant virus, a rescued virus was constructed by recombining in1814 DNA with pMC1. If, as desired, the phenotype of in1814 depended on the insertion mutation, then a rescued virus should behave as wt HSV-1. Initial observation of the properties of in1814 suggested that it grew poorly in comparison with wt HSV-1, thus it was expected that rescued recombinants would outgrow in1814 during successive passages of a mixed population. This turned out to be the case since after a single passage of the progeny virus from the initial cotransfection of in1814 DNA and pMC1, 25 out of 33 of the plaques screened had the wt DNA structure (figure 17). These viruses were unlikely to result from spontaneous reversion of in1814 since no reversion was detected at any stage during the passaging and propagation of mutant virus stocks. A stock of rescued virus, 1814R, was prepared after plaque purification. Figure 18 shows a Southern blot of wt HSV-1, in1814 and 1814R DNA which was digested with BamHI and probed with pMC17, a plasmid containing the Vmw65 coding sequences. The BamHI f fragment of 8 kilobase pairs (kbp) was seen in wt HSV-1 (lane 1)and 1814R (lane 3) whereas in in1814 (lane 2) this fragment was replaced by two fragments of predictable sizes (5 kbp and 3 kbp) expected from the presence of the BamHI linker insertion. Overexposure of the autoradiograph (figure 18, bottom) revealed no detectable BamHI f fragment in in1814 and therefore that the stock of mutant virus was essentially pure.

6.4.3 The efficiency of plaque formation by in1814 is markedly reduced and dependent on cell type

The successful isolation and propagation of in1814 confirms that the insertion mutation is compatible with virus growth in BHK cells. When in1814 was titrated on BHK

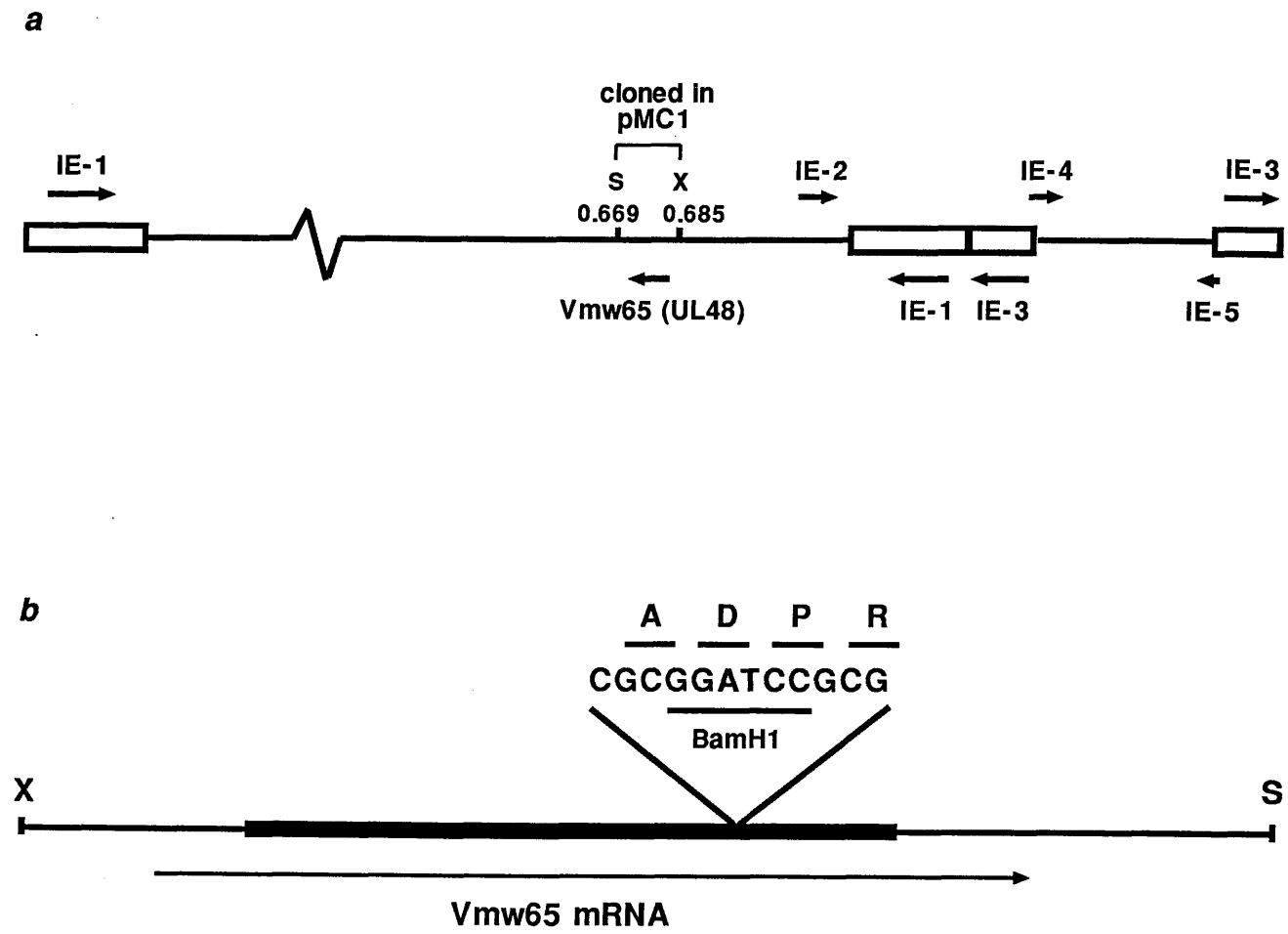
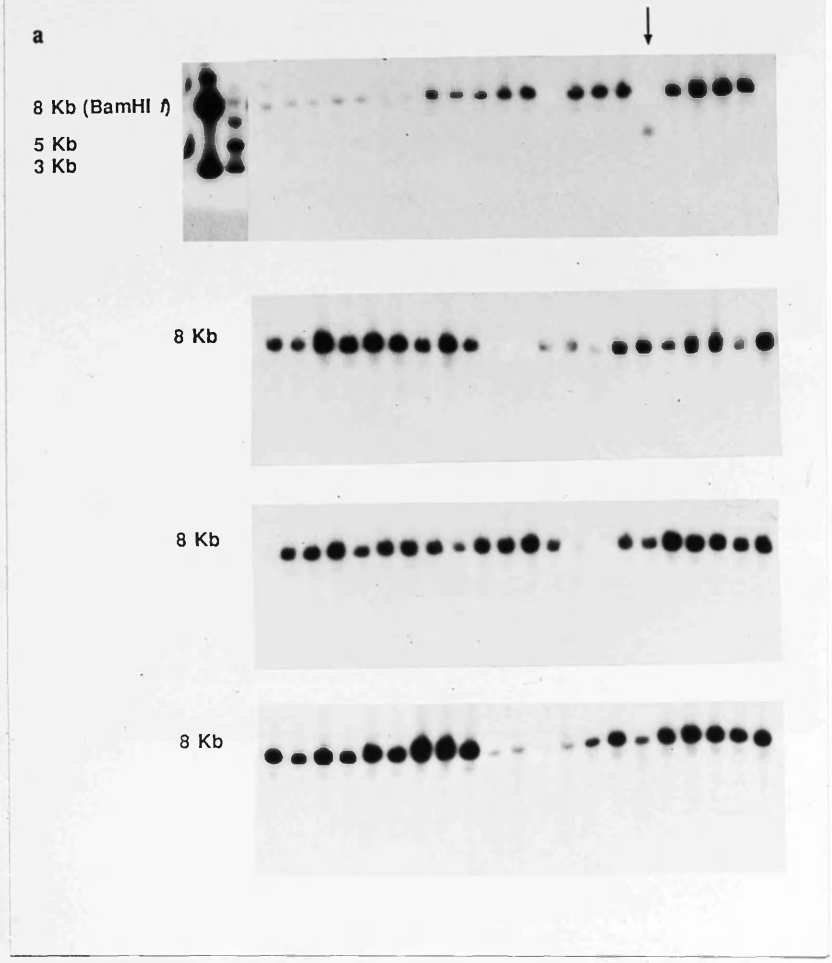

a
cloned in
pMC1
S
X
0.669
0.685
IE-1
IE-2
IE-4
IE-3
Vmw65 (UL48)
IE-1
IE-3
IE-5
b
A
D
P
R
CGCGGATCCGCG
BamH1
X
S
Vmw65 mRNA

Fig. 15. (a) Map location of the Vmw65 gene and position of IE genes in the HSV-1 genome. (b) DNA and amino acid sequence of the insertion mutation in Vmw65 encoded on a SalI(S)/XhoI(X) fragment in plasmid pMC1.in14.


a
8 Kb (BamHI f)
5 Kb
3 Kb
8 Kb
8 Kb
8 Kb

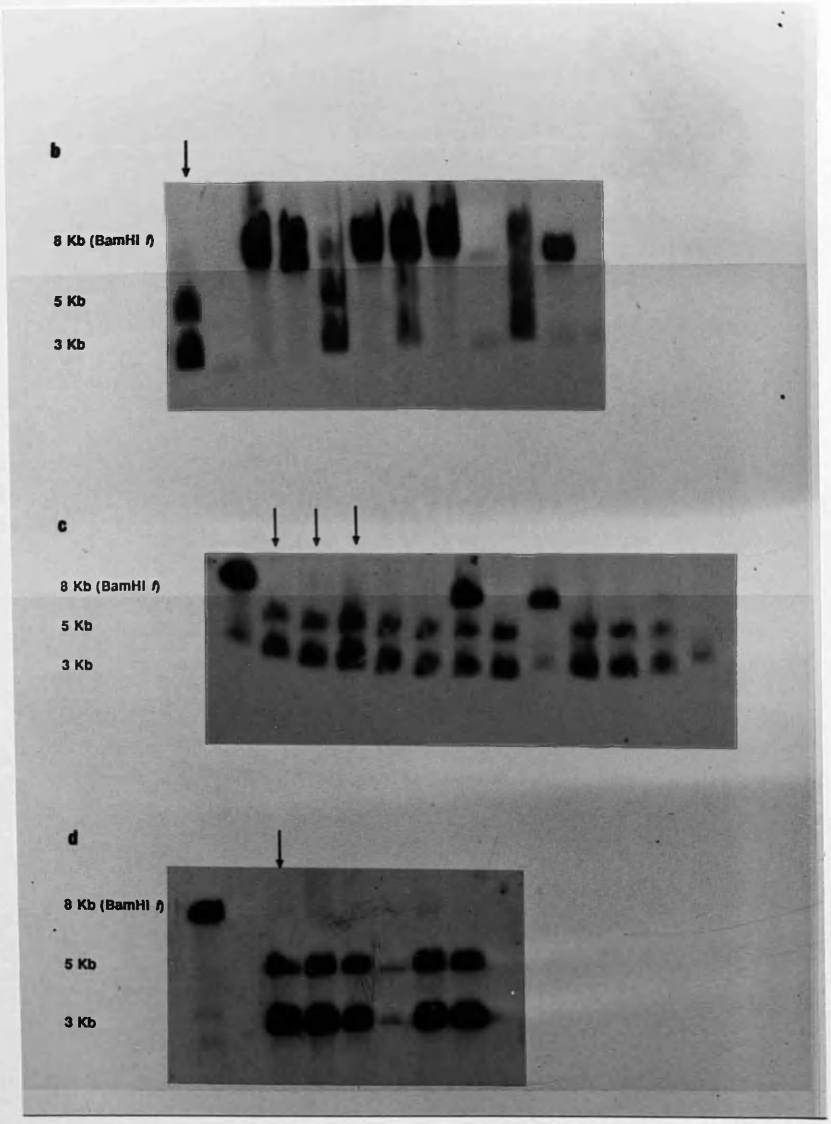

b
8 Kb (BamHI f)
5 Kb
3 Kb
c
8 Kb (BamHI f)
5 Kb
3 Kb
d
8 Kb (BamHI f)
5 Kb
3 Kb

Fig. 16. Screening for mutant recombinants by Southern blot analysis. (a) DNA was prepared from sub-cultures of 84 plaques isolated from the initial co-transfection event, cleaved with BamHI, the fragments separated on a 1.5% agarose gel, transferred to nitrocellulose and hybridised to ^{32}P-labelled pMC17 (which contains the DNA sequence of the Vmw65 gene). The position of the wt HSV-1 BamHI f fragment (8 Kbp), which encodes the entire Vmw65 gene, and the fragments expected to result from the presence of the mutation (5 Kb and 3 Kb) are indicated. One recombinant, indicated by the arrow, was identified. The recombinant was purified by successive rounds of titrating and screening of progeny plaques (b, c and d); the plaques isolated at each stage are indicated by arrows.

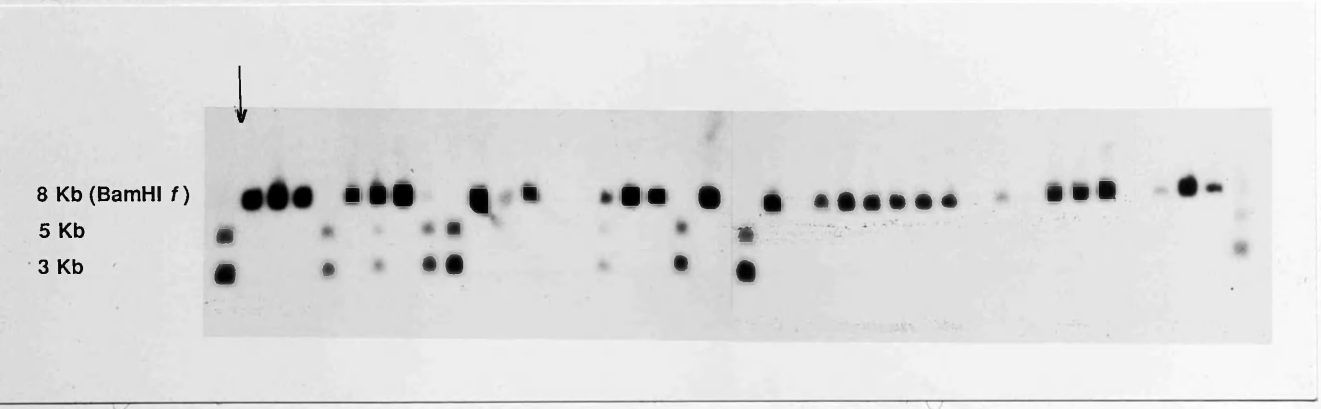
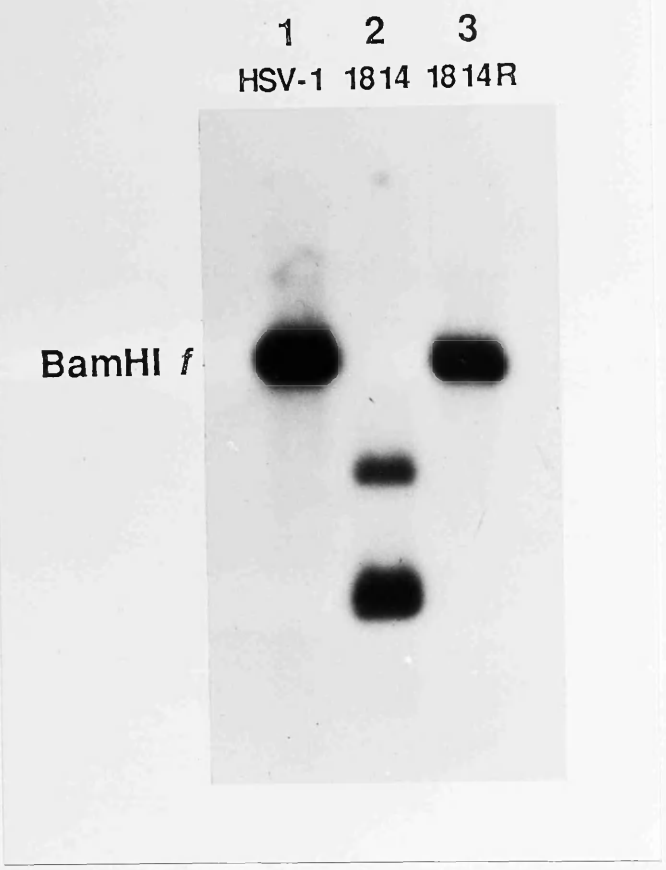

8 Kb (BamHI f)
5 Kb
3 Kb

Fig. 17. Screening for "revertants" constructed by marker rescue of the in1814 mutation. Plaques were isolated from the initial co-transfection, grown up and DNA prepared. The infected cell DNA was cleaved with BamHI, the fragments separated on a 1.5% agarose gel, transferred to nitrocellulose and hybridised to ^{32}P-labelled pMC17. The position of wild-type (8 Kb) and mutant (5 Kb and 3 Kb) DNA fragments are shown. One recombinant (arrowed) was grown up and used as a stock of 1814R.


1 2 3
HSV-1 1814 1814R
BamHI f

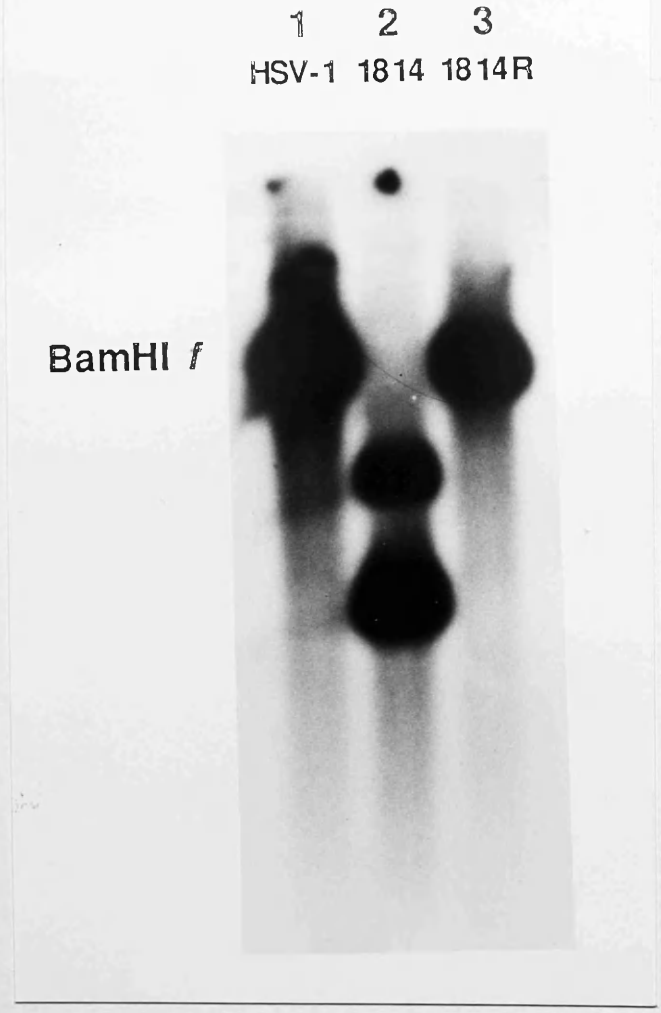

1 2 3
HSV-1 1814 1814R
BamHI f

Fig. 18. Structure of in1814 genome. (top) Wt HSV-1 (lane 1), in1814 (lane 2) or 1814R (lane 3) DNA was cleaved with BamHI and the fragments separated on a 1.5% agarose gel, transferred to nitrocellulose and hybridised to ^{32}P-labelled pMC17. The position of HSV-1 BamHI f is indicated. (bottom) Overexposure of this autoradiograph reveals that there are no detectable BamHI f fragments in in1814

Table 6.

Titration of in1814 on BHK, HFL, Vero and Hela cells

Virus	Particles/ml	Titre BHK (pfu/ml)	Titre HFL (pfu/ml)	Titre Vero (pfu/ml)	Titre HeLa (pfu/ml)
wt HSV-1	1.9×10^{11}	5.0×10^{9}	1.7×10^{10}	1.2×10^{10}	4.0×10^{9}
in1814	1.2×10^{11}	1.3×10^{7}	7.0×10^{5}	1.2×10^{7}	5.0×10^{6}
1814R	4.6×10^{10}	4.0×10^{9}	ND	ND	ND

Plaqueing efficiencies were determined by titrating virus stocks on cells as indicated.

cells it was observed that the morphology of the mutant plaques differed from that of wt HSV-1. Plaques of in1814 were smaller and their borders more defined. The plaque morphology of the rescued virus, 1814R, was indistinguishable from that of wt HSV-1. It was also noticed that the titre of the mutant virus was significantly lower than wt HSV-1 stocks, and therefore virus particle concentrations were determined (table 6). It was found that the particle concentrations of wt HSV-1, in1814 and 1814R stocks were comparable. However, titration of the virus stocks on BHK, Vero, HeLa and HFL cell monolayers revealed that although wt HSV-1 and 1814R plaqued with similar efficiencies, the ability of in1814 to form plaques was reduced by >100-fold on BHK, Vero and HeLa cells and as much as 25,000-fold on HFL cells (table 6). Consequently the particle/pfu ratio of in1814 was $>2.0x10^3$ on BHK, Vero and HeLa cells and $>1.0x10^5$ on HFL cells. The severely reduced titre of in1814 on HFL cells is not easily explained by the particles being defective, since when the same number of particles were titrated on the other cell types, a significantly increased proportion of these particles were infectious.

The apparent titre of a given preparation of in1814 on BHK cells varied by as much as 10-fold on different batches of cells, whereas the titres of wt HSV-1 and 1814R were much more consistent, suggesting that the cellular metabolic state affects the efficiency of plaque formation by in1814. In view of the variation in titre of in1814 when expressed in terms of pfu, cell monolayers were infected with equal numbers of particles of wt HSV-1, in1814 or 1814R in subsequent experiments.

6.4.4 Adsorption and penetration of cells by in1814

Vmw65 is a major structural protein of the virion and therefore the mutation in in1814 may affect adsorption, penetration or uncoating of the virus at the onset of infection. Consequently, the high particle/pfu ratio exhibited by the mutant may reflect the presence of a large number of structurally defective particles in the virus population. Therefore the early stages of infection by

Table 7.

Binding of in1814 to cells

Time (h)	cpm bound to cells (percent input)	
	wt HSV-1	in1814
0	5.0	2.5
2	10.0	6.0
4	16.0	10.0

Labelled virus particles were adsorbed to cells at 37°C.

The input radioactivity was 270 and 1200 cpm per 30mm plate for wt HSV-1 and in1814 respectively (100 particles/cell in each case).

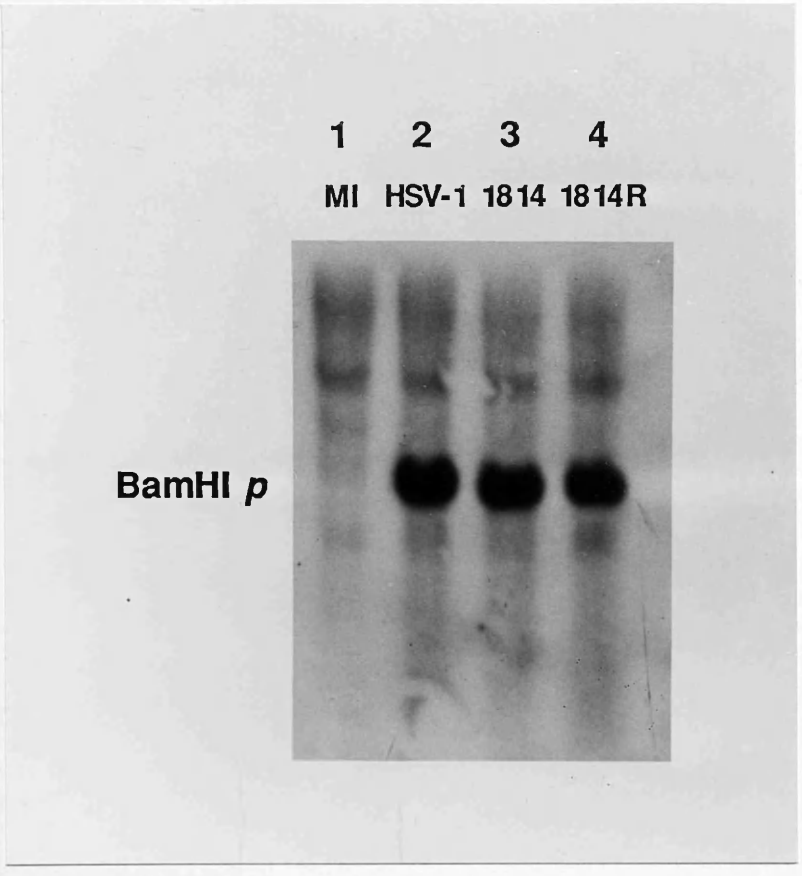

1
2
3
4
MI
HSV-1
1814
1814R
BamHI p

Fig. 19. DNA migration to the nucleus. DNA isolated from nuclei of cells mock infected (lane 1) or infected with wt HSV-1 (lane 2), in1814 (lane 3) or 1814R (lane 4) was cleaved with BamHI and the fragments separated on an agarose gel, transferred to nitrocellulose and probed with pTK1. The position of BamHI p is indicated.

in1814 were examined. In an initial experiment, the rate of adsorption of wt HSV-1 or in1814 preparations (radiolabelled by incubation with [^{3}H]-thymidine during virus propagation) to BHK cell monolayers was investigated. The adsorption rates of wt HSV-1 and in1814 particles were not significantly different (table 7).

The efficiency of DNA migration to the cell nucleus was also determined. BHK cell monolayers were infected in the presence of cycloheximide (CH) with 100 particles of wt HSV-1, in1814 or 1814R per cell, nuclei were prepared at 3 h post infection, and nuclear DNA was analysed by Southern blot hybridisation (figure 19). No differences were detected in the levels of HSV DNA, showing that the nuclear migration of in1814 DNA is not impaired and thus the particles are not defective for penetration.

This result reemphasises the requirement to use equal numbers of particles rather than pfu as a basis for the design of experiments with in1814, since preparations of in1814 that exhibit the same titre as wt HSV-1 and 1814R contain significantly more viruses capable of penetrating cells and releasing DNA to the nucleus; 1 pfu of wt HSV-1 and in1814 represent 38 and 10,000 particles, respectively.

6.4.5 Vmw65 of in1814 does not direct the formation of IEC

The ability of Vmw65, encoded by in1814, to form the protein-DNA complex IEC was investigated since this property was disrupted in pMC1.in14 (section 6.3.6)

Extracts of wt HSV-1, in1814 and 1814R virions were prepared and analysed by SDS-PAGE (figure 20). The levels of Vmw65 in these extracts were very similar and a slightly increased molecular weight (mol.wt) of the mutant polypeptide, presumably due to the 4 amino acid insertion, was apparent (lane 2). The virion extracts were incubated with HeLa cell nuclear extract and a ^{32}P-labelled 74 bp DNA fragment containing the TAATGAGAT sequence motif of IE gene 4/5. As shown in figure 21, the complex IEC was readily detected using extracts of wt HSV-1 (lane 2) and 1814R (lane 4) virions, but not with extracts of in1814 (lane 3) or when no virion extract was present (lane 1). This result

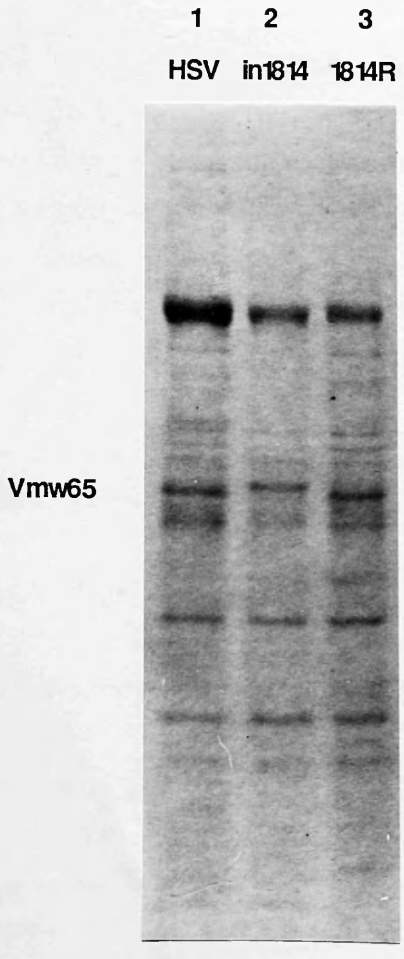

1
2
3
HSV
in1814
1814R
Vmw65

Fig. 20. Proteins extracted from virions of wt HSV-1 (lane 1), in1814 (lane 2) and 1814R (lane 3) and used as a source of Vmw65 for gel retardation analysis. A gel stained with coomassie brilliant blue is shown.

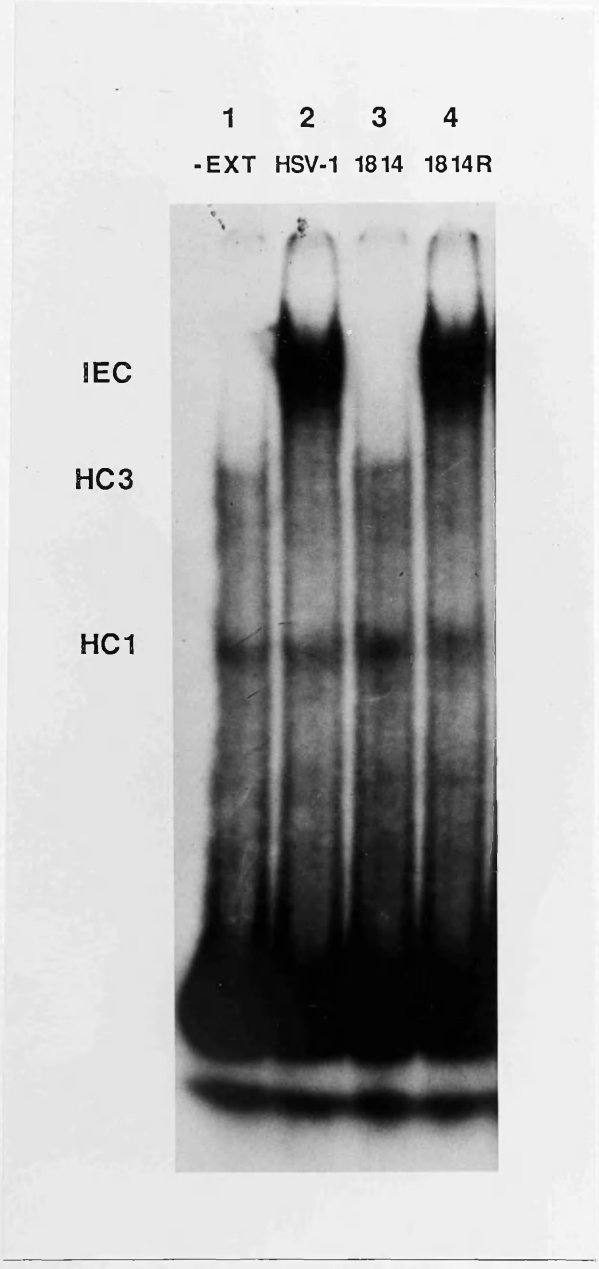

1
2
3
4
-EXT
HSV-1
1814
1814R
IEC
HC3
HC1

Fig. 21. IEC formation by Vmw65. A 74 bp DNA fragment containing the IE gene 4/5 TAATGAGAT sequence (see figure 14 legend) was incubated with HeLa cell nuclear extract alone (lane 1) or with virion extract from wt HSV-1 (lane 2), in1814 (lane 3) or 1814R (lane 4) and analysed by gel electrophoresis. The position of IEC and the cell-specific complexes HC1 and HC3 (Preston et al., 1988) are indicated.

demonstrates that Vmw65 specified by in1814 is not capable of binding the cellular protein(s) required for IEC formation due to the mutation in the viral polypeptide.

6.4.6 in1814 does not exhibit virion-mediated transinduction of IE genes

The ability of in1814 to transinduce IE gene expression was investigated since this property correlates with IEC formation in plasmid pMC1.in14. The level of activation from a transfected IE promoter in the presence or absence of superinfecting virus was investigated (figure 22). BHK cells were transfected with pIE3CAT (which contains the HSV-1 IE-3 gene promoter and regulatory sequences linked to the CAT gene (Stow et al., 1986)) and infected with 1000 particles of wt HSV-1, in1814 or 1814R per cell. An increase of approximately 6-fold in CAT activity was observed when cells were superinfected with wt HSV-1 (lane 2) or 1814R (lane 4), but infection with in1814 (lane 3) gave no stimulation over the level in mock-infected cells (lane 1).

Taken together, these results and those from sections 6.4.5 confirm that the properties of the mutant Vmw65 polypeptide in the viral context reflect the observations and expectations implicit in the initial characterisation of the mutation in cloned copies of the gene; that is, the mutation in in1814 disables the ability of the virus to direct the formation of IEC and consequently abolishes its transinducing activity.

6.4.7 Immediate early gene expression in in1814-infected cells

It would be expected that the abolition of transinduction by Vmw65 would affect the expression of viral genes, especially IE genes. The accumulation of IE RNA was quantitated by dot-blot analysis using IE gene-specific probes for hybridisation. BHK cells were infected in the presence of CH with 1000 particles of wt HSV-1, in1814 or 1814R per cell for 4h, and cytoplasmic RNA was applied to nitrocellulose and separately hybridised with radiolabelled DNA fragments corresponding to the IE-1, IE-2, IE-3 or IE-4

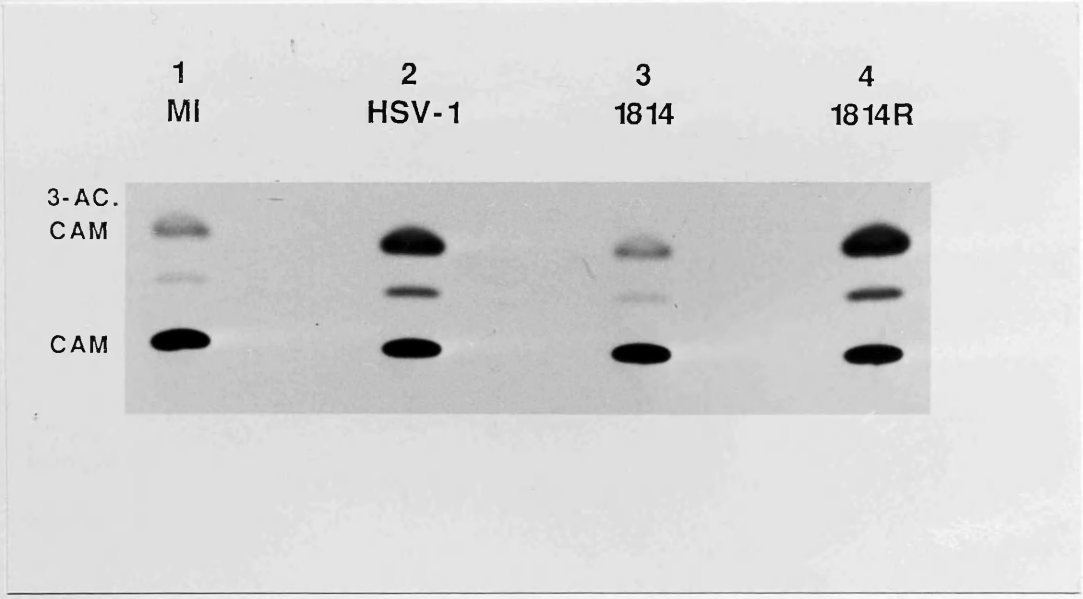

1
MI
2
HSV-1
3
1814
4
1814R
3-AC.
CAM
CAM

Fig. 22. Transinduction of IE transcription. Cells were transfected with pIE3CAT and mock infected (lane 1) or infected with wt HSV-1 (lane 2), in1814 (lane 3) or 1814R (lane 4) for 3h at 38.5°C. CAT assays were carried out on cytoplasmic cell extracts.

genes (figure 23). The levels of IE-1 and IE-2-specific RNA, as determined by densitometric analysis, were reduced 4 to 5-fold in in1814-infected cells compared to wt HSV-1 and 1814R-infected cells (figure 23, a and b), whereas the reduction in IE-4-specific RNA was only 2-fold (figure 23, d) and no significant effect on IE-3-specific RNA was detected (figure 23, c). The accumulation of correctly initiated IE-4/5 mRNA was also determined by S1 nuclease mapping of the 5'-terminus (figure 24). Infections were carried out as described above, and in addition infections using equal numbers of pfu were compared. Again, it was apparent that the levels of IE-4/5 RNA species were reduced by only 2 to 3-fold in in1814-infected cells during infections using equal numbers of virions (lanes 2 to 4). As expected, the level of RNA accumulation in in1814-infected cells was approximately 10-fold greater than in wt HSV-1 when infections were performed using equal pfu (lanes 5 to 7), since this criterion dictates that a much larger number of in1814 virus particles are used.

The expression of IE polypeptides in BHK (figure 25) and HFL (figure 26) cells was also investigated. Cells were infected as described above, but after 4h CH was washed from the cells and polypeptides were radiolabelled in the presence of actinomycin D and separated by SDS-PAGE. Densitometric analysis was used to determine the relative rates of synthesis of individual IE polypeptides, and the values were normalised to that of actin.

In BHK cells, the rates of synthesis of Vmw110 and Vmw63, the products of IE genes 1 and 2 respectively, were reduced by 4 to 5-fold in in1814-infected cells, whereas the rate of synthesis of Vmw175, the product of IE gene 3, was equal for the three viruses (figure 25). It was not possible to measure accurately the rate of synthesis of Vmw68, the product of IE gene 4, since this polypeptide migrated as a diffuse band. In addition to those already mentioned, a low molecular weight polypeptide was present in wt HSV-1 and 1814R but not in1814-infected cell extracts. The origin of this protein species is unknown, but it may be a degradation product of one of the IE polypeptides that is synthesised at higher levels in wt virus.

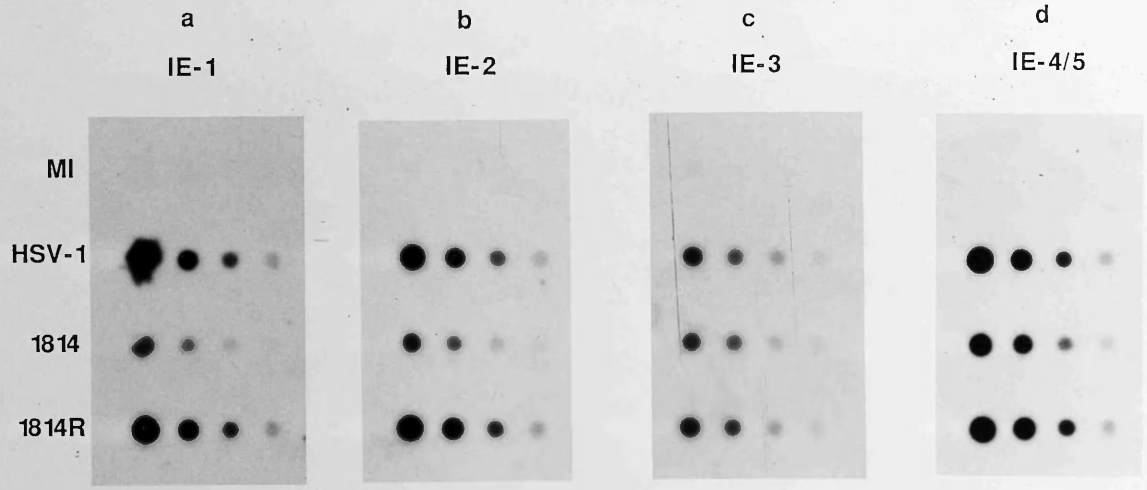

a
IE-1
b
IE-2
c
IE-3
d
IE-4/5
MI
HSV-1
1814
1814R

Fig. 23. Production of IE RNA. Cells were mock infected (row 1) or infected with wt HSV-1 (row 2), in1814 (row 3) or 1814R (row 4) in the presence of CH. RNA was prepared after 4 h and applied to nitrocellulose filters in 4 sequential dilutions (3ug, 1ug, 0.3ug and 0.1ug). Filters were separately hybridised with ^{32}P-labelled DNA probes corresponding to IE genes 1, 2, 3 and 4/5 (panels a, b, c and d respectively). Gene-specific probes were DNA fragments that corresponded to IE genes 1 (a 1367 bp SalI/NruI fragment from pJR3 [Everett, 1984b), 2 (a 2760 bp MluI/BamHI fragment from BamHI b [McGeoch et al., 1988]), 3 (a 3210 bp HincII fragment from XhoI c [McGeoch et al., 1986]) and 4 (a 2200 bp NruI/MluI fragment from BamHI n hybridising predominantly to IE RNA 4 [McGeoch et al., 1985]).

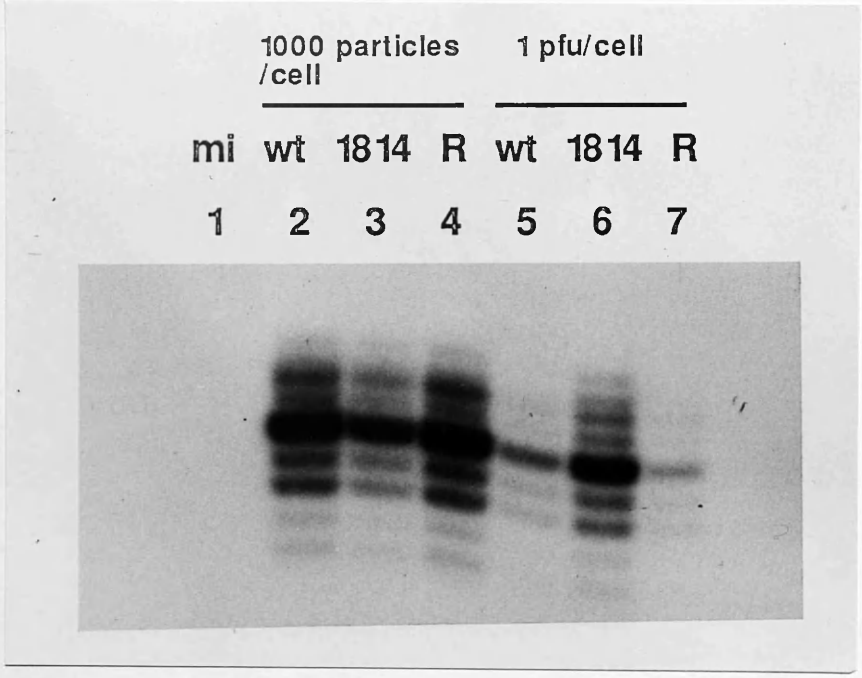

1000 particles /cell
1 pfu/cell
mi wt 1814 R wt 1814 R
1 2 3 4 5 6 7

Fig. 24. S1 5'-terminus analysis of IE4/5 mRNA. Cells were mock infected (lane 1) or infected with wt HSV-1, in1814 or 1814R at a moi of 1000 particles / cell (lanes 2 to 4 respectively) or 1 pfu / cell (lanes 5 to 7 respectively). Infections were done in the presence of 200ul/ml of cycloheximide for 4h at 38.5°C.

RNA was hybridised to a 5'-terminally labelled AvaII d fragment derived from pGX35 (Preston et al., 1984).

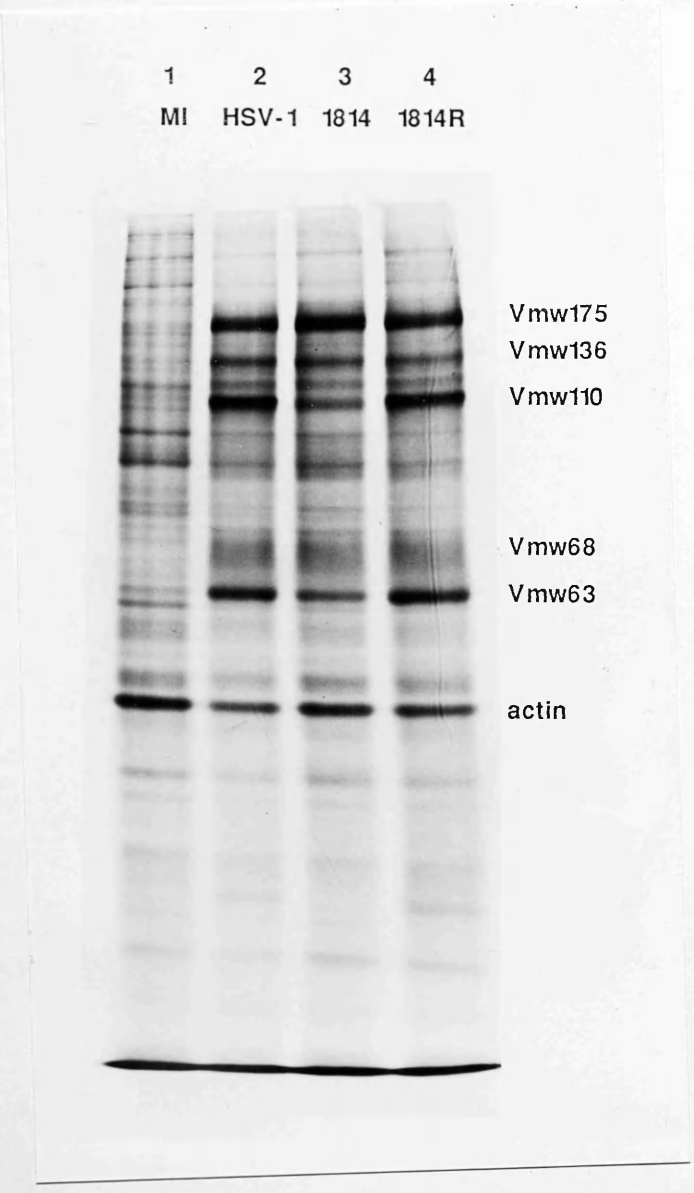

1
MI
2
HSV-1
3
1814
4
1814R
Vmw175
Vmw136
Vmw110
Vmw68
Vmw63
actin

Fig. 25. IE polypeptide synthesis in BHK cells. Cells were mock infected (lane 1) or infected with wt HSV-1 (lane 2), in1814 (lane 3) or 1814R (lane 4) in the presence of CH. Proteins were labelled with [^{35}S]-methionine after removal of CH by washing at 4 h post infection. The position of viral IE polypeptides and cellular actin are indicated.

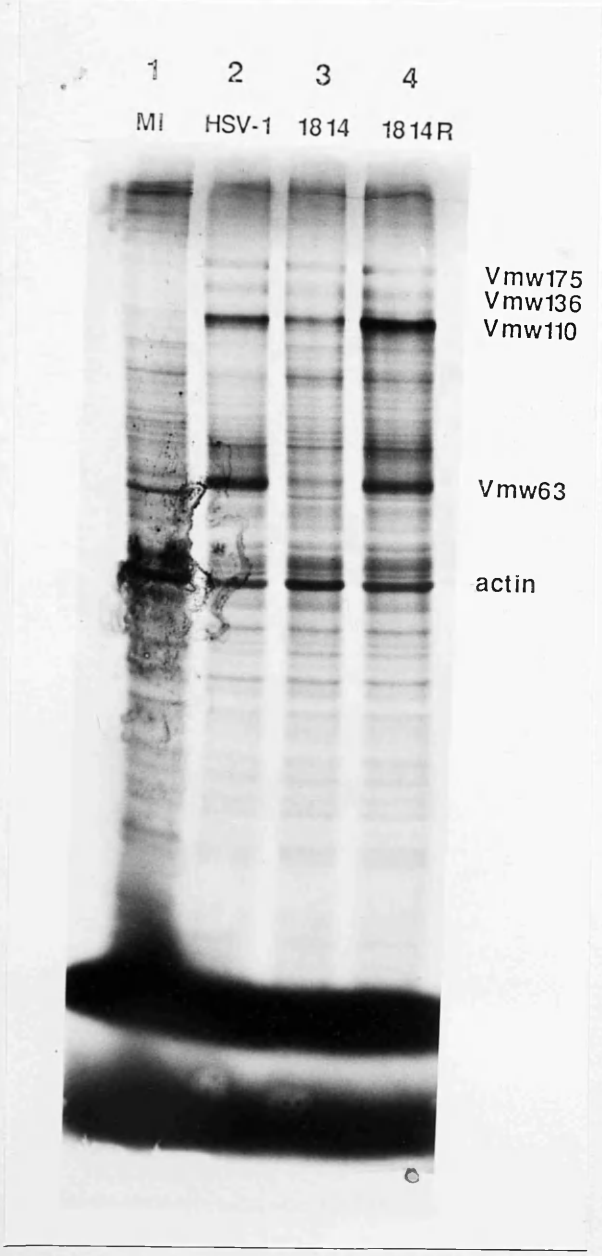

1
2
3
4
MI
HSV-1
1814
1814R
Vmw175
Vmw136
Vmw110
Vmw63
actin

Fig. 26. IE polypeptide synthesis in HFL cells. The procedure and lane arrangement are as described in the legend to figure 25.

In HFL cells, the level of Vmw110 in in1814 was reduced by approximately 10-fold and the reduction in Vmw63 would appear to be even greater, since its synthesis was not readily detectable (figure 26). The greater reduction in IE protein synthesis in in1814-infected HFL cells may explain the mutant's lower plaqueing efficiency on this cell type.

It is clear, however, that the data on IE RNA levels and IE protein synthesis rates in BHK cells are in good agreement and show that the expression of IE genes 1 and 2, and to a lesser extent IE genes 4 and, presumably, 5, is reduced in in1814-infected cells but the expression of IE gene 3 is essentially unaffected. Expression of the large subunit of ribonucleotide reductase (Vmw136), seen in "immediate-early" conditions, also appears to be unaffected in in1814 (figures 25 and 26).

6.4.8 Early and late gene expression in in1814-infected cells

Protein synthesis at 8 h post infection, a time at which early and, especially, late polypeptides are synthesized, was also examined (figure 27). The profiles of wt HSV-1, in1814 and 1814R-infected cells were very similar, the increased mol.wt of Vmw65 specified by in1814 being the only major difference.

Thus, upon infection of BHK cells with 1000 particles of in1814 per cell, the level of expression of IE genes 1, 2, 4 and, presumably, 5 is reduced but infection procedes normally to the late stage, suggesting that there is no overall effect of reduced IE gene expression. From the high particle/pfu ratio, however, it appears that the growth of in1814 is inefficient when cells are infected with 1 particle of virus per cell. To investigate whether the incapacity of in1814 at low multiplicity of infection is reflected in reduced gene expression, synthesis of TK, an early enzyme that can be detected with high sensitivity, was examined. BHK cells were infected with 1000, 100, 10 or 1 particle of wt HSV-1, in1814 or 1814R per cell, and incubation was continued for 15 h in the presence of PAA, to prevent secondary spread of virus. Table 8 shows the results of TK assays performed on the cell extracts. The

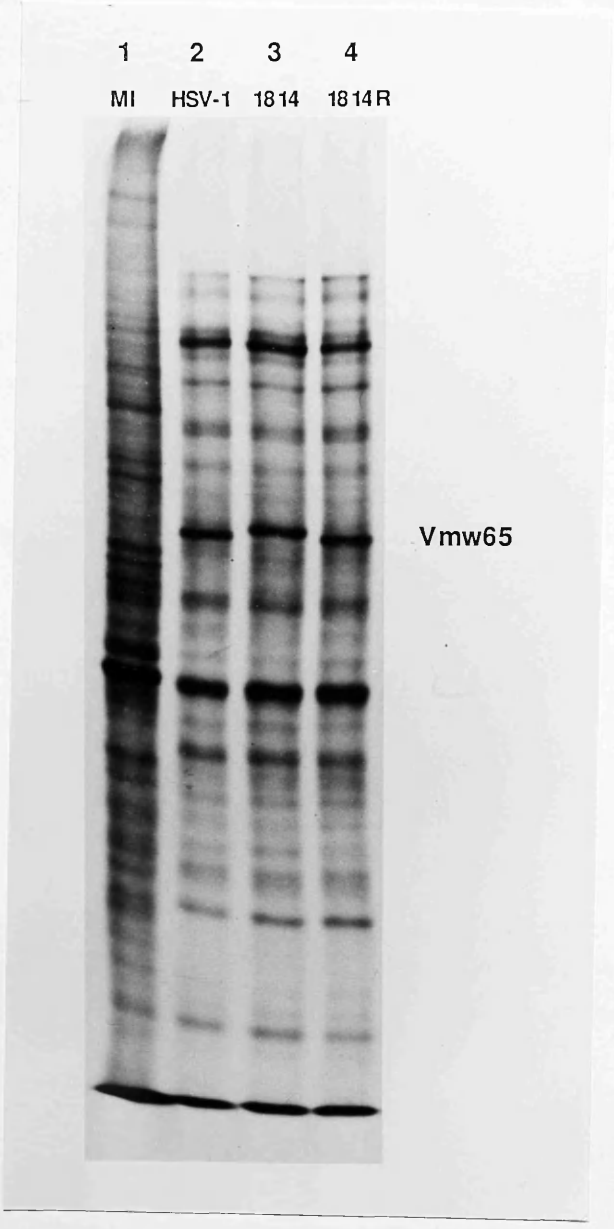

1
2
3
4
MI
HSV-1
1814
1814R
Vmw65

Fig. 27. Late polypeptide synthesis. Cells were mock infected (lane 1) or infected with wt HSV-1 (lane 2), in1814 (lane 3) or 1814R (lane 4) and proteins labelled with [^{35}S]-methionine at 8 h post infection. The position of Vmw65 is indicated.

Table 8.

TK production by in1814 at high and low multiplicity of infection

moi particles/cell	Viral TK activity (cts./min./min. assay/ug protein) wt HSV-1	in1814	1814R	Ratio wt HSV-1/in1814
1000	2693	2285	2789	1.2
100	1988	2549	2478	0.8
10	935	161	729	6.0
1	100	3	63	33.3

BHK cells were infected at the multiplicities indicated. Cytoplasmic extracts were diluted as necessary to ensure that TK determinations were within the linear response range of the assay. A background level of 3 cts./min/min. assay/ug protein (from mock infected cells) was subtracted from all values.

level of TK after infection with 1000 or 100 particles per cell was indistinguishable for wt HSV-1, in1814 and 1814R, reemphasizing that in1814 is not detectably impaired at high moi. At 10 particles per cell, the TK level in in1814-infected cells relative to that in wt HSV-1-infected cells was reduced by 5-fold, and at 1 particle per cell the decrease was 30-fold. Therefore the expression of TK (and presumably of other early and late genes) is more strictly dependent on moi for in1814 than for wt HSV-1 or 1814R, and it is likely that the observed reduction in expression, if exhibited by all early and late genes, is large enough to account for the inefficiency of plaque formation by the mutant.

6.4.9 Complementation of in1814 by Vmw110

If in1814 fails to form plaques at low moi due to the reduction in IE gene expression, then complementation of this state should increase the efficiency of plaque formation and consequently the apparent titre of the mutant virus. In contrast, a compensating increase in IE gene expression would not complement in1814 growth if the mutant phenotype resulted from a defect at a stage before the onset of IE transcription, ie. cell penetration. To test these possibilities, BHK cells were transfected with pl11 (a plasmid encoding the HSV-1 transactivator Vmw110) or pUC9 and then separately used for titration of wt HSV-1, in1814 or 1814R (table 9). Although the titre of wt HSV-1 and 1814R was constant in both cell samples, the apparent titre of in1814 increased approximately 10-fold on cells transfected with pl11. Therefore, raising the level of Vmw110 can (at least partially) rectify the defect of in1814, and thus the restricted growth of the mutant at low moi correlates with a failure to express IE gene products at sufficient levels.

6.4.10 in1814 has reduced virulence in mice

The following study was performed by J.M.Ryan and J.M.Cameron (Glaxo Group Research Ltd., Middlesex).

An assessment of the in vivo properties of in1814 was made by studying virulence after inoculation of mice

Table 9.

Complementation of in1814 by Vmw110

	wt HSV-1	in1814	1814R
+ pUC9	1.2×10^{10}	4.0×10^{6}	1.7×10^{9}
+ p111	9.5×10^{9}	4.2×10^{7}	2.2×10^{9}

BHK cells were initially transfected with p111 or pUC9 and plaqueing efficiencies were subsequently determined by titrating virus stocks onto the monolayers as indicated.

Table 10.

Virulence of in1814 in mice

Virus	LD_{50}[a]			
	ip		ic	
	pfu	particles	pfu	particles
wt HSV-1	9.7×10^2	4.0×10^4	3.1	1.3×10^2
in1814	$>7.4 \times 10^4$	$>2.4 \times 10^8$	$>7.4 \times 10^3$	$>2.4 \times 10^7$
1814R	3.0×10^3	9.2×10^4	19.1	5.8×10^2

[a] LD_{50} values are expressed as pfu or particles per mouse.

either ic or ip. The results (table 10) show that in1814 was much less virulent than wt HSV-1 or 1814R regardless of the method of inoculation. In fact, all mice challenged with in1814 survived, with the exception of 3 mice injected ic with undiluted virus. In these cases, death was atypically rapid, occurring within 12 h as opposed to the usual 3 to 5 days, and it is suspected that the effect was due to the large number of virus particles injected. The LD_{50} values in terms of particles per mouse, the more relevant value, show that virulence of in1814 was reduced by a factor of at least $3x10^3$ for ip or $2.5x10^4$ for ic inoculation, compared with wt HSV-1 or 1814R. The reduction was much greater when results were calculated on the basis of pfu per mouse. The reason for the small reduction in virulence of 1814R with respect to wt HSV-1 is unknown, and similar observations have been made for revertants of other mutants (Cameron et al., 1988). However, it is suspected that wt HSV stocks are composed of viruses of heterogeneous virulence (Taha et al., 1988), which is likely to be a dominant trait, and that a pfu picked at random (for instance when isolating mutants and revertants of them) will be less virulent than the virulence of the original population.

7. DISCUSSION

7.1 FUNCTIONAL REGIONS OF THE VMW65 POLYPEPTIDE

Insertion of a 12 bp oligonucleotide into the coding sequences is a convenient method for making relatively small changes in the amino acid sequence of a protein. Among the ten mutations constructed in plasmid encoded Vmw65 described here, three different amino acid sequence changes were possible depending upon the reading frame at the site of insertion, as shown in figure 28. Alteration in the properties of a protein could result from local or overall physical distortion or charge imbalance, but in a similar study of the HSV-1 IE polypeptide Vmw110 Everett concluded that the properties of the mutated protein correlated more strongly with the position than with the nature of small insertions (Everett, 1987). A summary of the phenotypes of mutant plasmids is presented in figure 28.

Three insertion mutants, in8, in9 and in14, were strongly reduced in their ability to transinduce IE transcription, and, since HaeIII sites 8 and 9 are only 15 bp apart, it is probable that these three mutants define only two important regions. The resultant structure of the deletion mutant d18-9 is a 1 amino acid deletion with a four amino acid change. This mutant is also defective for transinduction, reemphasising the importance of this region of the polypeptide. In addition, the predicted secondary structure (Chou and Fasman, 1978) of in9 indicates that the insertion at this position causes a strong alteration in structure in this region of the protein (F.Ramsay, thesis). However, such correlations may be of little consequence since the predicted structure at the site of in11 was also disrupted but transinduction was unaffected, and activity was lost in in14 but the insertion did not alter the predicted secondary structure (F.Ramsay, thesis). If the mutations exert their effects only at the site of insertion rather than by disruption of gross secondary or tertiary structure, it is possible that the regions defined by in8/9 and in14 are in close proximity to form a single domain in the properly folded protein. The observation that all of the deletion mutants are non-functional, including d14-7

IEC formation:	nd	nd	+	−	−	+	±	−	+	nd
Transinduction:	+	+	+	−	−	+	±	−	+	+
Rescue of ts2203 :	+	−	+	−	−	−	−	+	+	+
Inserted aa.s:	a	b	b	a	a	c	a	b	a	a
in. :	2	4	7	8	9	11	12	14	15	17

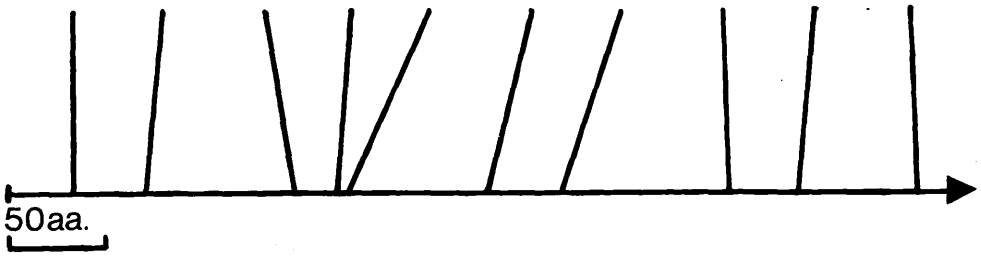


Fig. 28. A summary of the phenotypes of mutated Vmw65 polypeptides. A (+) sign denotes that the polypeptide has wt phenotype, a (-) sign indicates that the function has been abolished, and ($\pm$) represents intermediate activity. The inserted amino acids, determined by the reading frame of the insertion site, are either (a) ARIR, (b) ADPR or (c) RGSA.

which lacks a region of the polypeptide not recognised as important by insertion mutagenesis, indicates that the overall structure of the protein as a whole is important for function.

No striking feature of the amino acid sequence at the sites of the insertions is obvious at present, and a computer-assisted search of the NBRF database did not reveal any proteins with clearly meaningful homologies to these regions. There is, however, a region of nine amino acids (ELRAREESY) only three amino acids upstream from the in8 site which is strongly conserved between Vmw65 and its VZV homologue (Dalrymple et al., 1985). However, recent evidence suggests that the VZV homologous polypeptide does not function as a transinducing factor (TIF) in transfection assays (T.McKee, personal communication). The VZV protein lacks an acidic carboxy-terminus (discussed below) and also does not appear to form a protein complex that associates with TAATGARAT sequences present upstream of the VZV major IE gene (T.McKee, personal communication). Therefore, unless the homology is an evolutionary vestigial remnant, it is unlikely that amino acids responsible for transinducing domains would be conserved between the two polypeptides. Rather, regions of homology may be more significant with regard to virion structural properties provided that this is the function of the VZV homologue.

In addition to the in8/9 (amino acids 172 and 177) and in14 (amino acid 379) regions, the region between amino acids 411 and 453 is also known to be important for transinduction, since deletion back to a SmaI site (amino acid 453) has no detectable effect whereas a frameshift insertion at a SalI site (amino acid 411) abolishes transinduction (C.M. Preston, personal communication). This C-terminal domain of the polypeptide contains a high proportion of acidic residues and corresponds to the "acid tail" of Vmw65, also shown by others to be important for transinducing activity (Sadowski et al., 1988; Triezenberg et al., 1988a).

Proteins containing insertions at sites 8, 9 and 14 all failed to form IEC, suggesting that these mutations prevented Vmw65 from interacting with the cellular

polypeptide(s) contained in IEC. It may be, therefore, that one or more of these sites defines amino acids involved in the protein-protein interactions that are essential for the production of IEC (Preston et al., 1988). The observation that the ability to transinduce IE transcription correlates well with the formation of IEC provides evidence that the complex is an essential intermediate in IE gene activation. The insertion mutants that abolish transinduction all lie within the more N-terminal, internal domain of Vmw65 implicated by others to be important for binding with celluar factors (Triezenberg et al., 1988; Sadowski et al., 1988). None of the insertion mutants isolated were located within the "acid tail" of the protein, but such mutations would probably not affect the function of this region since activating regions of other eukaryotic polypeptides appear only to require the presence of acidic residues rather than conserved structural amino acid sequences (see section 3.2). Therefore, there appear to be two essential domains of Vmw65 involved in transinduction: first, the N-terminal and internal region which is responsible for binding cellular factors, and, second, the C-terminal acidic "tail". One model to explain these observations is that Vmw65 is targeted to TAATGARAT sequences in the form of the IEC such that the "acid tail" comes into close proximity of basic transcriptional machinery (e.g. TFIID and/or RNA polymerase) which it subsequently modulates to promote the rate of transcription. Both targeting and modulation of transcription factors would therefore be required for transinduction, and so deletion or disruption of either the N-terminal or the C-terminal domain would abolish the activity of the polypeptide. Other phenotypes of Vmw65 mutants can be envisaged, for example, a protein unable to mediate transinduction might form an aberrant IEC which could not interact correctly with additional transcription factors.

The experiments described here show that the structural requirements of Vmw65 for transcription activation and virion assembly differ (summarised in figure 28). The mutants in4 and in11 define sites which are important for virion structure but not transinduction, and

the mutation of ts2203 also falls into this category. Presumably these observations reflect the fact that Vmw65 is a multifunctional protein which interacts with virus structural proteins during virion assembly, whereas it is complexed with one or more host cell proteins when acting as a transinducing factor. Insertions which affect both functions may disrupt a domain important for both properties, or they may change severely the secondary and tertiary structure, resulting in a grossly altered protein. Mutant in14 is unable to transinduce but can rescue ts2203, suggesting that it is defective only for transinduction. In turn, this interpretation implied that viable HSV-1 viruses containing the in14 mutation could be constructed.

7.2 CONSEQUENCES OF TRANSINDUCTION IN HSV-1 IN TISSUE CULTURE

The isolation of a virus mutant defective in transinduction of IE transcription is a crucial step in determining the biological role of Vmw65. The 12 bp insertion mutation in in1814, constructed using plasmid pMC1.in14, appears to be stable since no revertants have been detected during passage and growth of virus stocks. Any reversion to the phenotype of wt HSV-1 would readily be detected, as shown by the ease with which the rescued virus, 1814R, was isolated.

A meaningful interpretation of the properties of in1814 hinges on the assertion that its phenotype is a consequence of the transcriptional defect rather than any additional block in the correct uncoating of the virus, and four lines of evidence suggest that an equivalent proportion of virus particles in wt HSV-1 and in1814 stocks proceed normally to the stage of IE gene transcription. First, direct measurement of DNA migration to the nucleus revealed no difference between wt HSV-1, in1814 and 1814R, indicating that the three viral genomes are uncoated and transported with similar efficiencies. Second, the fact that synthesis of IE gene 3-specific RNA, and hence Vmw175, is indistinguishable in wt HSV-1, in1814 and 1814R-infected cells under IE conditions suggests that the number of genomes available for transcription is comparable. Third,

the progression of wt HSV-1, in1814 and 1814R to an equivalent stage of infection after 8 h again shows that the number of active genomes is not very different. Fourth, complementation of in1814 by transfection with plasmids which encode Vmw110 indicates that the inability to form plaques is the result of inefficient IE gene expression rather than an uncoating problem. In addition, in1814 can be complemented to wt levels of growth in HeLa cells with UV-inactivated wt HSV-1, indicating that wt Vmw65 can fully rectify the defect in in1814 in trans (T.McKee, personal communication). This observation would be unexpected if the mutation caused a structural aberration of the virion since the nature of such a defect is more likely to be exerted in a cis-dominant fashion at the initiation of infection. From these considerations, it is concluded that the abolition of transinduction is the only significant effect of the insertion mutation.

Although the results presented here suggest that transinduction by Vmw65 is not essential for HSV replication, this interpretation must be taken cautiously as the assays available are of limited sensitivity. The degree of impairment of transinduction is difficult to assess because the stimulation of expression from a transfected IE promoter is only 5 to 10-fold, and thus it is possible to state only that in1814 is reduced by at least 90% in its ability to stimulate IE transcription. Analysis of the ability to form IEC, as shown in figure 21, is more sensitive and by this criterion in1814 is disabled by 95% or more. Nevertheless, each HSV particle contains approximately 1000 molecules of Vmw65 (Heine et al., 1974), and therefore a cumulative effect of a low residual activity might be sufficient to endow in1814 with the ability to form plaques at the observed low efficiency.

In the absence of transinduction by Vmw65, the IE genes would be expected to be transcribed according to the inherent strengths of their promoters, a feature that is presumably determined by interaction with cellular transcription factors. For IE genes 1 and 2, the 4 to 5-fold reduction in RNA accumulation and protein synthesis correlates well with the 5 to 10-fold stimulation of

transcription in BHK cells from transfected IE promoters by Vmw65 (C.M.Preston, personal communication). The expression of IE genes 3 and 4, however, is greater than would be anticipated from transfection studies since these promoters are also activated by more than 5-fold (Preston et al., 1984; Bzik & Preston, 1986; results in this thesis), and it is difficult to offer an obvious explanation for this apparent discrepancy. One possibility is that the enhancer-like sequence which lies between the promoters of IE genes 3 and 4 (Lang et al., 1984; Preston and Tannahill, 1984), rather than cis-responsive TAATGARAT elements, is the major requirement for transcription of these genes in the context of the viral genome. It is also noteworthy that the four upstream nucleotides of the TAATGARAT elements which control IE genes 1 and 2 confer a strong homology to the NFIII/OTF-1 binding site, the 'octamer' element ATGCAAAT, whereas this is not the case (with one exception) for the TAATGARAT elements located between IE genes 3 and 4/5 (table 2). If this correlation were meaningful it could be argued that the octamer sequences, rather than TAATGARAT elements, confer the response to Vmw65 in the context of the viral genome. Indeed, it has recently been shown that the virus induced complex that binds to TAATGARAT sequences of IE gene 1 is composed of Vmw65 and OTF-1, and that the OTF-1 specific binding site nucleotides (ATGC) are required for complex formation at this promoter region (Gerster and Roeder, 1988). However, this observation is not entirely consistent with the results presented here and by Preston et al. (1988) which demonstrate that IEC formation occurs at the IE4/5 TAATGARAT element (GCGGTAATGAGAT), a sequence which does not contain a strong octamer homology.

Alternatively, the discrepancy between observations made from transfections and virus infections may result from considerable differences in stoichiometric relationships between DNA and protein factors under these different conditions. The findings using virus-infected cells are probably the more relevant.

Even though in1814 lacks transinducing activity, the major polypeptides synthesized under 'IE' conditions are still the IE proteins. Activation by Vmw65 is therefore not

a definitive characteristic of IE genes, and other features must distinguish them from early and late genes. It may be that the presence of strong promoters and enhancer-like sequences determines the relatively high efficiency of IE gene transcription in the absence of IE proteins, but equally the TAATGARAT or other IE-specific elements might be responsible. It is known that cellular proteins bind to various sequences in IE gene upstream regions (Kristie and Roizman, 1987; Kristie and Roizman, 1988; Treizenberg et al., 1988b, O'Hare and Goding, 1988), and these factors might increase the availability of IE promoters to transcription components in the absence of Vmw65. Thus, the involvment of cellular transcription factors with IE-specific DNA sequences, rather than Vmw65, may be the primary determinant of an IE gene.

Transinduction by Vmw65 is important for infection only at low moi. At a superficial level, it is straightforward to view this property as a reasonable adaptation, since the initial interaction of HSV with an organism is likely to involve a small number of virus particles. The inability to replicate at low moi appears to result from the failure to produce IE proteins at levels sufficient to initiate infection, and it is probable that the reductions in Vmw110 and Vmw63 are crucial, since these polypeptides are required for gene expression (Sacks et al., 1985; Sacks and Schaffer, 1987; Stow and Stow, 1986). Thus, it seems that "threshold" levels of IE polypeptides must be attained before the lytic cascade ensues, and the role of Vmw65 is to ensure that such levels are reached, especially at low moi. The degree of stimulation of transcription of IE genes by Vmw65 at high moi is at best 4 to 5-fold in BHK cells. It is not clear whether this relatively small effect is wholly responsible for the greatly increased plaqueing efficiency that Vmw65 confers on virus. It is possible that transinduction is significantly greater than 4 to 5-fold at low moi. At high moi the transinducing function of Vmw65 appears to be redundant, presumably because the basal expression of a higher number of IE genes per cell can override or compensate for an uninduced state. It is not clear whether the few cells in which infection with in1814

results in the formation of a plaque represent a subpopulation in a particular metabolic state or simply random variation in the number of genomes per cell in response to infection.

Recently, Friedman et al. (1988) have shown that a transformed cell line which expresses the protein binding portion of Vmw65 supports virus growth poorly, presumably because the expressed protein sequesters the cell factor required to mediate transinduction. In essence, transinduction by Vmw65 is thought to be abrogated in the transformed cell line. The experiments dealt only with infection at low moi (0.1 or 0.3 pfu per cell), but the results are similar to those found with in1814, namely a big reduction in the efficiency of plaque formation, inefficient virus growth and a decrease (by 12-fold) but not abolition of IE RNA accumulation (Friedman et al., 1988). From the results reported here, it would be predicted that virus replication in the transformed cells should not be as severely affected at high moi.

HSV-1 mutants with insertions and deletions within IE gene 1 (encoding Vmw110) have been constructed (Stow and Stow, 1986; R.D.Everett, personal communication), and the phenotypes of these mutants resemble in1814 to an extent. Mutants lacking functional Vmw110 grew efficiently in HeLa cells, inefficiently in BHK and Vero cells and very inefficiently in HFL cells. in1814 grew inefficiently in BHK, HeLa and Vero cells and very inefficiently in HFL cells. Therefore, although these mutants exhibit some similarities in plaqueing characteristics, qualitative differences also exist. Thus it is unlikely that the sole consequential function of Vmw65 is to control the level of Vmw110 gene expression.

7.3 SPECULATIONS ON THE BIOLOGICAL RELEVANCE OF VMW65 DURING IN VIVO INFECTION

The basis for the difference in behaviour of in1814 in BHK and HFL cells remains undefined. However, in1814 IE polypeptide synthesis appears to be more reduced in HFL cells than it is in BHK cells, especially with regard to Vmw63 and Vmw110. This might explain why in1814 growth is more

restricted in HFL cells, since both of these proteins are known to be important for growth (Sacks et al., 1985; Sacks and Schaffer, 1987; Stow and Stow, 1986). Thus it may be that IE transcription in the absence of Vmw65 is significantly less in HFL cells than in BHK cells at low moi. Alternatively, uninduced IE transcription may not be significantly different in the two cell-types, but HFL cells may be worse at compensating for reduced levels of IE proteins. If this were the case, then the more "permissive" cell-types may contain a factor(s) that can partially complement the defect in Vmw65, such as a cellular Vmw65 homologue, IE protein homologues or a ubiquitous transactivator. A speculative analogy would be that certain in vivo cellular environments which lack such complementing factors would be less prone to lytic infection by viruses lacking Vmw65. Whatever the mechanism, it is clear that Vmw65 alleviates the cell-type dependent, cytopathic restrictions that in1814 exhibits in tissue culture. If this phenomenon was related to in vivo infections, then the presence of a Vmw65 gene may promote "infectivity" thereby increasing the chances of a virus's ability to infect a wider range of hosts, especially considering that OTFI/NFIII exists in a wide range of cell types. However, it is difficult to find any good evidence to support the correlation implied in this statement when comparisons with other herpesviruses are made. For instance, pseudorabies virus (PRV) does not apppear to possess a TIF since superinfection with this virus does not transinduce the PRV major IE gene promoter when transfected (Campbell and Preston, 1987), and yet PRV grows efficiently in a wide variety of tissue cultures and host organisms. As disscussed in section 7.1, current evidence suggests that VZV also lacks a TIF and, although its growth is greatly reduced in tissue culture, the virus is very infectious in vivo. In addition, complementation of VZV with Vmw65 (UV-irradiated HSV-1) does not increase the cytopathicity of the virus in tissue culture (T.McKee, personal communication) suggesting that its restricted growth is not simply due to the absence of a functional TIF. It is interesting, however, that both PRV and VZV (alpha

herpesviruses) contain good consensus TAATGARAT homologies upstream of their immediate early genes and that these genes, although unable to respond to components within their own respective virions, are stimulated by Vmw65 of HSV-1 (Campbell and Preston, 1987; T.McKee, personal communication). HCMV (a beta herpesvirus) does not contain TAATGARAT elements but has been reported to respond to HSV-1 virion components (Spaete and Mocarski, 1985), although this claim is not supported by other workers (Stinski and Roehr, 1985; C.M.Preston, personal communication). HCMV does not transinduce HSV-1 IE genes (Batterson and Roizman, 1983; Stinski and Roehr, 1985) but does possess a virion factor capable of stimulating its own major IE gene (Spaete and Mocarski, 1985; Stinski and Roehr, 1985). Thus although HCMV is analagous to HSV-1 in that it possesses a TIF, the host range of HCMV is limited in comparison. Therefore, the evolutionary molecular events that have led to these situations and their relevance, if any, to host range characteristics remain obscure.

The effect of Vmw65 on virus growth in tissue culture is only manifest at low moi. However, these conditions are probably a truer reflection of the initial stages of natural infections and so Vmw65 may contribute significantly to the pathogenicity of infections *in vivo*. Indeed this suggestion is supported by the finding that *in*1814 is avirulent in mice, even after injection of high doses. Vmw65 may therefore be a good target for the design of new antiviral drugs. Furthermore, synthetic oligopeptides that correspond to the host factor binding domain of the polypeptide might interfere with the formation of the IE complex and consequently the function of Vmw65. Analogous studies have been used to disrupt the interaction between ribonucleotide reductase subunits (Dutia *et al.*, 1986; Cohen *et al.*, 1986).

It is interesting to speculate on the role of Vmw65 in HSV latency in the light of the phenotype of *in*1814, since the majority of genes, including IE genes, are silent during latency (Stevens *et al.*, 1987; Spivack & Fraser, 1987), suggesting that an early transcriptional block may operate. One hypothesis is that Vmw65 may be lost or

rendered inactive during transport of the HSV nucleocapsid from the neuronal cell surface to the nucleus (Kristie and Roizman, 1988). Since the function of Vmw65 as an IE gene inducer is manifest at the earliest stages of infection, it is possible that the biological circumstances of the polypeptide dictates wether a lytic or latent infection will be pursued. For instance, if Vmw65 was absent or disabled in infected nuclei, then the viral DNA may become more susceptible to mechanisms that operate to establish the latent state (conceivably the suppression of IE genes). Conversely, the availability of functional Vmw65 in infected nulei could predispose HSV DNA to the mechanisms that operate to establish the lytic state (i.e. full expression of IE genes). From the results presented here, it is possible to predict that under circumstances of low moi, virus replication would not ensue and thus latency might be established. Support for this view comes from a recent finding that 'noninfectious' particles of in1814 can be retained by tissue culture cells after infection at low moi and can subsequently be reactivated to form plaques (C.M.Preston, personal communication). A similar observation was first made in studies with a mutant lacking Vmw110 (N.D. Stow & E.C. Stow, 1989). Therefore, the failure to transinduce IE transcription by interference with Vmw65 function is worthy of serious consideration as a basic precondition for latency.

7.4 FUTURE PROSPECTS

Future work concerns further understanding of the functional aspects of Vmw65 and its interaction with cellular proteins, and expanding the knowledge of the biological relevance of transinduction in HSV.

Having defined certain regions of Vmw65 important for the formation of the transcription complex, it is possible to construct oligopeptides corresponding to these regions and to test their ability to competitively inhibit the association of the proteins involved. Inhibitive oligopeptides would be predicted to have antiviral effects. Alternatively, a detailed mutational analysis of targeted regions of Vmw65 may be required to localise more accurately

specific amino acids involved in cell protein interaction. In addition, it remains unknown whether Vmw65 assembles into dimers or multimers in the transcription complex. Using the insertion and deletion mutants described here, it may be possible to determine whether Vmw65 polypeptides function as a multimer, since mutant polypeptides with different aberrations might then be expected to complement each other when present together. This possibility could be investigated in cotransfection assays and by gel retardation analysis using mixtures of mutant proteins.

Regarding the biological relevance of transinduction in HSV, the possession of the mutant virus, in1814, will facilitate many further aspects of investigation. For instance, the basis for cell type dependency which in1814 exhibits remains obscure and warrants further study. Similarly, the events which determine "silent infections" by in1814 at low moi are of significant interest but are at present unknown. It will also be important to investigate the latency properties of in1814 since the role of Vmw65 may influence the establishment of the latent state. In addition, the potential for Vmw65 to regulate cellular genes could be investigated since such events may influence HSV infections. Since in1814 is the first Vmw65 transinducing mutant to be reported in the literature, the virus should provide a unique opportunity to the study of IE gene induction.

"Occam is the root of all knowledge"
Tony Cullen

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Key words: *HSV-1*/trans-*inducing factor*/*mutagenesis* in vitro

Mutational Analysis of the Herpes Simplex Virus Type 1 *Trans*-inducing Factor Vmw65

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SUMMARY

The herpes simplex virus type 1 (HSV-1) polypeptide Vmw65 is a structural component of the virus particle and is also responsible for *trans*-induction of immediate early (IE) transcription. Functional domains of this polypeptide were investigated by constructing a series of 10 plasmids each with a 12 bp insertion in the gene encoding Vmw65. Plasmids were analysed for their ability to stimulate IE transcription in short term transfection assays, and the altered Vmw65 polypeptides were assayed for the ability to form an IE-specific protein–DNA complex (IEC) *in vitro*. A direct correlation was observed between stimulation of transcription and formation of IEC, strongly suggesting that IEC is an important intermediate in transcription activation. Plasmids were also tested for their ability to rescue the temperature-sensitive mutation in the HSV-2 assembly mutant *ts*2203, since marker rescue analysis indicated that this mutation maps within the gene encoding Vmw65. Five plasmids failed to rescue *ts*2203, thereby defining regions of Vmw65 required for virus assembly. The results show that distinct domains exist in Vmw65 for activation of transcription and assembly of virus.

INTRODUCTION

Herpes simplex virus (HSV) gene expression can be classified into three phases, immediate early (IE), early and late. The IE genes are the first to be transcribed after infection and the gene products (IE polypeptides) are essential for early and late gene expression (Honess & Roizman, 1974; Clements *et al.*, 1977). A distinctive feature of HSV gene activation is the stimulation of IE transcription by the late polypeptide Vmw65, the major tegument protein otherwise referred to as *trans*-inducing factor (TIF) (Post *et al.*, 1981; Batterson & Roizman, 1983; Campbell *et al.*, 1984; Pellett *et al.*, 1985). The *trans*-induction of IE gene transcription by Vmw65 is dependent on the *cis*-acting regulatory element TAATGARAT (where R represents a purine residue) which is located upstream of the mRNA cap sites of all IE genes (Mackem & Roizman, 1982*a*, *b*; Kristie & Roizman, 1984; Preston *et al.*, 1984; Gaffney *et al.*, 1985; Bzik & Preston, 1986; O'Hare & Hayward, 1987).

Polypeptide Vmw65 is the only viral protein required for recognition of TAATGARAT, since *trans*-induction can be achieved by transfection of plasmids which encode Vmw65 alone (Campbell *et al.*, 1984; Pellett *et al.*, 1985). It has recently been shown that Vmw65 interacts with cell factors, probably including nuclear factor III, to form a complex (IEC) which binds specifically to DNA sequences containing TAATGARAT (McKnight *et al.*, 1987; O'Hare & Goding, 1988; Preston *et al.*, 1988), and it is thought that IEC is an important intermediate in the induction of IE gene transcription. Thus, Vmw65 is involved in initiation of viral gene expression at a very early stage of infection.

Apart from its role in stimulating IE transcription, Vmw65 is an abundant viral structural protein, estimated to be present at approx. 1000 molecules per virion (Heine *et al.*, 1974;

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Roizman & Furlong, 1974). It is the major polypeptide of the tegument, and therefore would be expected to be important for the maintenance of virus structure.

To define functional domains of Vmw65 and hence obtain further information on its mode of action both as a transcription activator and as a structural protein, we have constructed a series of in-frame oligonucleotide linker insertion mutants in the gene that encodes the polypeptide. This approach was possible because the gene has been identified, cloned and its nucleotide sequence determined (Campbell *et al.*, 1984; Dalrymple *et al.*, 1985; Pellett *et al.*, 1985). The results from an analysis of 10 mutants indicate that the ability of Vmw65 to form IEC correlates with its *trans*-inducing activity, and that specific domains required for *trans*-induction and correct virion assembly can be distinguished within the polypeptide.

METHODS

Cells and viruses. BHK-21 (C13) cells (Macpherson & Stoker, 1962) were used throughout. The HSV type 2 (HSV-2) temperature-sensitive (*ts*) mutant *ts*2203 was derived from strain HG52.

Plasmids. Plasmid pIE4/5CAT was constructed by replacing the HSV-2 promoter in pLW2 (Gaffney *et al.*, 1985) with the promoter and upstream regulatory fragments of the HSV-1 IE gene 4/5. The IE4/5 upstream fragment used was defined by *Eco*RI and *Sal*I sites in plasmid pTKN7 (Preston *et al.*, 1984) which represent nucleotides −402 and −240 respectively. The *Eco*RI site was converted to a *Cla*I site by insertion of an oligonucleotide linker (CATCGATG). The IE4/5 promoter was contained in a *Sal*I (converted from *Sma*I)/*Nru*I fragment spanning nucleotides −69 to +99 in plasmid pS20TK (Murchie & McGeoch, 1982; Preston *et al.*, 1984). The upstream and promoter fragments were ligated together at the *Sal*I site, and the resultant 330 bp *Cla*I/*Nru*I product was inserted into pLW2 between the *Acc*I site and a filled-in *Bam*HI site. Thus the HSV-2 IE4/5 promoter in pLW2 was replaced by the upstream and promoter sequences of the HSV-1 IE4/5 gene, without the HSV-1 ori_S sequences (Stow & McMonagle, 1983; Preston *et al.*, 1984).

Plasmid pMC17 was constructed from pMC1, a plasmid that consists of a 2609 bp fragment defined by map coordinates 0·669 to 0·685 on the prototype HSV-1 genome (Campbell *et al.*, 1984) cloned into pUC9 (Fig. 1). A 1521 bp *Eco*RV/*Fok*I fragment of pMC1, containing the entire coding sequence for Vmw65, was cloned into the *Hin*cII site of pUC9 to give pMC17 (Fig. 2). The *Hin*dIII/*Eco*RI fragment from pMC17 was inserted into the corresponding sites of the multilinker in pGEM2 (Promega Biotech, Madison, Wis., U.S.A.) to give pGEMTIF (Fig. 2). Plasmid pGEMTIF therefore contained the entire coding sequence of Vmw65 under the control of the bacteriophage T7 promoter. Insertion mutations of Vmw65 were introduced into pGEMTIF by exchanging DNA fragments of pGEMTIF containing coding sequence with corresponding fragments of the pMC1, in series of plasmids that contained the insertion (Fig. 2). Only those mutations between the unique *Apa*I and *Sst*I restriction sites were introduced into pGEMTIF, and the resulting constructions were designated pGEMTIF.in(X).

Construction of insertion mutants. Plasmid pMC1 was cleaved with *Hae*III in the presence of ethidium bromide using conditions found empirically to produce a maximum of singly cut linear molecules (Fig. 1). Plasmid DNA (2 μg) was digested with 2 units of *Hae*III for 30 min at 37 °C with concentrations of ethidium bromide varying from 10 to 500 μg/ml, in a total volume of 20 μl. Samples containing a high proportion of singly cut molecules were identified, and 0·2 μg of DNA was ligated with a 50-fold molar excess of phosphorylated 12 bp *Bam*HI linker oligonucleotide (CGCGGATCCGCG). DNA ligase was inactivated by heating for 5 min at 60 °C and the DNA was digested with *Bam*HI. Linear molecules were resolved by electrophoresis on a 1% agarose gel, eluted from the gel and religated. Plasmids containing linker insertions were identified by analysis of small scale plasmid preparations. The locations of linker insertion sites were determined by restriction endonuclease mapping.

Transfections of BHK cells and chloramphenicol acetyltransferase (CAT) assay analysis. Monolayers of 10^6 BHK cells were co-transfected at 37 °C with 1 μg of insertion mutant plasmid and 1 μg of pIE4/5CAT by the calcium phosphate precipitation method (Campbell *et al.*, 1984). The total amount of DNA present was adjusted to 3·0 μg by the addition of pUC9.

CAT assays were carried out as described by Gorman *et al.* (1982). The radioactivity in the substrate and the acetylated product spots was determined by scintillation counting to calculate the percentage conversion of substrate to product. The protein concentration of each extract was determined (Bradford, 1976) and the percentage conversion per mg of protein calculated. The amount of extract tested was varied to ensure that the linear response range of the assay was used.

In vitro *transcription of pGEMTIF.in plasmids and translation of RNA templates.* The pGEMTIF.in plasmids were cleaved with *Eco*RI before the transcription reaction. *In vitro* transcription was performed using the Riboprobe system (Promega Biotech) following the manufacturer's protocol and incubating 1 μg of plasmid DNA, 0·5 mM-G(5′)ppp(5′)G (Pharmacia) and 0·4 mM of each nucleoside triphosphate in a total volume of 25 μl at 37 °C for 1 h.

In vitro translation was carried out by addition of 2·5 μl of transcription reaction mixture to 20 μl of rabbit

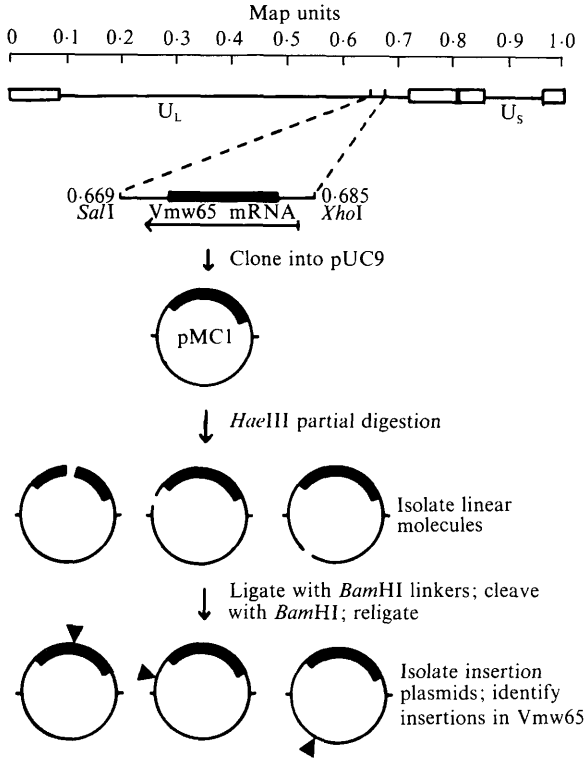

Fig. 1. A map of the HSV-1 genome in the prototype orientation, and position of Vmw65 (upper section). U_L and U_S are the long and short unique regions bounded by repeated sequences represented by open boxes. pMC1 contains the HSV-1 sequence between *Sal*I and *Xho*I sites cloned into pUC9. The lower section shows the construction of insertion mutations within the gene encoding Vmw65, as described in Methods.

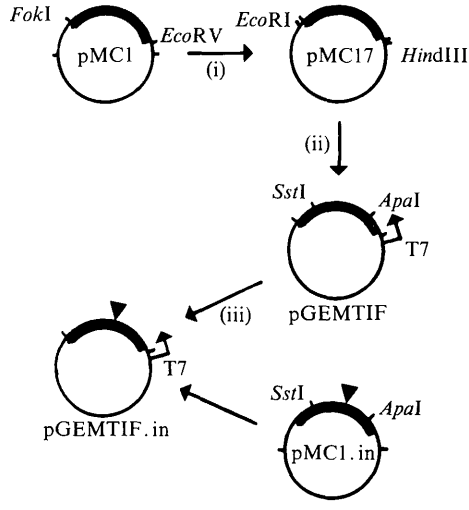

Fig. 2. Construction of templates for *in vitro* transcription. (i) pMC17 was constructed by insertion of a 1521 bp *Eco*RV/*Fok*I fragment from pMC1 into the *Hin*cII site of pUC9. (ii) The 1521 bp fragment was excised from pMC17 with *Hin*dIII and *Eco*RI and cloned between the *Hin*dIII and *Eco*RI sites of the pGEM2 transcription vector, to give pGEMTIF. (iii) Construction of insertion mutants. A 1048 bp *Apa*I/*Sst*I fragment from the pMC1.in plasmids was excised and exchanged with the corresponding fragment in pGEMTIF to give the pGEMTIF.in series.

reticulocyte lysate (Amersham). Duplicate samples were incubated for 90 min at 30 °C either in the presence or in the absence of 25 to 50 μCi [^{35}S]methionine (sp. act. >800 Ci/mmol) in a 25 μl reaction mixture. Non-radioactive samples were stored at −70 °C for use in gel retardation assays. The radiolabelled translation mixture was processed for SDS–PAGE as described by Preston (1979).

Gel retardation analysis of mutant polypeptides. Formation and analysis of protein–DNA complexes was performed as described by Preston *et al.* (1988). A 77 bp DNA fragment, containing IE4/5-specific sequences located between *Sma*I sites at −362 and −297 relative to the transcription start site plus extra nucleotides due to the presence of *Bam*HI and *Bgl*II linkers, was end-labelled with ^{32}P and purified from a polyacrylamide gel. Reaction mixtures contained the following components: 10 mM-HEPES pH 7·9, 0·6 mM-dithiothreitol, 2·3 mM-$MgCl_2$, 85 mM-NaCl, 0·1 mg/ml bovine serum albumin, 4 μg poly(dI)·poly(dC), 0·2 ng ^{32}P-3′ end-labelled fragment, 5·0 μg mock-infected HeLa cell nuclear extract and 5 μl reticulocyte lysate which had been incubated with Vmw65 mRNA synthesized *in vitro*. Incubation was at 25 °C for 30 min, and reaction mixtures were loaded directly onto a 3·5% polyacrylamide gel. After electrophoresis for 3·5 h at 160 V, the gel was dried and exposed for autoradiography.

Marker rescue analysis. Marker rescue was carried out as described previously (Stow *et al.*, 1978). DNA (0·2 μg) of the HSV-2 *ts* mutant *ts*2203 and linearized plasmid DNA (0·5 μg) was co-transfected onto monolayers of 10^6 BHK cells by the calcium phosphate method (Campbell *et al.*, 1984). The total amount of DNA added was adjusted to 3·0 μg using calf thymus DNA. After 3 to 4 h the monolayers were treated with 25% dimethyl sulphoxide and incubated at 31 °C for 3 days. Virus was harvested and titrated at the permissive temperature (PT) of 31 °C and the non-permissive temperature (NPT) of 38·5 °C. The efficiency of marker rescue was determined from the formula (titre at NPT/titre at PT) × 100.

RESULTS

Isolation of insertion mutants within the Vmw65-coding region

Plasmid pMC1 contains the gene encoding Vmw65 and no other complete reading frames from HSV-1 of acceptable codon usage (Dalrymple *et al.*, 1985; Pellett *et al.*, 1985). The approach used to mutagenize Vmw65 was to construct a series of mutants each with a 12 bp *Bam*HI oligonucleotide inserted into pMC1 at a different location within the Vmw65-coding region, as described in Methods (Fig. 1).

Plasmids with insertions at 10 of the 17 *Hae*III sites within the gene were isolated. A summary of the mutants obtained, together with their positions and the resultant amino acid changes, is shown in Fig. 6.

Trans-*inducing activity of insertion mutants*

Previous studies have shown that cloned HSV DNA fragments which encode Vmw65 can stimulate transcription from IE promoters in short term co-transfection assays (Campbell *et al.*, 1984). This approach was used to analyse the *trans*-inducing phenotype of insertion mutants, and the results of a representative experiment are shown in Fig. 3. When a plasmid containing the HSV-1 IE4/5 promoter and upstream regulatory sequences linked to the CAT gene (pIE4/5CAT) was transfected alone into BHK cells a basal level of CAT expression from this promoter was detected (Fig. 3, lane 5). When pMC1 was included in the transfection, however, CAT activity increased by six- to eightfold (Fig. 3, lane 1). The insertion in *Hae*III site 4 (in4) did not affect *trans*-induction (Fig. 3, lane 2), whereas the insertion in *Hae*III sites 8 (in8) and 14 (in14) (Fig. 3, lanes 3 and 4 respectively) essentially abolished the effect. A summary of the results from three to six experiments using the complete series of plasmids is shown in Table 1.

Six of the mutant plasmids (in2, in4, in7, in11, in15 and in17) were indistinguishable from pMC1 in their ability to stimulate IE transcription, showing that insertions of four amino acids can be tolerated in certain regions of Vmw65. Three mutants, in8, in9 and in14, were reduced by 90% or more in their *trans*-inducing ability and thus represent plasmids in which the insertion caused a strong impairment of activity, within the limits of sensitivity of the assay. Any residual activity in in8 and in14 amounted to no more than 5% of the pMC1 level. One plasmid, in12, gave an activity which was reduced by about 50%.

In an additional co-transfection experiment plasmid pIE2TK, which contains the HSV-1 IE gene 2 promoter and upstream regulatory region linked to the HSV-1 thymidine kinase (TK)-coding sequences, was substituted for pIE4/5CAT and transfections were carried out at both 31

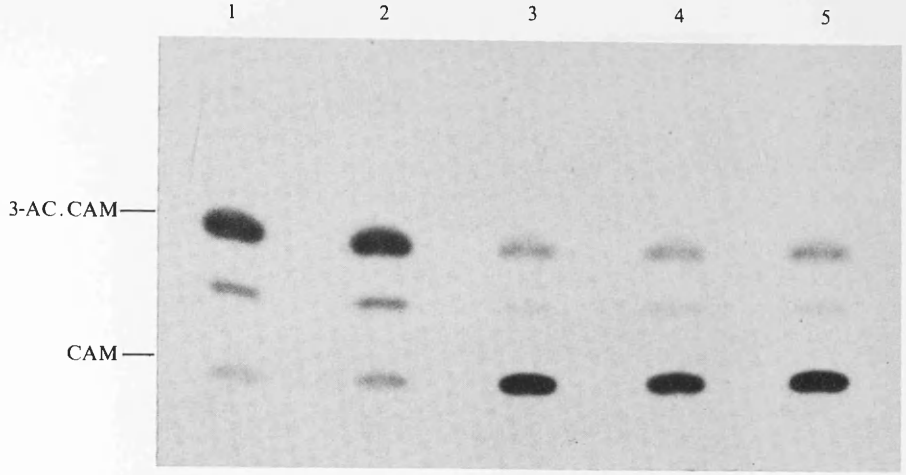

Fig. 3. *Trans*-induction of IE transcription by mutant plasmids. CAT assays were carried out on extracts of BHK cells co-transfected with pIE4/5CAT plus wt plasmid (lane 1), mutant plasmids in4 (lane 2), in8 (lane 3) or in14 (lane 4), or pUC9 (lane 5). The positions of 3-acetyl chloramphenicol (3-AC.CAM) and chloramphenicol (CAM) are indicated.

Table 1. Trans-*inducing activity of Vmw65 mutants*

Plasmid	*Trans*-inducing activity (%)*
pMC1	100
in2	88 (13)
in4	104 (34)
in7	78 (19)
in8	4 (1)
in9	10 (5)
in11	112 (13)
in12	46 (13)
in14	3 (4)
in15	111 (13)
in17	105 (30)
No pMC1	0

* The stimulation of expression from the IE4/5 promoter is given as a percentage of that obtained in parallel experiments with pMC1. The mean of at least three independent determinations was calculated and the standard error is presented in parentheses.

and 38·5 °C. The effect of the insertion mutant plasmids on TK expression from pIE2TK (results not shown) was analogous to the effect on CAT expression from pIE4/5CAT at either temperature, showing that the nature of the 'reporter' gene or IE promoter did not affect the conclusions and that none of the insertions resulted in a protein *ts* for *trans*-activation.

Thus the 10 insertion mutants exhibited *trans*-inducing phenotypes ranging from no alteration to abolition of activity.

Formation of a protein–DNA complex directed by Vmw65 synthesized in vitro

Vmw65 associates with cellular factors to form a complex, IEC, which binds specifically to TAATGARAT elements (McKnight *et al.*, 1987; Preston *et al.*, 1988). The supposition inherent in these findings, that IEC is important for IE gene induction, was investigated by correlating the ability of mutated forms of Vmw65 to produce the complex with their *trans*-inducing phenotypes. Wild-type (wt) and mutant Vmw65 were synthesized by coupled *in vitro* transcription and translation, using the pGEM system. The products were incubated with HeLa

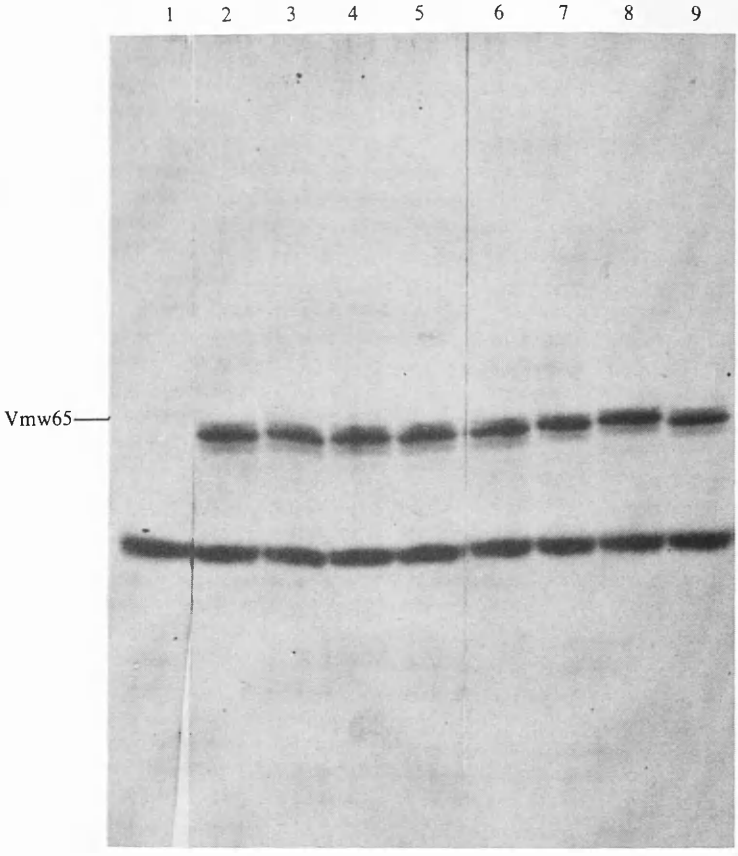

Fig. 4. *In vitro* translation of Vmw65 polypeptides. RNA transcribed from the pGEMTIF. in plasmid series was translated *in vitro* in the presence of [^{35}S]methionine. Lane 1, lysate without added RNA; lane 2, the translated products of RNA transcribed from pGEMTIF; lanes 3 to 9 show the translated products from pGEMTIF. in7, -8, -9, -11, -12, -14 and -15 respectively.

cell nuclear extracts and IEC was detected by analysis of retardation of the electrophoretic mobility of a 77 bp DNA fragment containing the regulatory region, including TAATGARAT, of IE gene 4/5. Fig. 4 shows that Vmw65 is the mRNA-dependent protein synthesized *in vitro* using the wt and mutant plasmid templates. This result rules out the possibility that frameshift mutations, resulting from deletion of a terminal nucleotide during cloning, occurred during construction of the insertion mutants.

The formation of IEC by Vmw65 synthesized *in vitro* was analysed by gel retardation assays (Fig. 5). When Vmw65 extracted from virus particles was added to reaction mixtures containing HeLa cell nuclear extract and the ^{32}P-labelled 77 bp DNA fragment, the slowly migrating complex IEC was formed (lane 1). Addition of reticulocyte lysate containing Vmw65 synthesized *in vitro*, in place of HSV virion extract, also resulted in the production of IEC (lane 2), whereas this complex was not formed when reticulocyte lysate incubated without mRNA was added (lane 3). The complex labelled HC3, present in all lanes, is the result of HeLa cell nuclear proteins binding to TAATGARAT (Kristie & Roizman, 1987; O'Hare & Goding, 1988; Preston *et al.*, 1988), and the band in lane 3 which migrated more rapidly than IEC probably represents the previously described complex HC4, which results from non-specific binding of HeLa cell proteins to DNA fragments (Preston *et al.*, 1988). HC4 was observed only when reticulocyte lysate without transcription mixture was used in the gel retardation assay, presumably because pGEM DNA from the transcription mix acts as an additional non-specific

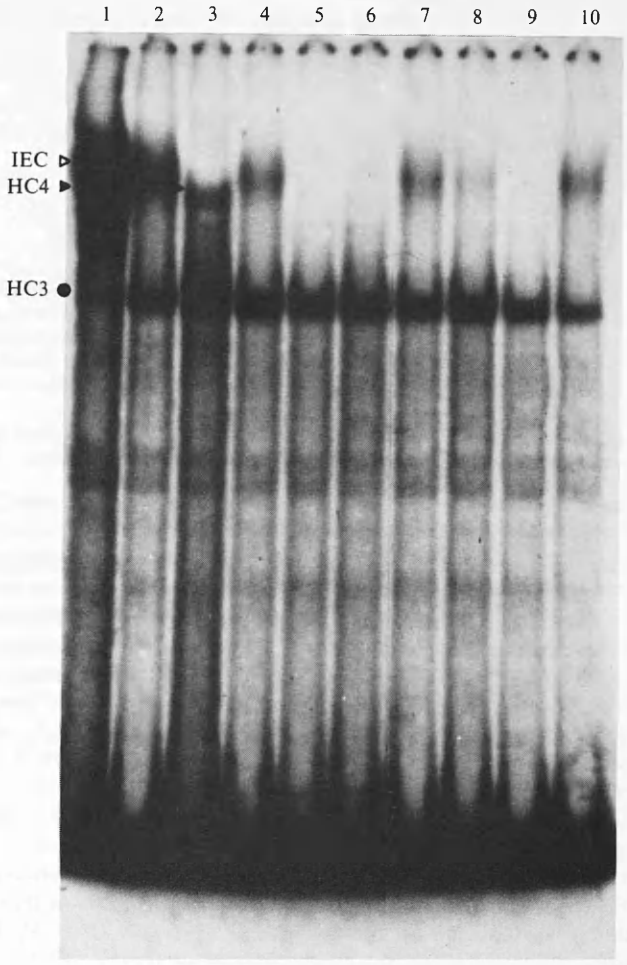

Fig. 5. Gel retardation assay using *in vitro* synthesized Vmw65 products. A 77 bp DNA fragment containing the IE4/5 regulatory sequence was incubated with HeLa cell nuclear extract and virion extract (lane 1), rabbit reticulocyte lysate (lane 3), lysate containing Vmw65 synthesized *in vitro* (lane 2), or lysate containing mutant proteins synthesized *in vitro* from pGEMTIF.in7, -8, -9, -11, -12, -14 and -15 (lanes 4 to 10 respectively). The positions of the complexes IEC, HC3 and HC4 are indicated.

competitor. In control experiments it was found that HeLa cell nuclear extract was necessary for the formation of IEC; thus the reticulocyte lysate did not contain significant amounts of the cell polypeptide that interacts with Vmw65 (results not shown).

When mutant Vmw65 synthesized *in vitro* was used in the gel retardation assay, a strong correlation between complex formation and *trans*-inducing activity was observed. Mutants in7, in11 and in15 all formed IEC (lanes 4, 7 and 10 respectively) and were also capable of stimulating transcription from IE promoters. Similarly mutants in8, in9 and in14 did not form IEC (lanes 5, 6 and 9 respectively) and did not *trans*-induce IE transcription. The correlation is highlighted by in12 which had intermediate *trans*-inducing activity and formed the IEC but in lesser amounts (lane 8). These results strongly suggest that the production of IEC is an important stage in the stimulation of transcription from IE promoters.

Marker rescue of ts*2203 by insertion mutants*

To investigate domains of Vmw65 important for the correct assembly of virions, mutant plasmids were analysed for the ability to rescue the *ts* mutation in the HSV-2 mutant *ts*2203. This

Table 2. *Marker rescue of* ts*2203 by mutant plasmids*

Mutation	Marker rescue (NPT/PT × 100)*
*Bgl*II i	22·0
*Bgl*II i (*ts*13)	<0·001
pMC1	2·6
in2	2·1
in4	<0·001
in7	0·2
in8	<0·001
in9	<0·001
in11	<0·05
in12	<0·001
in14	0·77
in15	1·2
in17	1·8
No pMC1	<0·02

* The ability of mutant plasmids to rescue the *ts* lesion in HSV-2 *ts*2203 is expressed as the percentage of resulting non-*ts* recombinants in the total virus progeny. The results are a mean of two independent determinations.

mutant was derived from the double mutant *ts*13 (Halliburton & Timbury, 1976), which is known to contain a *ts* mutation in the alkaline exonuclease and a second *ts* mutation located between genome coordinates 0·64 and 0·70 which affects the virus particle (Moss *et al.*, 1979). The *Bgl*II i fragment (genome location 0·62 to 0·72) of *ts*13 was subcloned, recombined with wt HSV-2 DNA, and a *ts* mutant, *ts*2203, was isolated and purified. The location of the mutation in *ts*2203 was established by marker rescue experiments using cloned HSV-2 DNA fragments, and was mapped to a DNA fragment which overlaps the 5′ end of the gene encoding Vmw65, as deduced from comparison with the known gene arrangement of HSV-1 (F. H. Ramsay & V. G. Preston, unpublished data).

The mutation in *ts*2203 did not affect the production of IE polypeptides at 38·5 °C over a range of input m.o.i. (results not shown), and cloned *Bgl*II i from *ts*13 was as effective as *Bgl*II i from wt HSV-2 in *trans*-inducing IE promoters at 31 or 38·5 °C in transient expression assays (results not shown). The mutation in *ts*2203 therefore appears to have no effect on IE transcription, and further studies have revealed a block in virus assembly at 38·5 °C (F. H. Ramsay & V. G. Preston, unpublished data).

Intertypic marker rescue was carried out using pMC1 and the insertion mutants, on the assumption that plasmids which failed to rescue *ts*2203 had defects incompatible with the proper assembly of virions, provided the *ts*2203 mutation is in the HSV-2 counterpart of Vmw65. It was found that pMC1 rescued *ts*2203 although, as expected, the efficiency was lower than with HSV-2 fragments (Table 2). Five mutants (in2, in7, in14, in15 and in17) rescued *ts*2203, whereas five (in4, in8, in9, in11 and in12) did not. One conclusion from this experiment is that the *ts*2203 mutation must lie within the coding sequences of the HSV-2 homologue of Vmw65, since this is the only gene affected by the oligonucleotide insertions in4, in8, in9, in11 and in12. Furthermore, the failure of these plasmids to rescue *ts*2203 indicates that the insertions affect regions essential for virus assembly. The interpretation of positive rescue is less clear, since two mechanisms might operate. Such a result may have been obtained because the insertion does not affect virion assembly, but it is also possible that the insertion lies sufficiently far from the *ts*2203 mutation that intragenic recombination, without incorporation of the mutation into viral DNA, could account for the observations. The latter argument does not apply to in7, since both in4 and in8 were unable to rescue *ts*2203, nor to in2 if the *ts*2203 mutation is truly at the 5′ end of the coding sequences.

Thus, of the five sites at which insertions prevent rescue only two (in8 and in9) are loci of major importance for both virus assembly and *trans*-induction of IE transcription. The features of Vmw65 required for virion assembly are not invariably the same as those involved in transcription activation.

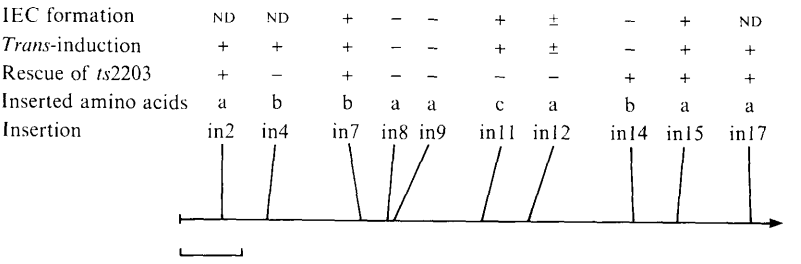

Fig. 6. A summary of the phenotypes of mutated Vmw65 polypeptides. A + sign denotes that the polypeptide has wt phenotype, − indicates that the function has been abolished, ± represents intermediate activity, and ND means that activity was not determined. The inserted amino acids, determined by the reading frame of the insertion site, are (a) ARIR, (b) ADPR or (c) RGSA. The scale bar represents a length of 50 amino acids.

DISCUSSION

Insertion of a 12 bp oligonucleotide into coding sequences is a convenient method for making relatively small changes in the amino acid sequence of a protein. Three different insertions were possible in the experiments described here, depending upon the reading frame at the site of insertion, as shown in Fig. 6. Alteration in the properties of a protein could result from local or overall physical distortion or charge imbalance, but in a study of the HSV-1 IE polypeptide Vmw110 it was concluded that the properties of the mutated protein correlated more strongly with the position than with the nature of small insertions (Everett, 1987). A summary of the phenotypes of mutant plasmids is presented in Fig. 6.

Three mutants, in8, in9 and in14, were strongly reduced in their ability to *trans*-induce IE transcription, and since *Hae*III sites 8 and 9 are only 15 bp apart it is probable that these three mutants define only two important regions. If the mutations exert their effects only at the site of insertion rather than by disruption of gross secondary or tertiary structure, it is possible that the regions defined by in8/9 and in14 are in close proximity and form a single domain in the properly folded protein. No striking features of the amino acid sequence at the sites of these insertions is obvious at present, and a computer-assisted search of the NBRF database did not reveal any proteins with clearly meaningful homologies to these regions. There is, however, a region of nine amino acids (ELRAREESY) only three amino acids upstream from the in8 site which is strongly conserved between Vmw65 and its varicella-zoster virus homologue (Dalrymple *et al.*, 1985). In addition to the in8/9 (amino acids 172 and 177) and in14 (amino acid 379) regions, the region between amino acids 411 and 453 is also known to be important for *trans*-induction, since deletion back to a *Sma*I site (i.e. amino acid 453) has no detectable effect, whereas a frameshift insertion at a *Sal*I site (amino acid 411) abolishes *trans*-induction (C. M. Preston & M. E. M. Campbell, unpublished observations; Triezenberg *et al.*, 1988).

Proteins containing insertions at sites 8, 9 and 14 all failed to form IEC, suggesting that these mutations prevented Vmw65 from interacting with the cellular polypeptide(s) contained in IEC. It may be, therefore, that one or more of these sites defines amino acids involved in the protein–protein interactions that are essential for the production of IEC (Preston *et al.*, 1988). The observation that the ability to *trans*-induce IE transcription correlates well with the formation of IEC provides evidence that the complex is an essential intermediate in IE gene activation. Other phenotypes of Vmw65 mutants can be envisaged; for example, a protein unable to mediate *trans*-induction might form an aberrant IEC that could not interact correctly with additional transcription factors.

The experiments described here show that the structural requirements of Vmw65 for transcription activation and virion assembly differ. The mutants in4 and in11 define sites which are important for virion structure but not *trans*-induction, and the mutation of *ts*2203 also falls into this category. Presumably these observations reflect the fact that Vmw65 is a multifunctional protein which interacts with virus structural proteins during virion assembly,

whereas it is complexed with one or more host cell proteins when acting as a *trans*-inducing factor. Insertions that affect both functions may disrupt a domain important for both properties, or they may change severely the secondary and tertiary structure, resulting in a grossly altered protein. Mutant in14 is unable to *trans*-induce but can rescue *ts*2203, suggesting that it is defective only for *trans*-induction. In turn, this interpretation implies that viable viruses containing the in14 mutation could be constructed, and thus that *trans*-induction is not essential for virus growth. It should be noted, however, that site 14 is distal to the proposed location of the *ts*2203 mutation, and therefore that recombination between the two mutations could occur. This interpretation cannot be ruled out, as it can in the case of in2 and in7, since both downstream insertions (in15 and in17) rescue successfully. Attempts to isolate virus mutants containing the in2, in7, in14 and in15 insertions are currently in progress.

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Construction and Characterization of a Herpes Simplex Virus Type 1 Mutant Unable To Transinduce Immediate-Early Gene Expression

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A herpes simplex virus mutant, *in*1814, possessing a 12-base-pair insertion in the gene encoding the transinducing factor Vmw65 has been constructed. The insertion abolished the ability of Vmw65 to transinduce immediate-early (IE) gene expression and to form a protein-DNA complex with cell proteins and the IE-specific regulatory element TAATGAGAT. Accumulation of IE RNA 1 and 2 was reduced four- to fivefold in *in*1814-infected cells, but the level of IE RNA 4 was reduced only by twofold, and IE RNA 3 was unaffected. Mutant *in*1814 had a high particle/PFU ratio, but many of the particles, although unable to form plaques, were capable of normal participation in the early stages of infection at high multiplicity of infection. The defect of *in*1814 was overcome partially by transfection of a plasmid encoding the IE protein Vmw110 into cells prior to titration and by prior infection with ultraviolet light-inactivated herpes simplex virus. Mutant *in*1814 was essentially avirulent when injected into mice. The results demonstrate that transinduction of IE transcription by Vmw65 is important at low multiplicity of infection and in vivo but that at high multiplicity of infection the function is redundant.

Herpes simplex virus type 1 (HSV-1) encodes 70 predicted genes which are expressed as three temporally regulated classes (35, 70). The five immediate-early (IE or α) genes are the first to be transcribed after infection, and their expression does not require de novo protein synthesis, whereas early (β) and late (γ) gene expression is dependent on the prior synthesis of IE polypeptides (8, 23). The products of IE gene 1 (polypeptide Vmw110 or ICP0) and IE gene 3 (Vmw175 or ICP4) are potent transactivators of early- and late-gene promoters in transient expression assays (12, 18, 40, 53). The IE gene 2 product (Vmw63 or ICP27) has also been implicated in the regulation of viral promoters (13, 54, 60). Analysis of temperature-sensitive mutants indicates that both Vmw175 and Vmw63 are essential for productive infection; Vmw175 is required for early- and late-gene expression (11, 47, 71), whereas Vmw63 appears to be required after the onset of early-gene expression and DNA replication (56). Viable mutants with deletions in Vmw110 exhibit restricted growth in certain cell types at low multiplicity of infection (MOI) but are apparently normal at high MOI (57, 65). Deletion mutations in IE gene 4 (which specifies Vmw68 or ICP22) also confer a host range phenotype to the virus (45, 59). The IE gene 5 product (Vmw12 or ICP47) appears to be unimportant for virus replication in tissue culture cells since deletions within the gene have little effect on growth of HSV (4, 29, 69).

A distinguishing feature of IE genes is the presence of the *cis*-acting element TAATGARAT (where R is a purine residue) in their 5′ regulatory regions. This element responds to the HSV-1 virion polypeptide Vmw65 (otherwise designated VP16 or αTIF), resulting in a stimulation of transcription from IE promoters (2, 5, 7, 9, 17, 25, 31–33, 41, 43, 44, 48). Although Vmw65 does not itself bind to DNA (34), the evidence currently available suggests that the polypeptide mediates transinduction of IE genes by associating with cellular proteins, including nuclear factor III, to form an IE complex (IEC) which is able to bind specifically to DNA sequences that contain TAATGARAT (1, 19, 38, 39, 49). Mutation analysis of cloned DNA fragments encoding Vmw65 suggests that the polypeptide contains at least two separable regions, both of which are necessary for transinduction of IE transcription. The amino-terminal 411 amino acids are sufficient for binding to the cellular factor (1; T. A. McKee, C. I. Ace, and C. M. Preston, manuscript in preparation), and the acidic carboxy-terminal domain defined by amino acids 411 to 490 (the "acid tail," a feature common to many other eucaryotic and procaryotic transactivators [3, 20, 30, 52, 58, 66, 67]) is required for stimulating transcription and may interact with fundamental transcription components, for example, the TATA binding factor TFIID, RNA polymerase, or both (24).

Because Vmw65 regulates the set of genes expressed at the earliest stages of infection, it is important to determine the role of the polypeptide during HSV growth. All information to date regarding the properties of Vmw65 has been obtained by transfection, using either stably transformed cell lines or transient expression assays. Such systems are clearly artificial, and the crucial biological question concerns the phenotype of virus mutants which lack the transinducing activity of Vmw65. A difficulty inherent in attempts to construct such mutants is that, apart from its role in transinducing IE genes, Vmw65 is also a major structural component which is required for virion assembly (1). To address this problem, functional domains of Vmw65 required for virion assembly and for transinduction were identified by insertion mutagenesis of a cloned gene fragment (1). The construction and characterization of a viable HSV-1 mutant that contains an insertion which affects only the transinducing activity of Vmw65 are described here. The results indicate that IE gene transinduction by Vmw65 is not essential for virus growth at high MOI but plays a critical role in determining whether infection is lytic or nonproductive at low MOI. Vmw65 is also important for the virulence of HSV-1 in mice.

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4/5 correct throughout.

MATERIALS AND METHODS

Cells and viruses. BHK cells (~~21~~ clone 13) were grown in Eagle medium with 10% newborn calf serum, 10% tryptose phosphate broth, and 100 U of penicillin and 100 μg of streptomycin per ml. Human fetal lung (HFL) cells (Flow Laboratories) were grown in Eagle medium with 10% fetal calf serum and 100 U of penicillin and 100 μg of streptomycin per ml. The wild-type (wt) HSV-1 virus used in these studies was Glasgow strain 17 syn$^+$. Virus particle concentrations were determined by comparison of virus stocks with ~~latex~~ bead preparations of known concentration.

Plasmids. Plasmid pMC1, which contains the coding sequences for Vmw65, has been described previously (7). The construction of pMC1.in14, which contains a 12-base-pair (bp) *Bam*HI oligonucleotide linker inserted in the Vmw65 gene, has also been described (1). Plasmid pIE3CAT contains the HSV-1 IE gene 3 promoter and regulatory sequences linked to the chloramphenicol acetyltransferase coding region (64), and p111 expresses wt HSV-1 Vmw110 (14).

Isolation of *in*1814 and Southern blot analysis. A BHK cell monolayer in a 35-mm-diameter petri dish was cotransfected with 0.5 μg of intact HSV-1 DNA, 0.5 μg of an *Eco*RI-cleaved plasmid with the mutation of pMC1.in14 in the larger plasmid pGX158 (which contains *Bam*HI *f* [7]), and 2.0 μg of calf thymus carrier DNA by the calcium phosphate precipitation method (51). After incubation for 3 days at 31°C, the progeny viruses were harvested and titrated on BHK cells. Single plaques were picked and used to infect BHK cells in multiwell plates containing 15-mm-diameter wells. After 2 days at 37°C, total DNA was prepared from infected cells (65) and the medium was retained as a viral stock. DNA samples were screened for the presence of viral genomes containing a *Bam*HI linker insertion within the Vmw65 gene. DNA was cleaved with *Bam*HI, and viral DNA analyzed by agarose gel electrophoresis and Southern blotting (61). Plasmid pMC17, which contains the Vmw65 coding sequences cloned in pUC9 (1), was radiolabeled with ^{32}P by nick translation (55) and used as a probe. Hybridization, membrane washing, and autoradiography conditions were as described previously (42). Progeny from a sample which contained viral DNA with a linker insertion was plaque purified and screened by hybridization twice more, and a working stock of virus was prepared from BHK cells. The virus was named *in*1814.

Marker rescue of *in*1814. A BHK cell monolayer was cotransfected with 0.1 μg of intact *in*1814 DNA, 0.5 μg of pMC1 cleaved with *Eco*RI, and 2.0 μg of calf thymus DNA, as described above. After 5 days at 31°C, progeny were harvested and used to infect a BHK cell monolayer on a 90-mm-diameter petri dish at 0.0001 PFU per cell. After 3 days, the progeny were harvested and titrated on BHK cells. Single plaques were picked and used to infect BHK cells on multiwell plates. Virus stocks were prepared from wells, and their genomes were analyzed as described above. Progeny from a sample that exhibited a wt HSV-1 DNA structure was plaque purified, and a working stock of virus was prepared in BHK cells. The virus was named 1814R.

Quantitation of viral DNA in nuclei. BHK cell monolayers in 90-mm-diameter petri dishes were infected in the presence of 200 μg of cycloheximide (CH) per ml. After incubation for 3 h at 38.5°C, cell nuclei were isolated (46) and DNA was extracted. Virus DNA was quantitated by Southern blotting as described above but by using ^{32}P-labeled pTK1, which contains the cloned HSV-1 *Bam*HI *p* fragment, in the hybridization procedure.

Radiolabeling of viral polypeptides. For IE polypeptides, BHK cell monolayers were infected in the presence of 200 μg of CH per ml. After 4 h at 38.5°C, CH was washed from the plates and proteins were radiolabeled for 1 h with [^{35}S]methionine in the presence of 1 μg of actinomycin D per ml and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (46). For early and late polypeptides, BHK cell monolayers were infected and proteins were radiolabeled for 1 h with [^{35}S]methionine and analyzed by SDS-PAGE ~~after incubation for 8 h at 38.5°C~~.

Gel retardation analysis. Virion extracts were prepared (49), and proteins were analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue. Virion extract was added to a mixture containing HeLa cell nuclear extract and a ^{32}P-labeled 74-bp DNA fragment containing HSV-1 IE gene 4/5 regulatory sequences (49). Reaction conditions for complex formation and analysis of protein-DNA complexes were as described previously (49).

Transinduction assay. BHK cell monolayers in 35-mm-diameter petri dishes were transfected with 3 μg of pIE3CAT by the calcium phosphate precipitation method (7), except that the dimethyl sulfoxide boost was performed 1 h after the medium overlay. The cells were incubated at 38.5°C for 1 h and then superinfected. After incubation for a further 3 h at 38.5°C, extracts were made from the cells and chloramphenicol acetyltransferase assays were performed (21).

Quantitation of IE RNA. BHK cells were infected in the presence of 200 μg of CH per ml. After incubation for 4 h at 38.5°C, cytoplasmic RNA was extracted and quantitated by dot blot analysis (72) by using DNA probes radiolabeled with ^{32}P by primer extension (15). Gene-specific probes were prepared from DNA fragments that correspond to IE genes 1 (a 1,367-bp *Sal*I-*Nru*I fragment from pJR3 [12]), 2 (a 2,760-bp *Mlu*I-*Bam*HI fragment from *Bam*HI *b* [35]), 3 (a 3,210-bp *Hin*cII fragment from *Xho*I *c* [36]), and 4 (a 2,200-bp *Nru*I-*Mlu*I fragment from *Bam*HI *n* hybridizing predominantly to IE RNA 4 [37]).

TK assay. BHK cells were infected in the presence of 200 μg of phosphonoacetic acid per ml. After incubation for 15 h at 38.5°C, cytoplasmic extracts were made and thymidine kinase (TK) assays were performed (9).

Complementation assay. BHK cell monolayers in 35-mm-diameter petri dishes were transfected with 3 μg of p111 or pUC9 by the calcium phosphate precipitation method (7) and treated with dimethyl sulfoxide 1 h later. After a further 1 h at 37°C, virus was titrated on the monolayers. Alternatively, monolayers were treated with the HSV-1 mutant *ts*K, which had been UV irradiated to reduce its titer by 5×10^5 (48). The MOI of UV-irradiated *ts*K corresponded to 0.1 PFU of unirradiated virus per cell. Wt HSV-1 or *in*1814 was titrated on the UV-irradiated-*ts*K-pretreated cells. After 2 days at 37°C, plates were stained and plaques were counted.

Virulence assay. Female Charles River mice, each weighing approximately 15 g, were inoculated either intracranially (ic) with 20 μl or intraperitoneally (ip) with 200 μl of 10-fold dilutions of virus stocks, as described previously (6). Ten mice were inoculated for each virus dilution, and the number of survivors after 21 days was recorded. The mean 50% lethal dose values from two experiments were calculated.

RESULTS

Isolation of a mutant containing an insertion within the Vmw65 gene. The gene encoding Vmw65 lies between map

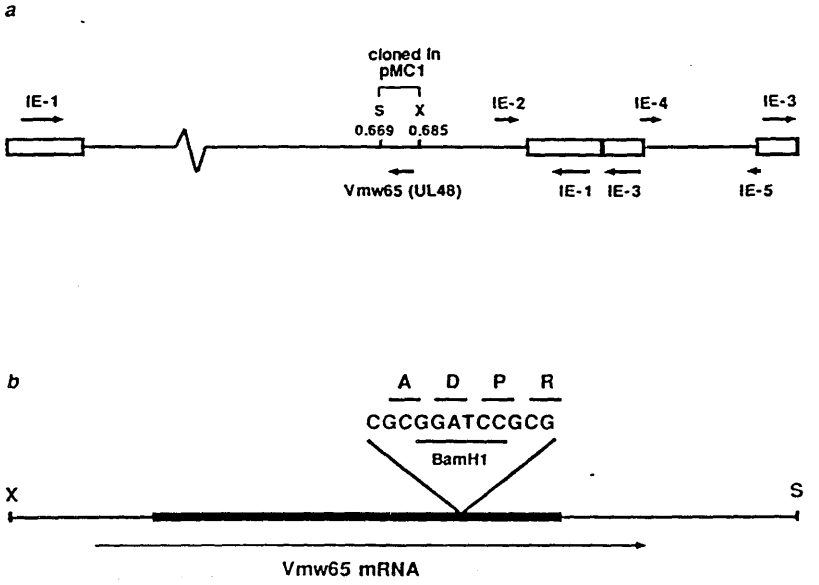

FIG. 1. (a) Structure of the HSV-1 genome showing the positions of the five IE genes (without introns) and Vmw65. The open boxes represent repeated sequences. (b) Structures of the insertion mutation in Vmw65 encoded on a *Sal*I(S)-*Xho*I(X) fragment in plasmid pMC1.in14.

coordinates 0.669 and 0.685 in the U_L region of the prototype HSV-1 genome and is contained within plasmid pMC1 (Fig. 1A; 7, 10). The isolation of a number of plasmids with in-frame *Bam*HI linker insertion mutations within the gene encoding Vmw65 has been described previously (1). In particular, a four-amino-acid insertion at codon 397, specified on plasmid pMC1.in14 (Fig. 1B), abolished the transinducing activity of the polypeptide in transfection assays. The mutation disabled the binding of Vmw65 to the host-cell factors and thus defined a region of the polypeptide involved in this interaction. The essential role of the polypeptide during virion assembly was not affected by the mutation, as inferred from its ability to rescue an HSV-2 mutant with a temperature-sensitive mutation in Vmw65 (1), suggesting that a viable virus could be constructed that contained the transinducing mutation specified by pMC1.in14. To construct such a mutant virus, a plasmid consisting of *Bam*HI *f* containing the pMC1.in14 mutation was cotransfected with intact wt HSV-1 DNA into BHK cells, and the structure of progeny virus DNA was examined by restriction enzyme analysis. One plaque from a total of 84 screened was identified as a recombinant that contained the *Bam*HI linker insertion. This mutant isolate, *in*1814, was plaque purified twice more, and a large scale stock of virus was prepared. To rule out the possibility of a second site mutation in *in*1814 that might affect the phenotype of the mutant virus, a rescued virus was constructed by recombining *in*1814 DNA with pMC1. If, as desired, the phenotype of *in*1814 depended on the insertion mutation, then a rescued virus should behave as wt HSV-1. Initial observation of the properties of *in*1814 suggested that it grew poorly in comparison with wt HSV-1; thus, it was expected that rescued recombinants would outgrow *in*1814 during successive passages of a mixed population. This turned out to be the case, since after a single passage of the progeny virus from the initial cotransfection of *in*1814 DNA and pMC1, 75% of the plaques screened had the wt DNA structure. These viruses were unlikely to result from spontaneous reversion of *in*1814, since no reversion was detected at any stage during the passaging and propagation of mutant virus. A stock of rescued virus, 1814R, was prepared after plaque purification. Figure 2 shows a Southern blot of wt HSV-1, *in*1814, and 1814R DNA which was digested with *Bam*HI and probed with pMC17, a plasmid containing the Vmw65 coding sequences. The *Bam*HI *f* fragment of 8 kilobase pairs was seen in both wt HSV-1 (lane 1) and 1814R (lane 3), whereas in *in*1814 (lane 2), this fragment was replaced by two fragments of the sizes (5 and 3 kilobase pairs) expected from the presence of the *Bam*HI linker insertion. Overexposure of the autoradiograph revealed no detectable *Bam*HI *f* fragment in

FIG. 2. Structure of the *in*1814 genome. Wt HSV-1 (lane 1), *in*1814 (lane 2), or 1814R (lane 3) DNA was cleaved with *Bam*HI, and the fragments were separated on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pMC17. The position of HSV-1 *Bam*HI *f* is indicated.

TABLE 1. Titration of wt HSV-1, *in*1814, and 1814R on BHK and HFL cells

Virus	Particles/ml	BHK titer (PFU/ml)	HFL titer (PFU/ml)
wt HSV-1	1.9×10^{11}	5.0×10^{9}	1.7×10^{10}
*in*1814	1.2×10^{11}	1.3×10^{7}	7.0×10^{5}
1814R	4.6×10^{10}	4.0×10^{9}	ND[a]

[a] ND, Not determined.

*in*1814 and therefore that the stock of mutant virus was essentially pure.

The efficiency of plaque formation by *in*1814 is markedly reduced and dependent on cell type. The successful isolation and propagation of *in*1814 confirms that the insertion mutation is compatible with virus growth in BHK cells. When *in*1814 was titrated on BHK cells, however, a low titer was obtained, and therefore virus particle concentrations were determined (Table 1). It was found that the particle concentrations of wt HSV-1, *in*1814, and 1814R stocks were comparable but that the particle/PFU ratio was approximately 100 times greater for *in*1814 than for wt HSV-1 and 1814R. The apparent titer of a given preparation of *in*1814 on BHK cells varied by as much as 10-fold on different batches of cells, whereas the titers of wt HSV-1 and 1814R were much more consistent, suggesting that the cellular metabolic state affects the efficiency of plaque formation by *in*1814. When titrations were performed on HFL cells, an even higher particle/PFU ratio, 1.7×10^5, was observed for *in*1814. In view of the variation in titer of *in*1814 when expressed in terms of PFU, cell monolayers were infected with equal numbers of particles of wt HSV-1, *in*1814, or 1814R in subsequent experiments.

DNA migration to the nucleus. The early stages of infection by *in*1814 were examined, since it was possible that the insertion mutation affected virus adsorption, penetration, or uncoating. In initial experiments, the rate of adsorption of wt HSV-1 or *in*1814 preparations, radiolabeled by incubation with [^{3}H]thymidine during virus propagation, to BHK cell monolayers was investigated. The adsorption rates of wt HSV-1 and *in*1814 particles were indistinguishable (results not shown). The efficiency of DNA migration to the cell nucleus was also determined. BHK cell monolayers were infected in the presence of CH with 1,000, 100, or 10 particles of wt HSV-1, *in*1814, or 1814R per cell, nuclei were prepared at 3 h postinfection, and nuclear DNA was analyzed by Southern blot hybridization (Fig. 3). No significant differences were detected in the levels of HSV DNA, showing that the nuclear migration of *in*1814 DNA is not impaired at either high or low MOI.

This result underlines the requirement to use particles rather than PFU as a basis for the design of experiments with *in*1814; 1,000 particles of wt HSV-1 and *in*1814 represent 26 and 0.1 PFU, respectively.

***in*1814 does not exhibit virion-mediated transinduction of IE genes.** The ability of Vmw65, encoded by *in*1814, to form the protein-DNA IEC and to transinduce expression from transfected IE promoters was investigated, since both of these properties were disrupted in pMC1.in14 (1).

Extracts of wt HSV-1, *in*1814, and 1814R virions were prepared and analyzed by SDS-PAGE (Fig. 4). The levels of Vmw65 in these extracts were very similar, and the slightly increased molecular weight of the mutant polypeptide due to the four-amino-acid insertion was apparent (lane 2). The virion extracts were incubated with HeLa cell nuclear extract and a 74-bp DNA fragment containing the TAAT GAGAT sequence motif of IE gene 4/5. As shown in Fig. 5, the IEC was readily detected with extracts of wt HSV-1 (lane 2) and 1814R (lane 4) virions but not with extracts of *in*1814 (lane 3) or when no virion extract was present (lane 1). This result demonstrates that Vmw65 specified by *in*1814 is not capable of binding the cellular proteins required for IEC formation because of the mutation in the viral polypeptide.

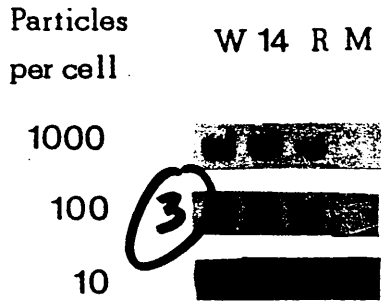

FIG. 3. DNA migration to the nucleus. DNA isolated from nuclei of cells infected with wt HSV-1 (W), *in*1814 (14), or 1814R (R) or mock infected (M), in the presence of CH, was cleaved with *Bam*HI, and the fragments were separated on an agarose gel, transferred to Genescreen Plus membrane, and probed with radiolabeled pTK1. The portion of the blot representing *Bam*HI *p* is presented. Exposure times were 0.4 h (1,000 particles per cell), 4 h (100 particles per cell), or 40 h (10 particles per cell).

The ability of *in*1814 to transinduce IE gene expression was investigated by comparing the level of activation from a transfected IE promoter in the presence or absence of superinfecting virus (Fig. 6). BHK cells were transfected with pIE3CAT and infected with 1,000 particles of wt HSV-1, *in*1814, or 1814R per cell. An increase of approximately sixfold in chloramphenicol acetyltransferase activity was observed when cells were superinfected with wt HSV-1 (lane 2) or 1814R (lane 4), but infection with *in*1814 (lane 3)

FIG. 4. Proteins extracted from virions of wt HSV-1 (lane 1), *in*1814 (lane 2), and 1814R (lane 3) and used as a source of Vmw65 for gel retardation analysis. The gel was stained with Coomassie brilliant blue.

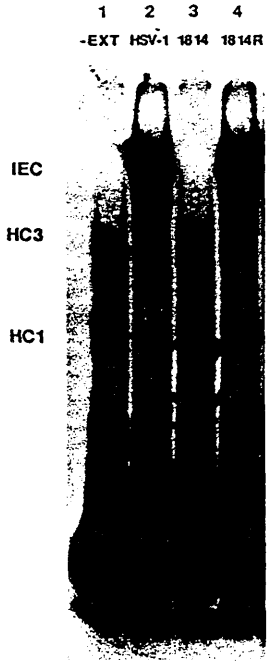

FIG. 5. IEC formation by Vmw65. A 74-bp DNA fragment containing the IE gene 4/5 TAATGAGAT sequence was incubated with HeLa cell nuclear extract (EXT) alone (lane 1) or with virion extract from wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and was analyzed by gel electrophoresis. The positions of IEC and the cell-specific complexes HC1 and HC3 (49) are indicated.

gave no stimulation over the level in mock-infected cells (lane 1).

Taken together, these results confirm that the properties of the mutant Vmw65 polypeptide in the viral context reflect the observations and expectations implicit in the initial characterization of the mutation in cloned copies of the gene; that is, the mutation in *in*1814 disables the ability of the virus to direct the formation of IEC and consequently abolishes its transducing activity.

Gene expression in *in*1814-infected cells. It would be expected that the abolition of transinduction by Vmw65 would affect the expression of viral genes, especially IE genes. The accumulation of IE RNA was quantitated by hybridization by using IE gene-specific probes. BHK cells were infected in the presence of CH with 1,000 particles of wt HSV-1, *in*1814, or 1814R per cell for 4 h, and cytoplasmic RNA was applied

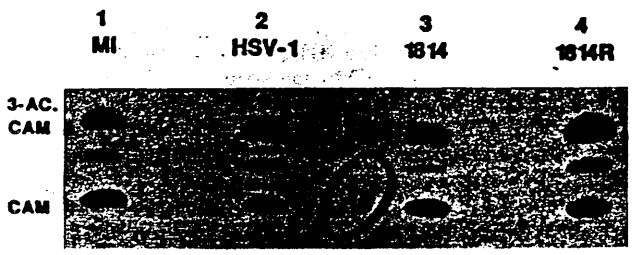

FIG. 6. Transinduction of IE transcription. Cells were transfected with pIE3CAT and mock infected (lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4). Chloramphenicol acetyltransferase assays were carried out on cytoplasmic cell extracts. The positions of chloramphenicol (CAM) and 3-acetyl chloramphenicol (3-AC. CAM) are shown.

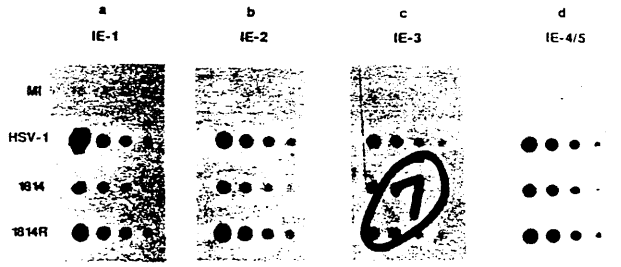

FIG. 7. Production of IE RNA. Cells were mock infected (MI; row 1) or infected with wt HSV-1 (row 2), *in*1814 (row 3), or 1814R (row 4) in the presence of CH. RNA was prepared after 4 h and applied to nitrocellulose filters in four sequential dilutions (3 μg, 1 μg, 0.3 μg, and 0.1 μg). Filters were separately hybridized with ^{32}P-labeled DNA probes corresponding to IE genes 1, 2, 3, and 4/5 (panels a, b, c, and d, respectively).

to nitrocellulose and separately hybridized with radiolabeled DNA fragments corresponding to the IE-1, IE-2, IE-3, or IE-4 genes. The levels of IE-1- and IE-2-specific RNA, as determined by densitometric analysis, were reduced four- to fivefold in *in*1814-infected cells compared with wt HSV-1- and 1814R-infected cells (Fig. 7, a and b), whereas the reduction in IE-4/5-specific RNA was only twofold (Fig. 7d), and no significant effect on IE-3-specific RNA was detected (Fig. 7c).

The expression of IE polypeptides was also investigated. BHK cells were infected as described above, but after 4 h CH was washed from the cells and polypeptides were radiolabeled in the presence of actinomycin D and separated by SDS-PAGE (Fig. 8). Densitometric analysis was used to

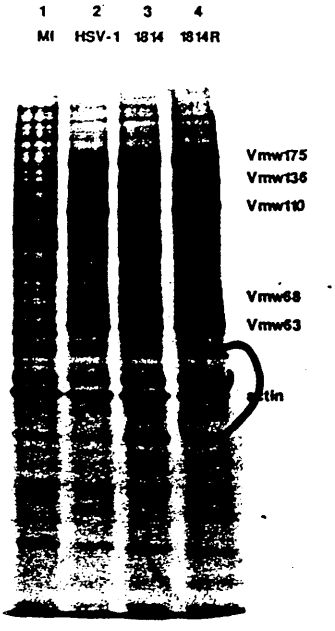

FIG. 8. IE polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4) in the presence of CH. Proteins were labeled with [^{35}S]methionine after removal of CH by washing at 4 h postinfection. The positions of viral IE polypeptides and cellular actin are indicated.

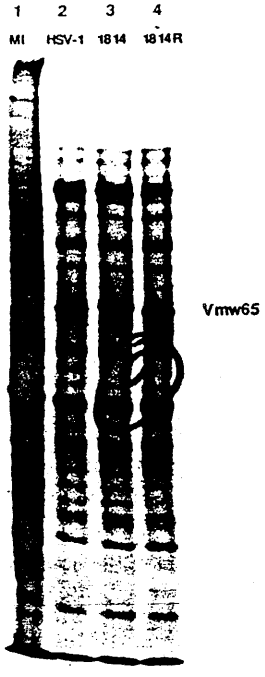

FIG. 9. Late polypeptide synthesis. Cells were mock infected (MI; lane 1) or infected with wt HSV-1 (lane 2), *in*1814 (lane 3), or 1814R (lane 4), and proteins were labeled with [^{35}S]methionine at 8 h postinfection. The position of Vmw65 is indicated.

determine the relative rates of synthesis of individual IE polypeptides, and the values were normalized to that of actin. The rates of synthesis of Vmw110 and Vmw63, the products of IE genes 1 and 2, respectively, were reduced four- to fivefold in *in*1814-infected cells, whereas the rates of synthesis of Vmw175, the product of IE gene 3, were equivalent for the three viruses. It was not possible to measure accurately the rate of synthesis of Vmw68, the product of IE gene 4, since this polypeptide ran as a diffuse band. It is clear, however, that the data on IE RNA levels and IE protein synthesis rates are in good agreement and show that the expression of IE genes 1 and 2 is significantly reduced in *in*1814-infected cells but that the expression of IE gene 3 is essentially unaffected.

Protein synthesis at 8 h postinfection, a time when early and, especially, late polypeptides are synthesized, was also examined (Fig. 9). The profiles of wt HSV-1, *in*1814-, and 1814R-infected cells were very similar, the increased molecular weight of Vmw65 specified by *in*1814 being the only major difference.

Thus, upon infection of BHK cells with 1,000 particles of *in*1814 per cell in the presence of CH, the level of expression of IE genes 1, 2, 4, and presumably 5 is reduced, but under normal conditions infection procedes to the late stage, suggesting that there is no overall consequence of reduced IE gene expression. From the high particle/PFU ratio, however, it appears that the growth of *in*1814 is inefficient when cells are infected with 1 virus particle per cell. To investigate whether the incapacity of *in*1814 at low MOI is reflected in reduced gene expression, the synthesis of TK, an early enzyme that can be detected with high sensitivity, was examined. BHK cells were infected with 1,000, 100, 10, or 1 particle of wt HSV-1, *in*1814, or 1814R per cell, and incubation was continued for 15 h in the presence of phosphonoacetic acid to prevent the secondary spread of virus. Table 2 shows the results of TK assays performed on the cell extracts. The level of TK after infection with 1,000 or 100 particles per cell was indistinguishable for wt HSV-1, *in*1814, and 1814R, reemphasizing that *in*1814 is not detectably impaired at high MOI. At 10 particles per cell, the TK level in *in*1814-infected cells relative to that in wt HSV-1-infected cells was reduced by 6-fold, and at 1 particle per cell the decrease was 30-fold. Therefore, the expression of TK (and presumably of other early and late genes) is more strictly dependent on MOI for *in*1814 than for wt HSV-1 or 1814R, and it is likely that the observed reduction in expression is large enough to account for the inefficiency of plaque formation by the mutant.

TABLE 2. TK production by wt HSV-1, *in*1814, and 1814R at high and low MOI[a]

MOI (particles/cell)	Viral TK activity (cpm/min of assay per μg of protein)			Ratio of wt HSV-1/*in*1814
	wt HSV-1	*in*1814	1814R	
1,000	2,693	2,285	2,789	1.2
100	1,988	2,549	2,478	0.8
10	935	161	792	6.0
1	100	3	63	33.3

[a] BHK cells were infected at the multiplicities indicated. Cytoplasmic extracts were diluted as necessary to ensure that TK determinations were within the linear response range of the assay. A background of 3 cpm per min of assay per μg of protein has been subtracted from all values.

Complementation of *in*1814 by Vmw110. If *in*1814 fails to form plaques at low MOI because of the reduction in IE gene expression, then complementation of this state should increase the efficiency of plaque formation and consequently the apparent titer of the mutant virus. In contrast, a compensating increase in IE gene expression would not complement *in*1814 growth if the mutant phenotype resulted from a defect at a stage before the onset of IE transcription. Two experiments were carried out to test these possibilities.

BHK cells were transfected with p111 (a plasmid encoding the HSV-1 transactivator Vmw110) or pUC9 and then used separately for titration of wt HSV-1, *in*1814, or 1814R (Table 3). Although the titers of wt HSV-1 and 1814R were constant in both cell samples, the apparent titer of *in*1814 increased approximately 10-fold on cells transfected with p111. Since only a proportion of cells (normally between 5 and 50%) in a BHK monolayer express Vmw110 after transfection of p111, it is likely that a higher level of complementation could potentially be obtained. Therefore, raising the level of Vmw110 can, at least partially, rectify the defect of *in*1814 in BHK cells.

Complementation of *in*1814 in HFL cells was achieved by infecting monolayers with UV-irradiated *ts*K, which supplied functional Vmw65 in *trans* (48), prior to titration of wt HSV-1 or *in*1814. The apparent titer of *in*1814 increased from 5.7×10^6 to 1.5×10^9 PFU/ml, whereas the titer of wt HSV-1 was 6.0×10^{10} on both cell monolayers. No plaques were

TABLE 3. Titration of wt HSV-1, *in*1814, and 1814R on BHK cells transfected with pUC9 or p111 (encoding Vmw110)

Plasmid	PFU/ml		
	wt HSV-1	*in*1814	1814R
+pUC9	1.2×10^{10}	4.0×10^6	1.7×10^9
+p111	9.5×10^9	4.2×10^7	2.2×10^9

[PFU (particles) per mouse]

TABLE 4. Virulence of wt HSV-1, *in*1814, and 1814R in mice

Virus	50% Lethal dose [PFU (particles per mouse)]	
	ip injection	ic injection
wt HSV-1	9.7×10^2 (4.0×10^4)[a]	3.1 (1.3×10^2)
*in*1814	$>7.4 \times 10^4$ ($>2.4 \times 10^8$)	$>7.4 \times 10^3$ ($>2.4 \times 10^7$)
1814R	3.0×10^3 (9.2×10^4)	19.1 (5.8×10^2)

[a] 50% lethal dose in terms of particles per mouse is shown in brackets

observed on monolayers treated only with UV-irradiated *ts*K. The titer of *in*1814 on UV-irradiated-*ts*K-treated cells represented a particle/PFU ratio of 74, and after account is taken of the fact that the MOI for UV-irradiated *ts*K was only 0.1 PFU per cell, it is clear that the efficiency of plaque formation was similar to that of wt HSV-1. Thus, the observed phenotype of *in*1814 on HFL cells was reversed by provision of Vmw65 in *trans*, arguing against a *cis*-acting defect, for example, inhibition of uncoating by the mutant protein.

***in*1814 has reduced virulence in mice.** An assessment of the in vivo properties of *in*1814 was made by studying virulence after inoculation of mice either ic or ip. The results (Table 4) show that *in*1814 was much less virulent than wt HSV-1 or 1814R, regardless of the method of inoculation. In fact, all mice challenged with *in*1814 survived, with the exception of three mice injected ic with undiluted virus. In these cases, death was atypically rapid, occurring within 12 h as opposed to the usual 3 to 5 days, and it is suspected that the effect was due to the large number of virus particles injected. The 50% lethal dose values in terms of particles per mouse, the more relevant value, show that virulence of *in*1814 was reduced by a factor of at least 3×10^3 for ip or 2.5×10^4 for ic inoculation, compared with wt HSV-1 or 1814R.

DISCUSSION

The isolation of a mutant defective in transinduction of IE transcription is a crucial step in determining the biological role of Vmw65. The 12-bp insertion mutation in *in*1814 appears to be stable, since no revertants have been detected during passage and growth of virus stocks; reversion to the phenotype of wt HSV-1 would readily be detected, as shown by the ease with which 1814R was isolated. Two features of *in*1814 are particularly noteworthy. At MOI of 100 or more particles per cell, no significant effect was observed on the overall pattern of virus gene expression, whereas at low MOI, the efficiency of plaque formation was severely reduced in a cell-dependent manner. The phenotype is similar to that exhibited by deletion mutants which do not express Vmw110 (57, 65).

Although the results presented here suggest that transinduction by Vmw65 is not essential for HSV gene expression at high MOI, this interpretation must be taken cautiously, as the assays available are of limited sensitivity. The degree of impairment of transinduction is difficult to assess because the stimulation of expression from a transfected IE promoter is only 5- to 10-fold, and thus, as argued previously (1), it is possible to state only that *in*1814 is reduced by at least 90% in its ability to stimulate IE transcription. Analysis of the ability to form IEC, as shown in Fig. 5, is more sensitive, and by this criterion *in*1814 is disabled by 99% or more. Nevertheless, each HSV particle contains approximately 1,000 molecules of Vmw65 (22), and therefore a cumulative effect of a low residual activity might be sufficient to endow *in*1814 with the ability to form plaques at the observed low efficiency.

In the absence of transinduction by Vmw65, the IE genes would be expected to be transcribed according to the inherent strengths of their promoters, a feature that is presumably determined by interaction with cellular proteins. For IE genes 1 and 2, the 4- to 5-fold reduction in RNA accumulation and protein synthesis correlates well with the 5- to 10-fold stimulation of transcription in BHK cells from transfected IE gene 1 and 2 promoters by Vmw65 (C. M. Preston, unpublished results). The expression of IE genes 3 and 4, however, is greater than would be anticipated from transfection studies, since these promoters are also activated by more than fivefold (5, 48), and it is difficult to offer an obvious explanation for this apparent discrepancy. One possibility is that the enhancer-like sequence which lies between the promoters of IE genes 3 and 4 (28, 50), rather than the TAATGARAT elements, is the major requirement for transcription of IE gene 3 in the context of the viral genome and that the strong proximal promoter suffices for IE genes 4 and 5 (48). It is also noteworthy that the four upstream nucleotides of the TAATGARAT elements which control IE genes 1 and 2 confer a strong homology to the nuclear factor III binding site, the octamer element ATGC AAAT, whereas this is not the case for the TAATGARAT elements located between IE genes 3 and 4/5. A further consideration is that the topology of the DNA template and the stoichiometric relationships between DNA and protein factors may vary considerably between transfected and infected cells. Clearly, the findings with virus-infected cells are the more relevant.

Even though *in*1814 lacks transinducing activity, the major polypeptides synthesized under IE conditions are the IE proteins. Activation by Vmw65 is therefore not a definitive characteristic of IE genes, and other features must distinguish them from early and late genes. It may be that the presence of strong promoters and enhancer-like sequences determines the relatively high efficiency of IE gene transcription in the absence of IE proteins, but equally, the TAATG ARAT or other IE-specific elements might be responsible. It is known that cellular proteins bind to various sequences in IE gene upstream regions (26, 27, 39, 58, 68), and these factors might increase the availability of IE promoters to transcription components in the absence of Vmw65. Thus, IE-specific DNA sequences, rather than Vmw65, may be the primary determinants of an IE gene.

Transinduction by Vmw65 is important for infection only at low MOI. At a superficial level, it is straightforward to view this property as a reasonable adaptation, since the initial interaction of HSV with an organism is likely to involve a small number of virus particles. The inability to replicate at low MOI appears to result from the failure to produce IE proteins at levels sufficient to initiate infection, and it is probable that the reductions in Vmw110 and Vmw63 are crucial, since these polypeptides are required for gene expression (56, 57, 65). Thus, it seems that threshold levels of IE polypeptides must be attained, and the role of Vmw65 is to ensure that such levels are reached, especially at low MOI. It is not clear whether the few cells in which infection with *in*1814 results in the formation of a plaque represent a subpopulation in a particular metabolic state or simply random variation in response to infection. Furthermore, the basis for the difference in behavior of *in*1814 in BHK and HFL cells remains undefined. It may be that IE transcription in the absence of Vmw65 is less efficient in HFL cells at low MOI, or that HFL cells are less able to compensate for low levels of IE proteins. The fact that deletion mutants in Vmw110 are also impaired for growth at low MOI and show

a relatively greater reduction in HFL cells than BHK cells (65; R. D. Everett, ~~personal communication~~) supports the latter proposal, but further work is needed to clarify this important point.

Recently, Friedman et al. have shown that a transformed cell line which expresses the protein-binding portion of Vmw65 supports virus growth poorly, presumably because the expressed protein sequesters the cell factors required to mediate transinduction (16). In essence, transinduction by Vmw65 is thought to be abrogated in the transformed cell line. The experiments dealt only with infection at low MOI (0.1 or 0.3 PFU per cell), but the results are similar to those found with *in*1814, namely, a significant reduction in the efficiency of plaque formation, inefficient virus growth, and a decrease (by 12-fold) in accumulation of IE RNA 1. From the results reported here, it is predicted that virus replication in the transformed cells should not be as severely affected at high MOI.

The avirulence of *in*1814 in mice, even after injection of high doses, demonstrates that transinduction is important for infection in vivo and emphasizes the importance of host-cell factors in the replication of *in*1814. Furthermore, Vmw65 may be a good target for the design of new antiviral agents.

It is interesting to speculate on the role of Vmw65 in HSV latency in the light of the phenotype of *in*1814, since the majority of genes, including IE genes, are silent during latency (62, 63), suggesting that an early transcriptional block may operate. One hypothesis is that Vmw65 may be lost or rendered inactive during transport of the HSV nucleocapsid from the neuronal cell surface to the nucleus (27). From the analysis of *in*1814 presented here it is possible to predict that under such circumstances, at low MOI, virus replication would not ensue, and thus latency might be established. Support for this view comes from our recent observation that noninfectious particles of *in*1814 can be retained by tissue culture cells after infection at low MOI and can subsequently be reactivated to form plaques (C. Ace and C. M. Preston, unpublished results), as found in studies with a mutant lacking Vmw110 (N. D. Stow and E. C. Stow, J. Gen. Virol., in press). Therefore, the failure to transinduce IE transcription by interference with Vmw65 function is worthy of serious consideration as a basic precondition for latency.

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The sequence of the gene for Vmw65

We present here 2609 base pairs of DNA sequence including the gene for Vmw65. The initiation site of the transcript for this gene is shown as O-----. The terminus of the mRNA is marked as -----X. Canonical sequences implicated in the control of transcription in other systems, and referred to individually in the text, are underlined. The single letter amino acid code is used to indicate the extent of the open reading frame for Vmw65. Insertion mutations are indicated.

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GAGCCCCAGCCCGCTCCGCTTCTCGCCCCAGACGGCCCGTCGAGTGAAAACTTCCGTACCCAGACAATAAAGCACCAACAGGGGTTCATTCGGTGTTGGCGTTGCGTGCCTTTG   114

TTTCCCAATCCGACGGGGACCGGGACTGGGTGGCGGGGGGTGGGTTGGACAGCCGCCCTCGGTTCGCCTTCACGTGACAGGAGCCAATGTGGGGGGAAGTCACGAGGTACGGGG   228

                                                    O--------->
CGGCCCGTGCGGGTTGCTTAAATGCGTGGTGGCGACCACGGGCTGTCATTCCTCGGGAACGGACGGGGTTCCCGCTGCCCACTTCCCCCCATAAGGTCCGTCCGGTCCTCTAAC   342

                                                                                                                    0
GCGTTTGGGGGTTTTCTCTTCCCGCGCCGTCGGGCGTCCCACACTCTCTGGGCGGGCGGGGACGATCGCATCAAAAGCCCGATATCGTCTTTCCCGTATCAACCCCACCCA   453

 M  D  L  L  V  D  E  L  F  A  D  M  N  A  D  G  A  S  P  P  P  P  R  P  A  G  G  P                                28
ATG GAC CTC TTG GTC GAC GAG CTG TTT GCC GAC ATG AAC GCG GAC GGC GCT TCG CCA CCG CCC CCC CGC CCG GCC GGG GGT CCC            537

 K  N  T  P  A  A  P  P  L  Y  A  T  G  R  L  S  Q  A  Q  L  M  P  S  P  P  M  P  V                                56
AAA AAC ACC CCG GCG GCC CCC CCG CTG TAC GCA ACG GGG CGC CTG AGC CAG GCC CAG CTC ATG CCC TCC CCA CCC ATG CCC GTC            621
                  ^in2
 P  P  A  A  L  F  N  R  L  L  D  D  L  G  F  S  A  G  P  A  L  C  T  M  L  D  T  W                                84
CCC CCC GCC GCC CTC TTT AAC CGT CTC CTC GAC GAC TTG GGC TTT AGC GCG GGC CCC GCG CTA TGT ACC ATG CTC GAT ACC TGG            705
                                                      ^in4
 N  E  D  L  F  S  A  L  P  T  N  A  D  L  Y  R  E  C  K  F  L  S  T  L  P  S  D  V                                112
AAC GAG GAT CTG TTT TCG GCG CTA CCG ACC AAC GCC GAC CTG TAC CGG GAG TGT AAA TTC CTA TCA ACG CTG CCC AGC GAT GTG            789

 V  E  W  G  D  A  Y  V  P  E  R  T  Q  I  D  I  R  A  H  G  D  V  A  F  P  T  L  P                                140
GTG GAA TGG GGG GAC GCG TAC GTC CCC GAA CGC ACC CAA ATC GAC ATT CGC GCC CAC GGC GAC GTG GCC TTC CCT ACG CTT CCG            873

 A  T  R  D  G  L  G  L  Y  Y  E  A  L  S  R  F  F  H  A  E  L  R  A  R  E  E  S  Y                                168
GCC ACC CGC GAC GGC CTC GGG CTC TAC TAC GAA GCG CTC TCT CGT TTC TTC CAC GCC GAG CTA CGG GCG CGG GAG GAG AGC TAT            957
                  ^in7
 R  T  V  L  A  N  F  C  S  A  L  Y  R  Y  L  R  A  S  V  R  Q  L  H  R  Q  A  H  M                                196
CGA ACC GTG TTG GCC AAC TTC TGC TCG GCC CTG TAC CGG TAC CTG CGC GCC AGC GTC CGG CAG CTG CAC CGC CAG GCG CAC ATG           1041
                  ^in8                ^in9
 R  G  R  D  R  D  L  G  E  M  L  R  A  T  I  A  D  R  Y  Y  R  E  T  A  R  L  A  R                                224
CGC GGA CGC GAT CGC GAC CTG GGA GAA ATG CTG CGC GCC ACG ATC GCG GAC AGG TAC TAC CGA GAG ACC GCT CGT CTG GCG CGT           1125

 V  L  F  L  H  L  Y  L  F  L  T  R  E  I  L  W  A  A  Y  A  E  Q  M  M  R  P  D  L                                252
GTT TTG TTT TTG CAT TTG TAT CTA TTT TTG ACC CGC GAG ATC CTA TGG GCC GCG TAC GCC GAG CAG ATG ATG CGG CCC GAC CTG           1209
                                                                                  ^in11
 F  D  C  L  C  C  D  L  E  S  W  R  Q  L  A  G  L  F  Q  P  F  M  F  V  N  G  A  L                                280
TTT GAC TGC CTC TGT TGC GAC CTG GAG AGC TGG CGT CAG TTG GCG GGT CTG TTC CAG CCC TTC ATG TTC GTC AAC GGA GCG CTC           1293

 T  V  R  G  V  P  I  E  A  R  R  L  R  E  L  N  H  I  R  E  H  L  N  L  P  L  V  R                                308
ACC GTC CGG GGA GTG CCA ATC GAG GCC CGC CGG CTG CGG GAG CTA AAC CAC ATT CGC GAG CAC CTT AAC CTC CCG CTG GTG CGC           1377
                        ^in12
 S  A  A  T  E  E  P  G  A  P  L  T  T  P  P  T  L  H  G  N  Q  A  R  A  S  G  Y  F                                336
AGC GCG GCT ACG GAG GAG CCA GGG GCG CCG TTG ACG ACC CCT CCC ACC CTG CAT GGC AAC CAG GCC CGC GCC TCT GGG TAC TTT           1461

 M  V  L  I  R  A  K  L  D  S  Y  S  S  F  T  T  S  P  S  E  A  V  M  R  E  H  A  Y                                364
ATG GTG TTG ATT CGG GCG AAG TTG GAC TCG TAT TCC AGC TTC ACG ACC TCG CCC TCC GAG GCG GTC ATG CGG GAA CAC GCG TAC           1545

 S  R  A  P  T  K  N  N  Y  G  S  T  I  E  G  L  L  D  L  P  D  D  D  A  P  E  E  A                                392
AGC CGC GCG CGT ACG AAA AAC AAT TAC GGG TCT ACC ATC GAG GGC CTG CTC GAT CTC CCG GAC GAC GAC GCC CCC GAA GAG GCG           1629
                              ^in14
 G  L  A  A  P  R  L  S  F  L  P  A  G  H  T  R  R  L  S  T  A  P  P  T  D  V  S  L                                420
GGG CTG GCG GCT CCG CGC CTG TCC TTT CTC CCC GCG GGA CAC ACG CGC AGA CTG TCG ACG GCC CCC CCG ACC GAT GTC AGC CTG           1713
                                                                  ^in15
 G  D  E  L  H  L  D  G  E  D  V  A  M  A  H  A  D  A  L  D  D  F  D  L  D  M  L  G                                448
GGG GAC GAG CTC CAC TTA GAC GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG           1797

 D  G  D  S  P  G  P  G  F  T  P  H  D  S  A  P  Y  G  A  L  D  M  A  D  F  E  F  E                                476
GAC GGG GAT TCC CCG GGG CCG GGA TTT ACC CCC CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT GAG           1881
                                                                  ^in17
 Q  M  F  T  D  A  L  G  I  D  E  Y  G  G  -                                                                        491
CAG ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGG TAG GGGGCGCGACCGGACCCGCATCCCCCGTCTGGGTTTTCCCCTCCCGTCACCGGT   1980
                                                --------X
TCGTATCCACAATAAACACGAGCACATACATTACAAAACCTGCGGTTGTCGTCTGATTATTTGGTGGTGGGGGAAAGAACTAGCCAGGAGACGGGACCGCGCAACCAACCCACT   2094

GGGGTCTGGGTTGCCGGCGTGTGTGTTAGCCGCGTCTGCGGGCCTGTCGTGTAGATTCGAAACCACGGACGGGTGATTGTGTCGCAGGGCGGCCCGCGTATAAAGGCGAGAGCG   2208

CGGGACCGTTTCCGCATTTGGCCGGGGGCTGGGGCGGCGGGTAGCCTTCGCGGGAGATACTGCGTTTTTTTGCGCCGGCCCCGTCGCTCCCGTCCATTCCCATCGCGAGGGGGT   2322

CCGGCGGCACCTACCCCGGCCTCCATCCGCGCTGTGGGGTCTTTTTCTTTTTTGGGGGGTAGCGGACATCCGATAACCCGCGTCTATCGCCACCATGTCGGCTCGCGAACCCGC   2436

GGGGCGCAGGAGGCGCGCATCCACCCGCCCCCGCGCCTCGCCCGTGGCGGACGAGCCAGCGGGCGATGGGGTGGGGTTCATGGGGTACCTGCGTGCGGTGTTCCGCGGGGATGA   2550

CGACAGCGAGCTAGAGGCTCTGGAGGAGATGGCGGGCGACGAGCCGCCCGTGCGCCGTC                                                          2609
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