A Functional Analysis of the Product of Varicella-Zoster Virus Gene 62

by

Graham Harvey Disney

A Thesis Presented For the Degree of Doctor of Philosophy

in the

Faculty of Science, University of Glasgow

Institute Of Virology,
University of Glasgow

December, 1990

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements

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

My project supervisor, Dr. Roger Everett, deserves particular mention, both for his tireless encouragement, advice and criticism throughout my studies, and for his prompt and thorough proof-reading of this thesis. I am grateful to other members of the scientific and support staff, especially the media, cytology and washroom services, Mr Jim Aitken for performing virus particle counts, Dr. John McLauchlan for synthesis of oligonucleotides, and Philip Taylor for his patient assistance with all aspects of computing.

My enjoyment of life outside the Institute owes much to Rikki and Rab, with whom I have had many good times. For the memorable days spent hillwalking (of which there were not enough) I would like to acknowledge John, who also appreciated the finer points of Theakstons ale. Finally, I am grateful to Joe for going to see all the bands, theatre productions and films that nobody else would consider, and to D.J. Caroline (our very own Queen Latifah) for the many Unity Reggae nights which will be sadly missed.

I am indebted to my parents, Colin and Barbara, and sister Philippa, for their moral and financial support through the years.

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

SUMMARY

In contrast to the increasingly sophisticated knowledge of herpes simplex virus type 1 (HSV-1), our understanding of varicella-zoster virus (VZV) gene regulation is at an early stage. VZV gene 62 is one of a number of VZV genes which, on the basis of homology with known HSV-1 regulatory genes, are predicted to encode gene regulatory polypeptides. The work presented in this thesis investigated the properties and functions of the product of VZV gene 62.

VZV gene 62 encodes a polypeptide with a predicted molecular weight of 140,000 (VZV 140k) which shares considerable predicted amino acid sequence homology with the HSV-1 polypeptide, Vmw175 (or ICP4), encoded by IE gene 3. Vmw175 is an essential viral polypeptide with a pivotal role in the activation of early and late viral gene expression, and also repression of immediate early gene expression. There is compelling evidence that VZV 140k is a functional counterpart of Vmw175, and as such may have a critical role in the regulation of VZV gene expression. This hypothesis was tested by inserting VZV gene 62 coding sequences (expressed from the HSV-1 IE-3 promoter) into both IE-3 gene loci in the short region repeats of the HSV-1 genome. The parent virus used for this manipulation was D30EBA, which is a variant of HSV-1 from which the majority of the Vmw175 coding sequences have been deleted. Like other HSV-1 viruses lacking Vmw175 function, D30EBA is able to grow only in cell lines which express Vmw175 constitutively. Significantly, the resulting recombinant virus, HSV-140, is able to propagate (but unable to form obvious plaques) on normal cell lines. The properties of HSV-140 were studied in comparison to HSV-1 by monitoring the time-course of polypeptide expression and DNA replication during normal infection. It was found that HSV-140 synthesises apparently normal amounts of many viral polypeptides during a high multiplicity infection, although the expression of certain late polypeptides (for example, Vmw65, and the major capsid protein) is reduced; this slight defect may be related to the decreased efficiency of

HSV-140 DNA replication. At low multiplicity HSV-140 expressed viral polypeptides inefficiently. Surprisingly, the VZV 140K polypeptide was produced in large amounts at later times of a normal infection, indicating that VZV 140k fails to repress the IE-3 promoter. Finally, infection of cells in the presence of actinomycin-D revealed that HSV-140 is much less efficient than wild type virus in the shut-off of host protein synthesis, suggesting that HSV-140 presents reduced amounts of the virion-associated host shut-off factor (encoded by gene UL41). These results indicate that VZV 140K is able to perform most of the functions of Vmw175 during growth of HSV-1, but that differences in detail lead to less efficient virus growth.

The properties of VZV 140k have also been studied in transient transfection assays. It was of particular interest to determine whether VZV 140k was able to repress expression from its own promoter (that of gene 62). To this end plasmid p140CAT was constructed in which VZV gene 62 upstream sequences from -1146 to +57 (which include the complete promoter) were linked to the coding sequences of the chloramphenicol acetyl transferase (CAT) gene. Co-transfection experiments utilizing p140CAT demonstrated that VZV 140k strongly represses expression from its own promoter, thus establishing further functional homology between the polypeptides. Interestingly, Vmw175 is competent in repression of the VZV gene 62 promoter whereas VZV 140k was unable to repress the HSV-1 IE-3 promoter in the reciprocal experiment. The failure of VZV 140k to repress the IE-3 promoter is consistent with observations made with HSV-140 (see above). Plasmids expressing mutant forms of Vmw175 which have small, in-frame insertions into region 2 of the polypeptide (e.g. pI13) are defective in repression of the IE-3 promoter. Similarly, co-transfection of pI13 fails to repress expression of p140CAT. The sequences comprising region 2 of Vmw175 and VZV 140k are among the most highly conserved between the polypeptides. Notably, an analogous plasmid to pI13 which expresses a mutant form of VZV 140k containing an in-frame insertion into homology region 2 (pC34) is likewise impaired in its ability to repress expression of p140CAT. These results

strongly suggest that repression of the VZV gene 62 promoter by VZV 140k and Vmw175 involves a similar mechanism to that of repression of the HSV-1 IE-3 promoter by Vmw175. That is, both polypeptides may effect repression of gene 62 by binding to specific sequences at or near the gene 62 capsite. Furthermore, pC34, like pI13 and other region 2 insertion mutants of Vmw175, is severely impaired in its ability to *trans*-activate the HSV-1 glycoprotein D promoter in transfection assays. Thus the mechanism of *trans*-activation might also be conserved between the polypeptides.

Vmw175 binds to DNA sequences containing the consensus, ATCGTC, present in some, but not all HSV-1 promoters. The transfection experiments described above suggest that VZV 140k may bind to specific sequences in the vicinity of the VZV gene 62 capsite, and thus it was of interest to investigate the DNA binding properties of the polypeptide. VZV 140k was found to bind non-specifically to calf-thymus DNA. In order to determine whether VZV 140k is able to bind to specific sequences in DNA, gel retardation assays were performed with nuclear extracts prepared from cells transfected with a plasmid expressing VZV 140k, or from cells infected with VZV or HSV-140, and a panel of probes representing the entire VZV gene 62 promoter-leader region. These analyses did not detect sequence-specific DNA binding by VZV 140k. This is discussed in relation to the mechanism whereby VZV 140k might represses VZV gene 62.

ABBREVIATIONS

A	adenine
Ac	acetate
Amp	Ampicillin
APS	ammonium persulphate
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BAP	bacterial alkaline phosphatase
BHK	baby hamster kidney cells
bp	base pair
BSA	bovine serum albumin
C	cytosine
^{14}C	carbon-14 radioisotope
cav	cell-associated virus
CAT	chloramphenicol acetyl transferase
CHX	cycloheximide
Ci	Curie(s)
CMV	cytomegalovirus
cpe	cytopathic effect
cpm	counts per minute
crv	cell released virus
CS	calf serum
CTP	cytidine-5'-triphosphate
Da	Daltons
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DBP	DNA binding protein
del	deletion
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
E	early (gene)
EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
EDTA	sodium ethylenediamine tetra-acetic acid

EHV	equine herpesvirus
EtBr	ethidium bromide
FCS	foetal calf serum
G	guanine
g	grams
gD	glycoprotein D
GMEM	Glasgow modification of Eagle's medium
gp	glycoprotein
h	hour(s)
HCMV	human cytomeglovirus
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSV	herpes simplex virus
Hz	hydrazine
IE	immediate early (gene)
IEC	immediate early complex (on TAATGARAT elements)
IPTG	isopropyl-D-thiogalactoside
IR	internal repeat
k	kilodalton
kb	kilobase
l	litre
L	late (gene)
LFP	large (Klenow) fragment of DNA polymerase
M	molar
min	minute(s)
ml	millilitre
mM	millimolar
moi	multiplicity of infection
MOPS	3-(N-morpholine)propanesulphonic acid
mRNA	messenger ribonucleic acid
M_r	molecular weight
N	unspecified nucleotide or amino acid
n	nano
NBC	newborn calf serum
NE	nuclear extract
ng	nanogram
NPT	non-permissive temperature
NP40	Nonidet p40
OD	optical density
ORF	open reading frame

ori	origin of DNA replication
^{32}P	Phosphorous-32 radioisotope
p.a.	post-absorption
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
PMSF	phenylmethylsulphonyl fluoride
poly(A)	polyadenylic acid
p	pellet
PRV	pseudorabies virus
PT	permissive temperature
R	purine moiety
RE	restriction enzyme
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
sec	seconds
syn	syncytial plaque morphology locus (*syn*$^{+}$ = non-syncytial, *syn*$^{-}$ = syncytial)
SV40	simian virus 40
T	thymidine
TEMED	n,n,n',n'-tetramethylethylene diamine
TK	thymidine kinase
TLC	thin layer chromatography
TR	terminal repeat
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
ts	temperature sensitive
TS	thymidylate synthetase
UAS	upstream activating sequences
UPE	upstream promoter element
UV	ultraviolet
V	volt
Vmw	apparent molecular weight of virus-induced protein
vol	volume(s)
v/v	volume/volume

VZV	varicella-zoster virus
wt	wild type
w/v	weight/volume
Y	pyrimidine moiety

Greek symbols

α	alpha
β	beta
μ	micro

Amino acid symbols

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

(The three letter code abbreviations are underlined)

CONTENTS

1. INTRODUCTION

VZV gene 62 encodes a polypeptide with a predicted molecular weight of 140,000 (VZV 140k) which shares extensive predicted amino acid sequence homology with the immediate early gene regulatory polypeptide, Vmw175, of HSV-1. The research presented in this thesis investigated the properties and functions of VZV 140k with a view to obtaining a better understanding of its role in the VZV life cycle. This introduction, therefore, includes an overview of the biology and molecular biology of VZV, and aims to review the current knowledge of gene regulation in the alphaherpesvirinae (of which VZV is a member), with particular emphasis on HSV-1 and the role and functions of Vmw175. In addition, since viral immediate early polypeptides regulate gene expression through interactions with the eukaryotic transcriptional machinery, relevant aspects of eukaryotic gene expression will be discussed.

1A. THE BIOLOGY OF VZV

1A.1. Definition and Classification of the Herpesviridae

The family herpesviridae is comprised of more than 80 distinct members which infect a wide range of higher eukaryotic hosts (Roizman and Batterson, 1985). Members of the herpesviridae are characterized by the inclusion of a linear double-stranded DNA genome in an enveloped virion particle 150-200nm in diameter. The herpesvirion is composed of four distinct structural elements:

i) The core is an electron dense structure within the capsid which contains the genomic DNA (Epstein, 1962; Furlong *et al.*, 1972; Nazerian, 1974).

ii) The capsid is icosahedral in structure, 100-110nm in diameter, and is composed of 150 hexameric and 12 pentameric capsomeres (Wildy *et al.*, 1960).

iii) The tegument is an ill-defined proteinaceous structure located between the capsid and the envelope (Roizman and Furlong, 1974).

iv) The envelope surrounding the capsid and tegument is

a tri-laminar membrane containing numerous virus-encoded protein spikes approximately 8nm in length (Wildy *et al.*, 1960; Wildy and Watson, 1963; Spear and Roizman, 1972). Asher *et al.* (1969) proposed that the envelope was derived from budding through the host cells nuclear membrane, but recent electron microscopic data suggests that the mature virus particle acquires its envelope by budding into cytoplasmic vesicles (Roffman *et al.*, 1990).

The herpesviruses as a family exhibit a diverse range of biological properties. However, common to all herpesviruses is the capacity to persist in a latent state in the infected host.

Herpesviridae have been classified into three subfamilies (alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae) on the basis of their biological properties including host range, length of reproductive cycle, cytopathology, and characteristics of latent infection (Matthews, 1982; Roizman, 1982). The herpesviruses have also been classified into five groups (A through E) based on the arrangement of the characteristic reiterated DNA sequences within their genomes (Roizman *et al.*, 1981).

Members of the alphaherpesvirinae are characterized by a short reproductive cycle, typically less than 24h in tissue culture, and capacity to establish latency, primarily in ganglia. This subfamily contains herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), pseudorabies virus (PRV), varicella-zoster virus (VZV), and equine herpes virus type 1 (EHV-1).

Betaherpesvirinae have a restricted host range, long reproductive cycles, and infection progresses slowly in tissue culture resulting in enlargement of infected cells (cytomegalia). Latency can be established in a number of tissues. This subfamily contains the cytomegaloviruses, including human cytomegalovirus (HCMV).

The gammaherpesvirinae, of which Epstein-Barr virus (EBV) is a member, have a restricted host range and

replicate in lymphoblastoid cell lines in tissue culture. Some members undergo lytic infections in epithelial and fibroblastic cells. Latent infections are frequently established in lymphoid tissues.

It should be noted that classification on the basis of biological properties alone may not always accurately reflect phylogenetic relationships. Human herpesvirus 6 (HHV-6), for example, was provisionally classified as a gammaherpesvirus since its tissue tropism closely mirrors that of gammaherpesviruses, and it shares some reiterated sequences with Marek's disease virus (Kishi *et al.*, 1988; Lopez *et al.*, 1988). Recently, however, more extensive sequence analysis of HHV-6 has revealed that it is in fact more closely related to HCMV than to EBV, HSV, or VZV, which suggests that it is a representative of a novel subgroup of the betaherpesvirus subfamily (Lawrence *et al.*, 1990; Martin *et al.*, and Dambaugh *et al.*, XVth International Herpesvirus workshop). It is clearly possible that a virus genetically related to one subfamily might, through having lost or gained non-homologous functions, have acquired a similar phenotype to viruses of another subfamily. Thus, there is no necessary contradiction in a virus (such as HHV-6) that may be a valid member of the biologically defined gammaherpesvirinae, having a betaherpesvirus as its closest relative in a molecular phylogeny.

Finally, Frenkel *et al.* (1990) have reported the isolation of a seventh human herpesvirus from CD4$^+$ T-cells of a healthy individual. Hybridization studies indicate limited homology of the new virus, designated HHV-7, with HHV-6 and HCMV. Analysis of the DNA sequence of HHV-7 will be required to determine the precise relationship of this new virus to previously defined human herpesviruses.

1A.2. Human Herpesviruses and Disease

The human herpesviruses are associated with a number of diseases.

HSV infection is widespread in the human

population. Following primary infection, which is often subclinical, HSV can establish a latent infection in the neurons of sensory ganglia or brain tissue (Baringer and Swoveland, 1973; Fraser *et al.*, 1981). Periodic reactivation may give rise to recurrent lesions at the primary site of infection or other peripheral sites. Infection with **HSV-1** is generally not serious, being primarily associated with "cold sore" lesions of the lips or eyes (herpes keratitis), although in immunocompromised hosts it can give rise to the more serious condition herpes encephalitis. **HSV-2** is generally associated with genital lesions (herpes genitalis) (Whitley, 1985).

Primary infection with VZV is usually associated with the childhood condition of chicken pox, a widespread vesicular rash present normally on the head and trunk, though mucous membranes can also be affected. Virus spread occurs by the respiratory route or through direct contact. VZV can become established in a latent state in dorsal root ganglia and the spinal column following primary infection. Reactivation from latency can lead to the painful cutaneous disease, shingles (herpes zoster) (Hope-Simpson, 1965), which occurs more commonly with advancing age. In adults and a small percentage of children, VZV infection can spread to the central nervous system (CNS) and cause the more serious conditions meningitis and encephalitis (Kennedy, 1987).

HCMV infects the majority of the human population. Although congenital infection is sometimes associated with neurological damage and mental retardation (Alford and Britt, 1984), infection is generally asymptomatic or causes only benign disease. Latent virus can be reactivated as a result of immunosuppression, particularly in transplant or blood transfusion patients (Ho, 1982). Outbreaks of HCMV infection can also occur in patients with acquired immunodeficiency syndrome (AIDS).

HHV-6 was first isolated from patients with lymphoproliferative disorders. The virus is typically acquired in early infancy and can cause the condition

exanthum subitum in a proportion of infected children (Yamanishi *et al.*, 1988).

Primary infection with EBV is often asymptomatic, though with increasing age the symptoms become increasingly severe resulting in infectious mononucleosis among adolescents. EBV is also associated with the development of two tumours, Burkitt's lymphoma and Nasopharyngeal carcinoma, though its precise role in these conditions is not clear (Neiderman *et al.*, 1976; Miller, 1985).

1A.3. BIOLOGY AND PATHOLOGY OF VZV INFECTIONS

As noted above, VZV is associated with two diseases in humans, chickenpox or varicella, and herpes zoster. The biology and pathology of these two diseases will be discussed in more detail.

Almost all persons in industrial society living within the temperate climate zone contract varicella, usually during childhood, whereas the incidence of herpes zoster is generally much lower. Historically, herpes zoster was recognized as a distinct clinical entity early in the medical literature. Varicella, however, was frequently confused with small pox, and it was not until the late eighteenth century that Heberden finally distinguished the two conditions (Gordon, 1962). That chickenpox and herpes zoster were associated with the same infectious agent was first proposed in 1888 by von Bokay who reported an occurence of chickenpox following exposure to zoster in a family setting (Gelb, 1990). Final definitive proof of this association was obtained when virus from both chickenpox and zoster vesicle fluid was isolated and propagated in tissue culture, and the recovered viruses shown to be identical (Weller *et al.*, 1958; Weller and Witton, 1958).

3.1. Varicella

Much of the knowledge of varicella pathogenesis is derived from comparison with other viral exanthems since the clinical disease is quite benign, and no suitable experimental models are available (Gelb, 1990). Fenner

speculated that the pathogenesis of VZV would follow the dual viremic model demonstrated for mousepox (Fenner, 1948), and clinical and experimental data has subsequently confirmed this (Grose, 1981).

The characteristic rash of varicella typically develops following an incubation period of 14 or 15 days from the primary infection. The virus appears to enter via the mucosa of the upper respiratory tract and oropharynx, or via the conjuctiva. Viral replication initiates at the site of inoculation giving rise to a primary viremia after 4-5 days. During primary viremia virus disseminates via the bloodstream and lymphatic system and is taken up by cells of the reticuloendothelial system, where it undergoes multiple rounds of replication. The host's immune response limits viral replication in the early stages of infection, but is soon inundated whereupon a more extensive secondary viremia develops. This results in release of virus into the bloodstream in higher titre, which then rapidly invades cutaneous tissue, culminating in the formation of the characteristic cutaneous lesions. Lesions continue to appear in crops over a period of 2-4 days which probably reflects a cyclic viremia. In addition, lesions are also found on all mucosal surfaces, including the respiratory and gastrointestinal tracts. Termination of the viremic phase usually occurs within three days of the appearance of lesions as a result of specific humoral and cell-mediated responses (in the immunocompetent host) (Asano *et al.*, 1985; Arvin *et al.*,1986).

Complications of varicella result from widespread dissemination of virus and the failure to limit replication. The most common serious complication is varicella pneumonia which is generally seen in adults (Guess *et al.*, 1986). Neurological complications are most often observed in children aged 5-14 (Guess *et al.*, 1986), the most common presentation being acute cerebellar ataxia. The most severe complications occur in immunosuppressed patients, particularly leukemics, but also in patients with other malignancies receiving immunosuppressive

chemotherapy. The persistent viremia in such cases leads to extensive invasion of the lungs, liver, and CNS, with fatal cases having varicella pneumonia and often encephalitis.

3.2. Herpes zoster

The pathogenesis of herpes zoster is poorly understood. The current model, proposed originally by Hope-Simpson in 1965, was derived largely from clinical and epidemiological data, and by analogy with recurrent HSV-1 infections. It is thought that following primary infection, VZV passes from the skin, via the sensory nerve fibres to the sensory ganglia, whereupon a latent infection is established. Reactivation from this quiescent state is considered to be the primary cause of herpes zoster, though exposure to exogenous varicella doubtless accounts for a minority of cases (particularly in immunosuppressed individuals) (Berlin and Campbell, 1970). The extensive viral replication in the ganglia which follows reactivation is associated with neuronal necrosis and inflamation, often accompanied by neuralgia. These events precede the development of cutaneous lesions.

The most common complication of herpes zoster is postherpetic neuralgia. As with varicella, the most severe complications of herpes zoster are associated with patients with immune defects, malignancies, and those on immune suppression therapy. Pneumonia in such patients accounts a significant number of mortalities. Persistent infection of the CNS also occurs and is associated with two distinct forms of encephalitis.

Comparisons between the characteristic distribution of zoster lesions and the architecture of the nervous system, together with histopathological evidence, implicated ganglia as the site of VZV latency (Head and Campbell, 1900; Hope-Simpson, 1965). In support of this view, both VZV DNA and RNA have been detected in human sensory ganglia obtained at autopsy from individuals with no recent history or clinical signs of herpes zoster (Gilden *et al.*, 1983; Hyman *et al.*, 1983). The site of

latency remains uncertain, however, with both neuronal (Gilden *et al.*, 1983: Hyman *et al.*, 1983) and non-neuronal cells (Croen *et al.*, 1988) having been suggested. HSV-1 establishes a latent infection in sensory ganglia from which it can be recovered by explantation and cultivation of the ganglia (Stevens and Cook, 1971); similar attempts to recover VZV from ganglia obtained at autopsy have thus far failed. However, Straus *et al.* (1984) employed restriction endonuclease analysis to demonstrate that a VZV isolate recovered from a patient with zoster was identical to that recovered from his primary varicella, thus inferring that zoster was a consequence of reactivation of virus which had established a latent infection following varicella.

The mechanisms underlying the maintenance of, and reactivation from latency are unclear. Hope-Simpson (1965) speculated that small subclinical reactivations occur periodically, but are contained by the immune system, thus serving to boost immunity to VZV, as well as marking the reactivation event. Asymptomatic rises in titre of antibodies to VZV consistent with such reactivations have been noted (Luby *et al.*, 1987). In this model, clinical disease is thought to arise when the host's immune defences deteriorate such that VZV replication is no longer contained. The original model considered antibody levels to be of prime importance in containment, although more recent data suggests that cell mediated immunity is in fact more important (Dolin *et al.*, 1978; Burke *et al.*, 1982).

Analysis of transcription during VZV latency has revealed that several areas of the latent genome are transcriptionally active (Croen *et al.*, 1988). This is in marked contrast to the situation with HSV-1 where a number of groups have detected only one family of transcripts during latency, the so called latency associated transcripts (or LATS) (Stevens *et al.*, 1987; Croen *et al.*, 1987; Deatly *et al.*, 1987; Spivack and Fraser, 1987; Rock *et al.*, 1987; Wagner *et al.*. 1988).

An animal model would clearly be useful in the

study of VZV latency. Attempts to develop such a model have been hampered by the fact that VZV is highly species-specific. Infection of both the guinea pig and common marmoset with VZV has been achieved, although the resulting clinical disease differs from that in humans, and latency has yet to be demonstrated (Myers *et al.*, 1980; Provost *et al.*, 1987). A latent simian varicella virus (SVV, the simian counterpart of VZV) infection has been established in african green monkeys in the absence of clinical disease (Mahalingam *et al.*, XVth International Herpesvirus Workshop), and although SVV and VZV are clearly different viruses, this system may nevertheless be useful as an animal model of VZV latency.

1A.4. Growth of VZV *in vitro*

The inability to isolate stable, high titer stocks of cell-free VZV has significantly impaired investigation of this important virus.

Weller *et al.* (1958) first noted that very little, if any infectious virus was released into the medium when VZV was propagated in tissue culture. In fact, infectious virus remains highly cell associated in all the cell types which are able to support VZV infection *in vitro*. Consequently, virus has routinely been propagated using infected cells as the inoculum. The infectivity of such cells is critically dependent upon their continued viability (Gold, 1965).

Cell-free virus can be obtained by sonic disruption of infected cells, although initially this produced stocks of very low titre (Caunt, 1963; Caunt and Taylor-Robinson, 1964). Schmidt and Lennette (1976) subsequently optimized the conditions for isolating cell-free virus and obtained yields of up to 10^6 pfu/ml, although this is at best only 10-fold greater than the initial inoculum. Furthermore, infection with cell-free virus is apparently an inefficient process and proceeds much more slowly than if infected cells are used as the inoculum (Schmidt and Lennette, 1976). Hence, the only advantages offered by cell-free VZV

are its increased stability during storage (relative to infected cells) and its ability to produce quantitatively controlled, synchronous infections *in vitro*.

The low infectivity of VZV in tissue culture was originally thought to reflect a defect in virions assembled *in vitro* (Achong and Meurise, 1968). Cook and Stevens (1968, 1970), however, suggested that the lack of infectivity was due to lability of the virion coat during its transit through the cytoplasm. Gershon *et al.* (1973) reported that VZV virions were packaged into vesicles which later coalesced to form larger vacuoles. Interestingly, they found acid phosphatase activity, a lysosomal marker, associated with these vesicles, which led them to speculate that the co-localization of VZV in vacuoles with lysosomal enzymes might be responsible for inactivation of the virus during egress from the cell. This same group went on to study this further with some particularly interesting findings (Gabel *et al.*, 1988). It had previously been demonstrated that an uncommon oligosaccharide component of lysosomal enzymes containing mannose 6-phosphate (Man 6-P) specifies their targetting to the lysosomal compartment (Kaplan *et al.*, 1977; Ullrich *et al.*, 1978; Von Figura and Weber, 1978). Significantly, both the immature and mature forms of glycoprotein gp3 (now gpII) were found to contain at least one Man 6-P, and may thus target VZV to lysosomes in an analogous fashion. These same Man 6-P residues may also facilitate entry of VZV into cells via Man 6-P receptors in the plasma membrane (Gabel *et al.*, 1988).

1A.5. THE VIRAL GENOME

5.1. Structure

In 1964 two groups reported that the growth of VZV in tissue culture was inhibited by 5-iodo-2-deoxyuridine, a compound known to inhibit DNA, but not RNA, containing viruses (Rapp and Vanderslice, 1964; Rawls *et al.*, 1964). Attempts to characterize the VZV genome were initially hampered by difficulties associated with isolation and purification of viral DNA. Nevertheless, Ludwig *et al.*

(1972) succeeded in purifying sufficient viral DNA to determine its G+C content as 46%. Dumas *et al.* (1980) subsequently determined the molecular weight of VZV DNA to be $80x10^6$ Daltons by electron microscopy measurements, and went on to report the first restriction enzyme maps and structure of the genome (Dumas *et al.*, 1981). The linear duplex DNA molecule was shown to comprise two covalently linked segments, L and S, L consisting of a unique sequence (U_L), and S of a unique sequence (U_S) bounded by inverted repeat sequences (IR_S and TR_S). VZV virion DNA was found to contain equimolar amounts of two arrangements of the genome resulting from inversion of S relative to L. A combination of restriction mapping, molecular cloning, and electron microscopy studies of self annealed single stranded virion DNA molecules confirmed and extended this model (Straus *et al.*, 1981, 1982; Ecker and Hyman, 1982; Gilden *et al.*, 1982; Davison and Scott, 1983; Mishra *et al.*, 1984). It was not until the sequence of the genome termini and the L-S joint region were determined that a small inverted repeat (IR_L and TR_L) of 88.5 bp flanking U_L was discovered (Davison, 1984). This work also established that the VZV genome is not terminally redundant (in contrast to HSV-1, Davison and Wilkie, 1981), and possesses an unpaired C residue at the 3' end of L, and an unpaired G residue at the 3' end of S. Furthermore, 2-5% of virions were found to have genomes in which L was inverted relative to S. This was subsequently confirmed by others (Kinchington *et al.*, 1985; Ruyechan *et al.*, 1985). This contrasts with HSV, where both the L and S segments invert relative to one another with equal frequency giving rise to equal amounts of the four genome isomers (Roizman, 1979). Hayakawa and Hyman (1987) showed that HSV superinfection of VZV infected cells did not increase inversion of L relative to S, and the infrequency of this event has yet to be explained. It was also proposed that the genome might occasionally circularize, though the significance of this is not clear (Straus *et al.*, 1981; Kinchington *et al.*, 1985). The DNA sequence of one isolate of varicella-zoster virus has been

determined (Davison and Scott, 1986). The total genome contained 124884 bp, with U_L containing 104836 bp, TR_L and IR_L 88.5 bp each, U_S 5232 bp, and TR_S and IR_S 7319.5 bp each.

Restriction enzyme analysis of a number of VZV isolates has demonstrated that the genome is relatively stable when passaged *in vitro* (Zweerink *et al.*, 1981), although small but significant variations exist between different isolates (Richards *et al.*, 1979; Martin *et al.*, 1982; Straus *et al.*, 1983; Hayakawa *et al.*, 1986). Straus *et al.* (1983) mapped regions of the genome exhibiting size variability between different virus isolates, and these correspond approximately to the locations of reiterated sequences (R2, R3, and R4) in the sequence of Davison and Scott (1986). More detailed analysis of variations in these regions has been performed. Casey *et al.* (1985) have reported variability in the copy number of the 27 bp element in the R4 reiteration (within IR_S/TR_S) between virus isolates. Similar variations in the 42 bp element of the R2 reiteration within gene 14 have been reported (Kinchington *et al.*, 1986). More recently, variations in the complex element, R1, within the gene 11 orf have been reported (Kinoshita *et al.*, 1988); the variations were all multiples of 3 bp, thus maintaining the translational reading frame.

5.2. Molecular Biology

Prior to the determination of the complete VZV DNA sequence, studies to identify virus-specific polypeptides and functions had adopted two approaches. The first of these, analysis of polypeptides in the VZV virion, was impeded by difficulties associated with virion purification. The second approach, analysis of viral polypeptides induced in VZV-infected cells, encountered problems of detection due to the high background of host cell proteins. Nevertheless, several virus-induced enzyme activities were demonstrated: Doberson *et al.* (1976) reported pyrimidine deoxyribonucleoside kinase (thymidine

kinase) activity in VZV infected cells; DNA polymerase activity was identified, purified and characterized (Miller and Rapp, 1977; Mar *et al.*, 1978); Cheng *et al.* (1979) confirmed the induction of a thymidine kinase activity, and also demonstrated a DNAse activity. Several groups reported SDS-PAGE analysis of polypeptides in partially purified virions (Wolff, 1978; Shemer *et al.*, 1980; Shiraki *et al.*, 1982). The results of these studies were largely in agreement and identified between 31 and 33 polypeptides, at least five of which were glycoproteins. Asano and Takahashi (1979) detected 33 virus induced polypeptides in VZV-infected cells by SDS-PAGE, including 13 glycoprotein species. The glycoproteins of VZV have been characterized by their reactivity with monoclonal antibodies into three major families (Davison *et al.*, 1986).

Analysis of the complete VZV DNA sequence (Davison and Scott, 1986) identified 71 genes predicted to encode polypeptides. Of these, 64 are single copy genes present in either U_L or U_S, and three are diploid genes, one copy being present in each of the repeat sequences, IR_S and TR_S. For a summary of the VZV gene layout, see Figure 1. Similarities in gene layout between VZV and HSV-1 permitted the assignation of functions to a number of the VZV genes. Additionally, genes 13 and 66 were predicted to encode a thymidylate synthetase and protein kinase, respectively, on the basis of their having homology with polypeptides in the NBRF database known to possess these functions. This information, together with functions subsequently assigned to a further eight genes, is summarized in Table 1. Smith and Smith (1989) found a number of sequence motifs characteristic of protein kinases in the predicted polypeptide product of gene 47. The predicted kinase is probably of the serine-threonine type. Genes encoding a uracil-DNA glycosylase were recently identified in both HSV-1 and HSV-2 (Worrad and Carradona, 1988; Mullaney *et al.*, 1989), and significant homology between the HSV-1 protein and the product of VZV gene 59 was noted (Mullaney *et al.*, 1989). Challberg (1986) identified a minimum of five

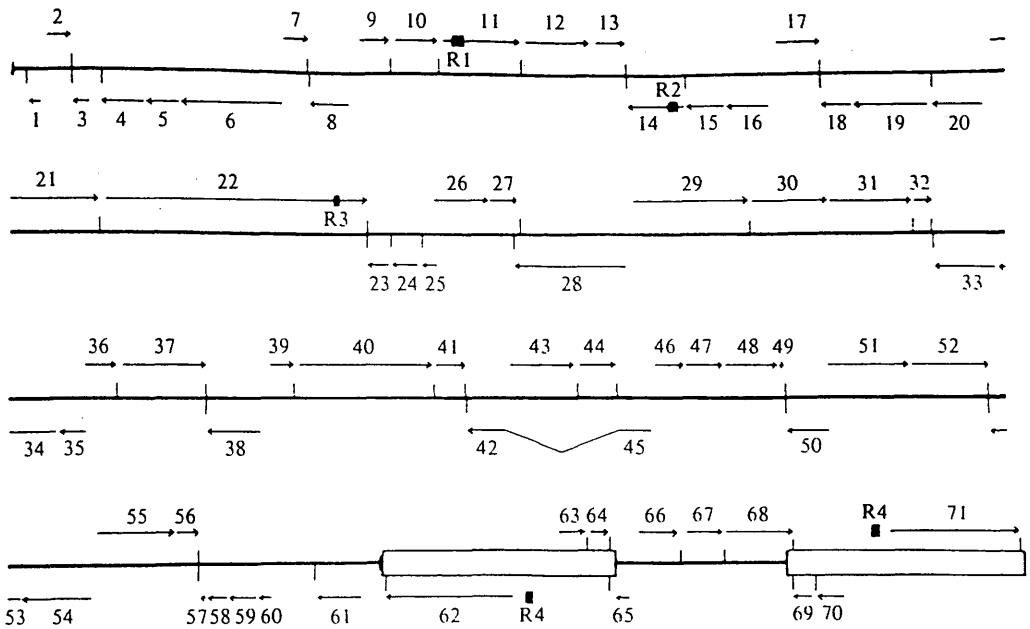


Figure 1. Summary of the VZV gene layout. The genome is represented as in four sections, heavy horizontal lines indicating the unique regions (U_L and U_S), the two large open rectangles depicting the inverted repeats flanking U_S (TR_S and IR_S), and the two heavy short vertical lines denoting the inverted repeats flanking U_L (IR_L and TR_L). ORFs 1 to 71 are illustrated as arrows against the appropriate strand, and correspond to the protein coding sequences shown in Table 1. Light vertical lines indicate potential polyadenylation sites in the appropriate strand. The locations of four reiterations (R1 to R4) are denoted by filled rectangles; R1, R2 and R3 are in the coding regions of ORFs 11, 14 and 22 respectively.

Reproduced from Davison and Scott, 1986.

Table 1

Gene[1]	Codons	Mol. wt.[2]	Function or properties[3]
1	108	12103	
2	238	25983	
3	179	19149	
4	452	51540	*trans*-activator-homologue of HSV-1 Vmw63
5	340	38575	possible membrane protein
6	1083	122541	component of DNA helicase-primase complex
7	259	28245	
8	396	44816	deoxyuridine triphosphatase
9	302	32845	
10	410	46573	possible tegument protein
11	819	91825	
12	661	74269	
13	301	34531	thymidylate synthetase
14	560	61350	predicted membrane glycoprotein-(gpV)
15	406	44522	
16	408	46087	associated with DNA polymerase
17	455	51365	virion host shut-off protein
18	306	35395	ribonucleotide reductase small subunit
19	775	86823	ribonucleotide reductase large subunit
20	483	53969	capsid protein
21	1038	115774	
22	2763	306325	tegument protein
23	235	24416	
24	269	30451	possible virion protein
25	156	17460	
26	585	65692	probable virion protein
27	333	38234	
28	1194	134041	DNA polymerase
29	1204	132133	single stranded DNA binding protein
30	770	86968	probable virion protein
31	868	98062	membrane glycoprotein (gpII)
32	143	15980	
33	605	66043	required for DNA packaging
34	579	65182	virion protein
35	258	28973	
36	341	37815	pyrimidine deoxyribonucleoside kinase
37	841	93646	membrane glycoprotein (gpIII)
38	541	60395	
39	240	27078	
40	1396	154971	major capsid protein
41	316	34387	capsid protein
42+45	747	82752 (spliced)	
43	676	73905	
44	363	40243	
46	199	22544	
47	510	54347	probable protein kinase
48	551	61268	exonuclease

49	81	8907	myristylated virion protein
50	435	48669	
51	835	94370	oris-binding protein
52	771	86346	component of DNA helicase-primase complex
53	331	37417	
54	769	86776	virion protein
55	881	98844	component of DNA helicase-primase complex
56	244	27166	
57	71	8079	
58	221	25093	
59	305	34375	uracil-DNA glycosylase
60	159	17616	
61	467	50913	possible transcriptional *trans*-repressor
62/71	1310	139989	*trans*-activator-homologue of HSV-1 Vmw175
63/70	278	30494	homologue of HSV-1 IE polypeptide-Vmw68
64/69	180	19868	virion protein
65	102	11436	tegument protein
66	393	43677	protein kinase
67	354	39362	membrane glycoprotein (gpIV)
68	623	69953	membrane glycoprotein (gpI)

1. Data taken from Davison and Scott, 1986.

2. All predicted molecular weight values, except those of the primary translation products of genes 14, 31 and 68, were calculated from the amino sequence commencing at the first in-frame ATG in the ORF.

3. References to appropriate DNA sequence data which confirm the assignment of VZV gene functions are largely given in Davison and Scott, 1986. Functions characterized since then are referenced in the text or in Davison, 1991.

This Table is adapted from an original Table in Davison (1991), with permission.

HSV-1 genes which, in combination with DNA polymerase and the major DNA binding protein, are sufficient for HSV origin directed DNA replication. On the basis of homology with these HSV-1 genes, five VZV genes with a potential role in VZV DNA replication have been identified. Finally, a weak but significant homology between the gene 61 polypeptide sequence and the HSV-1 IE protein, Vmw110 (Perry *et al.*, 1986), has been identified. Thus the elucidation of the complete VZV DNA sequence has significantly increased our knowledge of the potential functions of VZV. The products of the relevant genes can now be studied in a variety of expression and transfection systems largely circumventing the hitherto encountered problems of growing VZV *in vitro*.

5.3. Transcript Mapping

Two groups have reported attempts to map viral transcripts isolated from infected cells. Maguire and Hyman (1986) analysed polyadenylated cytoplasmic transcripts, and identified between 41 and 67 transcripts. In a similar study, VZV-infected total cellular RNA was analysed and a total of 77 RNAs identified and mapped (Ostrove *et al.*, 1985; Reinhold *et al.*, 1988). Due to a lack of resolution in these studies, it was not possible to assign unequivocally the majority of the transcripts to a specific reading frame, although a 4.3 kb transcript was mapped to gene 62. In addition, Sawyer *et al.* (1986) have mapped a 1.8 kb transcript to a region of the genome between 0.52 and 0.54 map units which contains the thymidine kinase gene, gene 36. The unambiguous mapping of remaining transcripts will require determination of the precise location of their 5' and 3' ends.

5.4. Mapping of Polypeptides to Specific Genes

A number of polypeptides have now been mapped to specific genes. The best characterized in this respect are the VZV glycoproteins, all of those identified to date having been mapped. The most abundant of the glycoproteins

are those of the gpI family. The gpI gene has been mapped to gene 68 (Ellis *et al.*, 1985), which has limited sequence homology with HSV-1 gE. The gpII gene has been mapped to gene 31 in the centre of U_L (Keller *et al.*, 1986) which has significant homology with HSV-1 gB. However, in contrast to gB, the mature gpII glycoproteins appear to be disulfide linked dimers (Montalvo and Grose, 1987). The gpIII glycoprotein has been purified and its N-terminal sequence determined. This information was used to design a family of degenerate oligonucleotides which were used to map the gpIII gene to gene 37 (Keller *et al.*, 1987). The gene for the gpIV (minor) family of glycoproteins has been mapped to gene 67 which displays limited homology to the HSV-1 U_S7 gene (Davison *et al.*, 1985). The VZV sequence contains an additional gene predicted to encode a polypeptide product (designated gpV) with homology to HSV-1 gC, though as yet a fifth serologically distinct glycoprotein species has not been detected.

That VZV encodes a thymidylate synthetase (TS) was confirmed when a plasmid expressing the predicted 8 gene product was shown to complement the growth of a strain of *E.coli* from which the endogenous TS had been deleted (Thompson *et al.*, 1987). The only other herpesvirus shown to specify a TS to date is herpesvirus saimiri (Honess *et al.*, 1986). Another enzyme involved in nucleotide metabolism, pyrimidine deoxyribonucleoside kinase, has been mapped to the viral genome between 0.52 and 0.54, and presumably corresponds to the predicted TK gene, gene 36 (Sawyer *et al.*, 1986). A DNA-binding protein has been detected in VZV-infected cells (Kinchington *et al.*, 1988). These workers utilized an antiserum directed against a synthetic oligopeptide representing 12 amino acids from the C-terminus of the predicted product of gene 29, which encodes a homologue of HSV-1 Vmw130, the major DNA-binding protein. The properties of the VZV protein were similar to those of its HSV-1 counterpart. Investigation of the potential functions of some of the predicted VZV IE genes has begun and is described in Section 1B.4.

1.B. GENE REGULATION IN THE ALPHAHERPESVIRINAE

This section will review the current knowledge of gene regulation in the alphaherpesvirinae, with particular emphasis on HSV-1.

1B.1. Regulation of Herpes Simplex Virus Gene Expression: An Overview

Analysis of viral polypeptide expression during HSV-1 infection of tissue culture cells in the presence of inhibitors of protein synthesis or DNA replication identified three broad, coordinately regulated, sequentially expressed groups of polypeptides (Honess and Roizman, 1974). The first of these, the immediate-early (IE) or alpha polypeptides, are detected as early as 1h pi, and comprise most of the viral gene regulatory functions. The second group, the early (E) or beta polypeptides, are maximally synthesised from 2-7h pi, and at decreasing rates thereafter. Polypeptides involved in viral DNA replication are of the E class. Synthesis of the late (L) or gamma polypeptides is first detected following the onset of DNA replication at around 2h pi, and continues at increasing rates late into infection. This group comprises many of the virus structural polypeptides.

The IE genes are defined as those which are transcribed by the unmodified host RNA polymerase in the absence of *de novo* protein synthesis (Honess and Roizman, 1974; Clements *et al.*, 1977; Costanzo *et al.*, 1977; Jones and Roizman, 1979). Functional IE gene products are absolutely required for the transition to E and L gene expression (Preston, 1979a, Watson and Clements, 1980). The E genes are those whose expression follows that of the IE gene products, but precedes DNA replication. The expression of both IE and E gene products is a prerequisite for L gene expression which, in addition, requires ongoing DNA replication for maximum efficiency (Wagner *et al.*, 1972; Ward and Stevens, 1975; Jones and Roizman, 1979; Holland *et al.*, 1980; Pedersden *et al.*, 1981; Conley *et al.*, 1981). Two subclasses of L genes have been defined

according to their sensitivity to inhibitors of viral DNA replication. Thus expression of the "leaky" late or beta-gamma (gamma 1) genes is reduced, but still clearly detectable, when DNA replication is inhibited, whereas expression of the "true" late or gamma (gamma 2) genes is dramatically reduced under these conditions (Powell *et al.*, 1975; Pereira *et al.*, 1977; Pedersden *et al.*, 1981).

1B.2. Control of HSV Gene Expression Involves Both Transcriptional and Post-Transcriptional Mechanisms

Synthesis of viral transcripts is also temporally regulated, and IE, E and L RNA species have been defined (Clements *et al.*, 1977; Jones and Roizman, 1979). Several groups have analysed the accumulation of specific transcripts and their protein products (Zhang and Wagner, 1987; Smith and Sandri-Goldin, 1988), from which they concluded that transcription is the major control mechanism in the temporal regulation of viral genes. Post-transcriptional mechanisms have also been implicated (Weinheimer and McKnight, 1987). Intriguingly, when DNA synthesis was inhibited, the rate of transcription of the gC gene (a true L gene) was indistinguishable from that of E genes. However, under these conditions E transcripts accumulated, whereas, in marked contrast, little or no accumulation of L mRNA was observed (Weinheimer and McKnight, 1987). It would then appear that the interplay of several regulatory mechanisms controls herpesvirus gene expression.

Finally, Silver and Roizman (1985) reported that a true late promoter (that of the UL42 gene) was regulated as an early promoter when incorporated into the cellular genome, suggesting that the environment of the viral genome may also influence gene regulation.

1B.3. REGULATION OF IMMEDIATE-EARLY, EARLY AND LATE GENE EXPRESSION

This section will describe the *cis*-acting sequences and *trans*-acting factors involved in the regulation of the various classes of HSV genes.

3.1. Immediate-Early (IE) Gene Regulation

(a) *Cis*-Acting Elements

A number of functionally distinct elements have been defined within IE promoters. A minimal promoter region of low constitutive activity contains the TATA element which is required for both basal and induced levels of transcription, and which ensures the fidelity of transcription initiation. Upstream of this minimal promoter is a regulatory region which comprises multiple *cis*-acting elements involved in both constitutive activity and IE gene specific induction.

Post *et al.* (1981) were the first group to demonstrate that IE gene regulation was determined by specific promoter sequences. They replaced the TK gene (an E gene) promoter with the IE gene 3 promoter, and showed that the resulting chimeric gene was regulated as an IE gene when present in either the viral or cellular genome. All HSV-1 IE genes have since been shown to contain an upstream regulatory region capable of conferring IE regulation on heterologous genes (Mackem and Roizman, 1982 a,b,c; Cordingley *et al.*, 1983; Kristie and Roizman, 1984; Preston *et al.*, 1984; Mosca *et al.*, 1985). Sequence comparisons between the upstream regions of the IE genes revealed that they all contain at least one copy of an A+T rich sequence element of consensus, TAATGARAT (where R is a purine), and it was proposed that this element might mediate the coordinate induction of IE promoters during virus infection. Gaffney *et al.* (1985) subsequently established that the TAATGARAT element was of itself sufficient for IE gene activation. The viral function responsible for IE gene induction was mapped to a genome fragment encoding Vmw65, a component of the virion tegument

(Campbell *et al.*, 1984). The functions of Vmw65 and its role in IE gene regulation are discussed in detail in Section 3.1.(b).

The IE gene 3 upstream promoter region, as well as conferring IE regulation, was shown to have many of the properties of an enhancer. It functioned in either orientation, exhibited cell-type specificity, and retained function when moved over 1000bp upstream of the TATA box (Lang *et al.*, 1984; Preston and Tannahill, 1984). However, it did not function well when located downstream of the TATA box. The sequences mediating induction by Vmw65 are distinct from those containing enhancer activity (Bzik and Preston, 1986).

A detailed analysis of the IE gene 3 upstream regulatory region identified multiple distinct *cis*-acting sequence elements. Enhancer activity was found to reside in three separable motifs; two of these contained GC boxes (Sp1 binding sites), and the third a GA element (Bzik and Preston, 1986). The three elements were able to function independently, and their effects in combination were approximately additive. Sp1, a cellular transcription factor, has been shown to bind the GC boxes in the IE3 promoter and activate IE3 gene expression *in vitro* (Jones and Tjian, 1985). Further functional complexity has been revealed in this region. Thus although the TAATGARAT element alone is sufficient to mediate induction by Vmw65, the GA element upstream of IE genes 2, 3 and 4/5, which does not of itself confer responsiveness to Vmw65, appears to be necessary for maximum activity of TAATGARAT (Bzik and Preston, 1986; Triezenberg *et al.*, 1988b; LaMarco and McKnight, 1989). What is more, the GC boxes also appear to influence induction by Vmw65 (Preston *et al.*, 1984). Therefore, the efficiency with which the TAATGARAT element mediates IE gene induction by Vmw65 is apparently modulated by the elements of the enhancer.

A homology to the octamer motif overlaps many TAATGARAT elements (O'Hare and Goding, 1988), and has been shown to bind Oct-1 (OTF-1, NFIII) (Pruijn *et al.*, 1986;

Fletcher *et al.*, 1987; Gerster and Roeder, 1988); the relevance of this to IE gene regulation is discussed in the next section.

(b) *Trans*-Acting Factors

Following the observation that IE gene induction was not dependent on *de novo* protein synthesis, it was proposed that induction is mediated by a virion component (Post *et al.*, 1981). Studies with a *ts* mutant, *ts*B7, which was able to penetrate cells but failed to uncoat and release viral DNA, led to a similar conclusion (Knipe *et al.*, 1981; Batterson and Roizman, 1983). The virion function was subsequently identified as Vmw65, the product of gene UL48 (Campbell *et al.*, 1984). The sequence of UL48 has been determined (Dalrymple *et al.*, 1985; Pellet *et al.*, 1985).

As discussed above, the TAATGARAT sequence element mediates induction of IE genes by Vmw65. That Vmw65 has a very low intrinsic affinity for DNA (Marsden *et al.*, 1987), suggested that it does not interact directly with the TAATGARAT *cis*-element. It has since been shown that Vmw65 induces formation of a specific complex (designated immediate-early complex or IEC) on TAATGARAT elements, but only in the presence of host cell factors (McKnight *et al.*, 1987; O'Hare and Goding, 1988; Preston *et al.*, 1988). Competition experiments suggested that the cellular factor, Oct-1, might be an essential component of IEC (O'Hare and Goding, 1988), and this has been confirmed by Gerster and Roeder (1988) using purified Oct-1. There is additional evidence that at least one other factor, possibly interacting with the GARAT portion of TAATGARAT, may be required for IEC formation (Gerster and Roeder, 1988; Kristie *et al.*, 1989).

In a series of elegant experiments, Stern *et al.* (1989) demonstrated that the ability of Oct-1 to form IEC with Vmw65 is dependent upon the integrity of residues within a helix-turn-helix motif of the Oct-1 homeodomain. This region of Oct-1 is closely related to a

helix-turn-helix motif in the bacterial lambda repressor which is involved simultaneously in DNA binding and protein-protein interactions. Moreover, they present evidence that the low efficiency with which Oct-2 forms IEC (Gerster and Roeder, 1988) reflects amino acid changes (compared to Oct-1) in at most seven of 60 residues in the corresponding homeodomain of Oct-2. The Oct-1 protein appears to have flexible sequence recognition characteristics inasmuch as it can bind both the octamer sequence, ATGCTAAT, and TAATGARAT motifs independently (ApRhys *et al.*, 1989).

Finally, a cellular polypeptide has been purified which binds to the GA *cis*-element of IE genes, and to related elements in other viral and interferon (IFN) inducible genes (LaMarco and McKnight, 1989). This is a particularly interesting finding since IFN treatment has been shown to inhibit IE gene expression during HSV-1 infection (Mittnacht *et al.*, 1988). LaMarco and McKnight (1989) present results of transfection experiments which show that IFN treatment reduces IE promoter activity to a similar extent as mutations within the GA element. They speculate that a fuller understanding of the function of their purified factor, especially its role, if any, in IFN induction of gene expression, may lead to a model for repression of IE gene expression by IFN.

(c) Immediate-Early Complex (IEC) Formation and IE Gene Induction

Mutational analysis of both the TAATGARAT element and of Vmw65 has defined the role of IEC formation in IE gene induction. O'Hare *et al.* (1988) demonstrated that IEC formation is a prerequisite for IE gene induction. They found that mutations within the GARAT portion of TAATGARAT did not affect Oct-1 binding, but abolished IEC formation and induction. Thus Oct-1 binding is not of itself sufficient for Vmw65 induction, and it appears that an octamer motif (or near relative) with appropriate flanking sequences is required. Studies by Kemp and Latchman

(1988a) support this view. Several mutants of Vmw65 have been described which fail to form IEC, and in agreement with the above results, they fail to stimulate transcription (Ace *et al.*, 1988). Another class of Vmw65 mutants have been characterized which fail to *trans*-induce IE genes, but are able to form IEC (Triezenberg *et al.*, 1988a; Greaves and O'Hare, 1989, 1990). Thus, while IEC formation is clearly necessary, it is not of itself sufficient for Vmw65 *trans*-induction.

(d) Mutational Analysis of Vmw65

An activation domain has been defined in the C-terminus of the Vmw65 polypeptide. Deletion mutagenesis identified sequences composed of a high proportion of acidic residues (the so called "acid tail") which were dispensable for IEC formation, but absolutely required for *trans*-induction (Triezenberg *et al.*, 1988a; Greaves and O'Hare, 1989, 1990). Furthermore, when appended to the GAL4 DNA binding domain, the acid tail of Vmw65 strongly activates transcription from promoters bearing GAL4 recognition sequences, and thus represents a separable activation domain (Sadowski *et al.*, 1988, Chasman *et al.*, 1989; Cousens *et al.*, 1989). The acidic nature of the Vmw65 activation domain is strikingly reminiscent of the activation domains of a number of prokaryotic and eukaryotic activators including GAL4 and GCN4 of *Saccharomyces cerevisiae* (Ma and Ptashne, 1987 a,b; Hope and Struhl, 1986; Hope *et al.*, 1988). This class of activators are thought to activate transcription through interaction with RNA polymerase or the TATA box factor, TFIID (Horikoshi *et al.*, 1988a; Ptashne, 1988). Vmw65 might then activate transcription by interacting with these same components of the transcription machinery. Consistent with this view, the acid tail of Vmw65 has now been shown to bind directly and specifically to TFIID *in vitro* (Stringer *et al.*, 1990). Significantly, mutations in this region of Vmw65 which inactivate the polypeptide *in vivo*, eliminate TFIID binding *in vitro*. It has been proposed that complex

formation between Oct-1 and Vmw65 apposes the latter's acidic tail with PolII or TFIID, resulting in activation (Stern *et al.*, 1989).

Thus Vmw65 appears to have two separable functional domains, one of which is involved in protein-protein interactions with Oct-1 (and possibly other factors) to form IEC, while the other is an activation domain which interacts with the transcription machinery when Vmw65 is present in IEC.

The HSV-2 mutant, *ts*2203, which has a lesion in Vmw65, is impaired in virion assembly, and thus Vmw65 is an essential structural polypeptide (Ace *et al.*, 1988). The structural requirements of Vmw65 for virion assembly differ from those for *trans*-induction (Ace *et al.*, 1988).

(e) The Biological Role of Vmw65

The role of Vmw65 in the viral life cycle has been investigated by constructing a virus, *in*1814, in which a 12bp insertion in Vmw65 eliminates its ability to form IEC and thus *trans*-induce IE gene expression (Ace *et al.*, 1989). Although not essential for virus growth in tissue culture during infection at high multiplicity, at lower multiplicities replication was severely impaired. Furthermore, the mutant was avirulent *in vivo*. Vmw65 appears then to be required in situations where infection involves very few particles per cell. This is reminiscent of the phenotype of other HSV-1 mutants which have deletions removing both copies of the IE1 gene (encodes Vmw110) (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989). Thus Vmw65 and Vmw110 might serve to commit the virus to a lytic infection, and perhaps the role of Vmw65 is to ensure efficient expression of Vmw110.

3.2. Autoregulation of IE Gene Expression

Following the isolation of a number of HSV-1 mutants with *ts* lesions in Vmw175, all of which overproduce IE polypeptides at the NPT, it was concluded that Vmw175 was involved in repression of IE gene expression (Preston,

1979; Dixon and Schaffer, 1980; Watson and Clements, 1980). The role of Vmw175 in repression has been investigated in transfection assays. Of the IE promoters, only that of IE gene 3 has unequivocally been shown to be repressed by Vmw175 (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b, 1987; Gelman and Silverstein, 1987a,b). The data for the other IE genes is less convincing. For example, the IE1 promoter is activated by low levels of Vmw175, but repressed by higher levels (Gelman and Silverstein, 1986). It should be emphasised that during a normal HSV-1 infection, only IE gene 3 RNA expression is clearly repressed at the level of transcription, and significant levels of the other IE RNAs persist late into infection (Harris-Hamilton and Bachenheimer, 1985; Weinheimer and McKnight, 1987). Furthermore, at least some of the IE polypeptides appear to accumulate as infection proceeds (Everett and Orr, 1991). Taken together, the above data suggest that possibly only IE gene 3 expression is effectively repressed during a normal virus infection. The overexpression of IE RNA and polypeptides which occurs in the absence of functional Vmw175 might simply result from increased availability (relative to a normal infection) of a limiting basic transcription factor(s) (such as TFIID) when E and L promoters are not being utilized. The functional aspects of repression by Vmw175 are discussed in Section 1C.2.4.

3.3. Early (E) and Late (L) Gene Regulation

(a) E-Gene Regulation: *Cis*-Acting Sequences

As discussed earlier (Section 1B.1), E genes are those whose expression requires the prior expression of functional IE polypeptides, but is not dependant on DNA replication. The DNA sequence requirements for basal and virus induced expression from the gD and TK promoters, both of the E class, has been studied in detail.

McKnight *et al.* (1981) created a series of 5' and 3' deletion mutations within the promoter region of a plasmid-borne TK gene. Analysis of these mutant plasmids by

microinjection into oocytes defined a minimal promoter-regulatory region between approximately -100 and -40 (relative to the transcription start site). These results were extended in the classic "linker scanning" mutational analysis of TK regulatory regions (McKnight and Kingsbury, 1982). A TATA box was identified which influenced the fidelity of transcription initiation, and the efficiency of expression was found to be governed by distal promoter elements (two GC-rich elements and a CAAT box). These results were duplicated in a HeLa cell derived *in vitro* transcription system, and in addition, the GC-rich elements shown to bind Sp1 (Jones *et al.*, 1985). The sequence requirements for efficient *trans*-activation by viral IE polypeptides were found to be largely identical to those for efficient basal expression (Eisenberg *et al.*, 1985; ElKareh *et al.*, 1985), although ElKareh *et al.* suggested that virus regulation utilized upstream sequences slightly differently. However, analysis of the linker scanning TK mutant promoters following their insertion into the normal TK locus of the viral genome did not identify any sequences specific to virus regulation; all mutations that reduced basal level promoter activity in oocytes reduced induction during infection (Coen *et al.*, 1986).

The DNA sequence requirements for basal level and regulated expression of the gD gene were defined using a series of plasmids in which the β-globin gene was expressed from portions of sequence derived from the gD promoter region. The plasmids were transfected into cells, which were subsequently infected with virus to supply IE polypeptides. Sequences located within 83 bp of the transcription start site were found to be sufficient for regulated expression (Everett, 1983). A more detailed analysis of this region identified a TATA box and two G-rich elements (Everett, 1984a). The efficiency of expression from this promoter region, activated in *cis* by the SV40 early enhancer or in *trans* by viral IE gene products, was governed by the G-rich elements, and in agreement with the analysis of TK promoter elements

discussed above, no virus specific E gene regulatory elements were identified (Everett, 1984a).

(b) L-Gene Regulation: *Cis*-Acting Sequences

The efficient expression of true L genes during virus infection requires DNA replication (see Section 1B.1). The promoter sequence requirements of two true L genes have been studied in detail.

The regulation of the late US11 gene has been reproduced in a transient expression system (Johnson and Everett, 1986a). The US11 promoter region (linked to a promoter-less β-globin gene) was cloned in a plasmid containing a functional HSV origin of DNA replication. This construct was transfected into cells which were then infected with virus to supply all IE polypeptides and functions required for DNA synthesis. Expression of the US11-β-globin chimera was activated to high levels concordant with the onset of DNA replication, mirroring the regulation of the US11 gene in the viral genome. Deletion analysis demonstrated that a TATA box/capsite region alone was sufficient for fully efficient L gene regulation when linked to a functional origin of DNA replication (Johnson and Everett, 1986b). This finding was supported by an experiment in which all distal promoter elements were removed from the gD promoter; when linked to an origin of DNA replication, this truncated promoter (with only a TATA box) was activated with true late kinetics (Johnson and Everett, 1986b).

Homa *et al.* (1986) reported essentially identical findings following deletion analysis of the gC promoter. In the context of the viral genome, sequences within -34 to +124 conferred late gene regulation on gC and on a promoter-less TK gene.

(c) The Importance of the TATA Box

Analyses of the promoter sequence requirements of several viral E and L genes for *trans*-activation by IE polypeptides during infection (discussed above) did not

identify any sequences which were specific to virus induction. Since the same sequence elements were involved in transcription in uninfected and infected cells, it was concluded that *trans*-activation by viral IE polypeptides is mediated via interaction with cellular transcription factors. Notably, Coen *et al.* (1986) found that the TATA element was quantitatively the most important sequence element for *trans*-activation. It has since been shown that the precise sequence of the TATA box can affect the efficiency of *trans*-activation, some sequences apparently being more responsive than others (Everett, 1988). Likewise, the TATA box has been implicated in *trans*-activation of the adenovirus EIB promoter (which consists of only a TATA box and Sp1 element) by the adenovirus E1a polypeptide (Wu *et al.*, 1987). In addition, analysis of *trans*-activation of the more complex *hsp70* gene promoter by E1a revealed that activation occurred through the basal transcription complex (Simon *et al.*, 1988; Williams *et al.*, 1989). Simon *et al.* demonstrated that only certain TATA box sequences were responsive to *trans*-activation by E1a.

Transcription of a limited subset of cellular genes is activated during infection (whereas a majority appear to be unaffected), and in at least some cases this requires Vmw175 (Latchman *et al.*, 1987; Kemp and Latchman, 1988b). The rabbit β-globin gene promoter has been shown to be *trans*-activated during infection of transfected cells and also of cells with integrated copies of a plasmid containing the β-globin gene (Everett and Dunlop, 1984; Everett, 1984b, 1985). Furthermore, a number of cellular genes (including β-globin) have been shown to be regulated with typical early or late kinetics when resident in the viral genome (Smiley *et al.*, 1987; Panning and Smiley, 1989). It is particularly noteworthy that a transduced rodent *aprt* gene, which lacks a TATA box (Dush *et al.*, 1986; Nalbantoglu *et al.*, 1986), was not transcribed during infection (Tackney *et al.*, 1984). These observations support the proposition that viral *trans*-activators utilize

the cellular transcription machinery, and again highlight the importance of the TATA box.

(d) *Trans*-Acting Factor Requirements

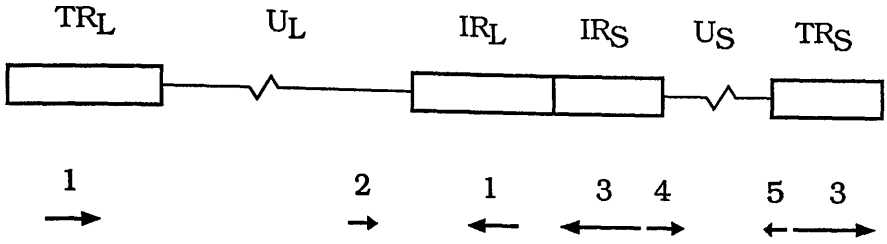
Expression of IE genes is an absolute requirement for E and L gene induction (see Section 1B.1), and this section will discuss the role of each of the IE gene products in this process. There are five IE genes in HSV-1; their approximate locations and orientations on the viral genome are shown in Figure 2, and their nomenclature and properties are summarized in Table 2.

Vmw175: The early isolation and characterization of a number of viruses with *ts* lesions in Vmw175 demonstrated that functional Vmw175 was an essential requirement for transition from IE to later classes of gene expression (Marsden *et al.*, 1976; Watson and Clements, 1978; Preston, 1979a; Dixon and Schaffer, 1980). What is more, Vmw175 function is required continuously rather than transiently since upshift to the NPT at later times in infection precludes L gene induction (Preston, 1979b; Watson and Clements, 1980). Several Vmw175 *ts* mutants have since been described which express significant amounts of many early polypeptides and synthesize near wild-type amounts of viral DNA, but underexpress or fail to express many L polypeptides (DeLuca and Schaffer, 1984; Paterson *et al.*, 1990). Vmw175 is thus directly implicated in the efficient expression of L genes. The properties and functions of Vmw175 are discussed in detail in Section 1C.

Vmw63: Vmw63 is an essential viral polypeptide. A *ts* mutant with a lesion in Vmw63 over-produces some IE and E polypeptides, yet despite synthesizing substantial amounts of viral DNA, fails to induce L gene expression (Sacks *et al.*, 1985). Mutant viruses with deletions in Vmw63 coding sequences have similar phenotypes (McCarthy *et al.*. 1989). Hence Vmw63 appears to be involved in the regulation of L genes, as well as some IE and E genes, though the mechanism by which this occurs is unclear. *Trans*-activation and *trans*-repression of viral promoters by Vmw63 in transient

Figure 2. A simplified map of the HSV-1 genome showing the positions and orientations of the IE genes. The internal and terminal repeat sequences are labelled IR and TR with the subscript denoting with which unique segment they are associated. The IE genes are labelled 1 to 5. (Not to scale.)

Table 2. Nomenclature and properties of the HSV-1 IE genes and their products. The Glasgow system of nomenclature for the HSV-1 IE genes and their products is shown with the sizes of the primary unmodified amino acid sequence deduced from the DNA sequence and the sizes of the polypeptides as estimated by SDS-polyacrylamide gel electrophoresis.



Gene	Product	Size on SDS-PAGE	Size From Sequence
IE-1	Vmw110	110kd	78452
IE-2	Vmw63	63kd	55376
IE-3	Vmw175	175kd	132835
IE-4	Vmw68	68kd	46521
IE-5	Vmw12	12kd	9792

expression assays has been reported. With one exception (Rice and Knipe, 1988), Vmw63 has not been shown to *trans*-activate any viral promoter (IE, E, or L) by itself in transient assays (Everett, 1984b; O'Hare and Hayward, 1985b; Mavromara-Nazos *et al.*, 1986), but is capable of modulating the activities of Vmw175 and Vmw110 (Everett, 1986; Sekulovich *et al.*, 1988). The effects of Vmw63 are probably confined to a restricted set of genes.

Vmw110: A number of viruses containing insertion and deletion mutations within IE gene 1 coding sequences have been constructed and characterized (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989). These studies established that viruses lacking Vmw110 function are able to grow in normal cell lines, although in certain cell types, they are impaired in plaque formation relative to wild type. This defect appears to reflect a reduction in the synthesis of viral polypeptides by mutant viruses during low multiplicity infections of less permissive cell types (Everett, 1989). These defects are overcome during infection at high multiplicity. This suggests that Vmw110 function is required during low multiplicity infections (such as those *in vivo* which, presumably, often initiate at low multiplicity) to ensure efficient expression of viral genes, thus committing the virus to a lytic infection. As such, Vmw110 might influence the outcome of infection *in vivo*, that is, whether the virus enters the lytic or latent cycle. Interestingly, viruses lacking Vmw110 fail to stimulate latent viral genomes to reactivate in an *in vitro* latency system (Russell *et al.*, 1987; Harris *et al.*, 1989). In accordance with its apparent role in modulating viral gene expression, Vmw110 activates viral promoters both by itself and in combination with Vmw175 in transient assays (Everett, 1984b; O'Hare and Hayward, 1985a,b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985, 1986).

Vmw68: A virus containing a deletion in IE gene 4 has been constructed (Post and Roizman, 1981). Characterization of this virus revealed that Vmw68 is dispensable for growth in tissue culture, although the mutant virus exhibited poor

growth in certain cell types, and at least one L gene was under-expressed (Sears *et al.*, 1985). This suggests that Vmw68, like Vmw63, might have a role in ensuring efficient L gene expression.

Vmw12: This is the least characterized of the IE polypeptides. A number of fortuitous HSV-1 and HSV-2 deletion mutants which lack IE gene 5 have been isolated. These viruses grow efficiently in tissue culture and it was concluded that Vmw12 is a non-essential polypeptide (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

(e) The Role of DNA Replication in L Gene Expression

The characterization of viruses containing lesions in the IE genes has demonstrated a requirement for Vmw175 and Vmw63 (and possibly Vmw68) in the efficient expression of L genes (see above). Nonetheless, these experiments have not defined the precise role of these IE polypeptides, inasmuch as reductions in L gene expression could result indirectly from the aberrant expression of other viral polypeptides. However, Johnson and Everett (1986a) demonstrated that abundant expression of a plasmid-borne promoter of a true-late gene during normal virus infection required DNA replication. Mavromara-Nazos and Roizman (1987) have also addressed the relative importance of *trans*-acting functions versus DNA synthesis in regulation of a L gene promoter present in the viral genome. They infected cells with a TK$^-$ virus to provide all *trans*-acting functions present during a normal infection. At early and late times following this, cells were infected with a second virus containing a chimeric TK gene under the control of an IE or L promoter, in the presence or absence of PAA. The TK gene under L control was not expressed when present in a non-replicating genome. Thus *cis*-activation by DNA synthesis, as opposed to *trans*-acting functions specified before and during DNA synthesis, is the principal requirement for efficient L gene induction.

The increase in L gene expression which follows the

onset of DNA replication is believed to be greater than can be accounted for by the increased template copy number alone (Johnson and Everett, 1986b). Rather, it is envisaged that DNA replication has a more direct effect, perhaps alleviating some constraint imposed by the secondary structure of the DNA. Interestingly, Workman *et al.* (1988) have investigated the effect of DNA structure on *trans*-activation by the PRV IE polypeptide which is closely homologous to Vmw175. Using an *in vitro* nucleosome assembly system, they demonstrated that PRV-IE was able to form a potentiated pre-initiation complex prior to, or during nucleosome assembly, but that once nucleosomes were assembled, the template remained refractory to induction by IE polypeptide. They suggested that once in this state, activation would require the destabilization of the nucleosome structure by, for example, DNA replication. Although HSV-1 genomic DNA is not thought to be assembled into a chromatin structure, that is not to say that occlusion of L promoters by other polypeptides does not occur.

1B.4 VZV GENE REGULATION

The genetic organization of VZV and HSV-1 is similar and, moreover, they appear to encode a number of closely homologous products. Consequently, it is generally assumed that the mechanisms whereby VZV gene expression is controlled will be similar to those described above for HSV-1. There is as yet little evidence to support this, however.

4.1. VZV Immediate-Early Genes

DNA sequence analysis identified four genes in VZV which are predicted to encode polypeptides with homology to HSV-1 IE polypeptides. The location and orientation of these genes is shown in Figure 1, and their known functions and properties are summarized below.

Gene 62: The product of VZV gene 62, which has a predicted molecular weight of 140,000 (VZV 140k), shares

extensive predicted amino acid sequence homology with the HSV-1 polypeptide, Vmw175 (McGeoch *et al.*, 1986), and thus it was suggested that 140k is the VZV counterpart of Vmw175. In support of this, expression of VZV 140k in a transformed cell line (F114), or from a transfected plasmid, is able to complement the growth of HSV-1 viruses with *ts* and deletion mutations in Vmw175 (Felser *et al.*, 1987, 1988). That treatment of F114 cells with cycloheximide increased accumulation of 140k mRNA indicates that 140k may, like Vmw175, autoregulate its own expression (Felser *et al.*, 1988). Consistent with its proposed role in VZV gene regulation, VZV 140k has been shown to *trans*-activate both HSV-1 and VZV promoters in transient assays (Everett, 1984b; Everett and Dunlop, 1984; Inchauspe *et al.*, 1989; Cabirac *et al.*, 1990). A structural comparison of VZV 140k and Vmw175 is shown in Figure 3.

Gene 4: Gene 4 encodes a product of predicted molecular weight 51,540 whose predicted amino acid sequence displays homology to Vmw63, the product of HSV-1 IE gene 2 (Davison and Scott, 1986; McGeoch *et al.*, 1988). Accordingly, VZV is able to complement the growth of HSV-1 viruses with *ts* lesions in Vmw63, though this is less efficient than complementation of Vmw175 mutant viruses (Felser *et al.*, 1987). The gene 4 product also activates VZV promoters in transfection assays, both alone and in synergy with VZV 140k (Inchauspe *et al.*, 1989).

Gene 61: The product of gene 61 has limited homology to the predicted product of HSV-1 IE gene 1, Vmw110 (Perry *et al.*, 1986). Sequence similarity is confined to a region with the potential to form a zinc finger structure (Berg, 1986). Interestingly, an HSV-1 virus (FXE) in which this finger region is deleted is as impaired in growth as a virus lacking the majority of the Vmw110 coding sequences (Everett, 1989). To date, the function of VZV gene 61 has not been discerned, although Inchauspe *et al.* (1989) reported preliminary results which suggest that the product of this gene can repress the VZV TK promoter, and modulate its activation by genes 4 and 62.

Gene 63: This gene encodes a product with homology to Vmw68, the product of HSV-1 IE gene 4. The function of this gene product is also unknown.

There is no direct evidence that these genes are regulated like HSV-1 IE genes during VZV infection. Two attempts to detect IE polypeptides in VZV infected cells in classic cycloheximide reversal experiments have been reported (Lopetegui *et al.*, 1985; Shiraki and Hyman, 1987). There were inconsistencies between the studies, but they indicate the presence of at least four VZV IE polypeptides. The largest of these, a phosphoprotein of M_r 185,000, was proposed to be the product of gene 62.

4.2. Regulation of VZV IE Genes

The *cis*-acting sequences and *trans*-acting factors regulating expression of VZV gene 62 have been investigated (McKee *et al.*, 1990). The VZV promoter exhibited low activity compared to the equivalent control sequences of HSV-1 or HCMV. It appears, therefore, that unlike these other viral IE promoters, the VZV IE promoter does not possess an efficient enhancer function, at least in the cell types studied. The promoter was strongly activated by the HSV-1 polypeptide Vmw65, confirming the earlier results of Felser *et al.* (1988). Within the sequences which conferred responsiveness to Vmw65 (-410 to -131), a strong homology to the HSV-1 TAATGARAT element was identified. The product of VZV gene 10, which shares homology with Vmw65 (Dalrymple *et al.*, 1985), failed to *trans*-activate the VZV promoter. This is not surprising since the VZV polypeptide failed to direct formation of an IEC complex with the TAATGARAT element and HeLa cell proteins, and furthermore, lacks the C-terminal acidic activation region that is essential for the activity of Vmw65 (Sadowski *et al.*, 1988; Triezenberg, 1988a; Greaves and O'Hare, 1989, 1990).

Interestingly then, a functional TAATGARAT element is conserved in this VZV IE promoter, yet the VZV homologue of Vmw65 is inactive. This is reminiscent of PRV which apparently does not encode a homologue of Vmw65 (Hampl *et*

al., 1984), but whose sole IE promoter contains a TAATGARAT motif and is *trans*-activated by Vmw65 (Campbell and Preston, 1987). Ace *et al.* (1989) found that during infection with HSV-1 *in*1814 (which lacks functional Vmw65) under conditions of cycloheximide reversal, the most abundantly expressed polypeptides were the IE polypeptides. They concluded that IE-specific promoter DNA sequences, possibly the TAATGARAT element itself, distinguish the kinetics of IE gene expression from those of E and L genes (rather than activation of IE promoters by Vmw65). It may be that within the natural host cells of VZV and PRV, binding of a host factor to TAATGARAT is sufficient to induce IE gene expression. Alternatively, certain cell types may contain a Vmw65 homologue. The intriguing possibility exists that differences in IE gene regulation in these alphaherpesviruses may be related to their biological properties.

4.3. Early and Late Genes?

VZV contains genes which are homologous to E and L genes of HSV-1, although as yet, no formal evidence exists that these genes are regulated with E and L kinetics (as described for HSV-1; Section 1B.1). The finding that at least two of the VZV IE polypeptides can *trans*-activate other viral promoters is clearly significant, though whether or not VZV contains E and L genes which are differentially regulated remains to be determined.

In an attempt to address this question, Asano and Takahashi (1979, 1980) analysed viral polypeptide expression during VZV infection of tissue culture cells in the presence and absence of PAA. Under conditions where DNA synthesis was inhibited, only a few of the polypeptides detected during a normal infection were observed, and it was suggested that these might represent E polypeptides. However, given the considerable difficulties associated with detection of viral polypeptides against the background of host cell polypeptides, these results should be viewed with caution. The detection of polypeptides which are of

low abundance in a normal infection might require that infection be allowed to spread, thus increasing the percentage of infected cells, with a corresponding increase in the ratio of viral to host polypeptides. Further analysis of gene expression during VZV infections will require the use of higher titre cell-free virus stocks (with which synchronous infections are possible). Since detection of viral polypeptides has previously been a limiting factor, it would undoubtedly be better to study transcript accumulation using gene specific probes which would greatly increase sensitivity. If this approach identified VZV genes which were regulated with E or L kinetics, their promoters could be analysed in transfection assays and *in vitro*. Studies of HSV-1 gene regulation have demonstrated that the results of such assays are largely consistent with observations during virus infection.

1B.5. PRV GENE REGULATION

PRV expresses a single IE RNA which is transcribed from the inverted repeat sequences of the genome (bounding U_S), and encodes a multi-functional polypeptide of M_r 180,000 (Ihara *et al.*, 1983). Molecular hybridization studies established that the sequences comprising this region of the genome are homologous to the corresponding repeat sequences of HSV-1, which contain IE gene 3 (Davison and Wilkie, 1983; Ben-Porat *et al.*, 1983). Subsequent determination of the PRV IE gene sequence has revealed extensive predicted amino acid sequence homology between PRV-IE and both Vmw175 of HSV-1, and VZV 140k (Cheung, 1989; Vlcek *et al.*, 1989).

The regulation of PRV IE polypeptide expression is discussed elsewhere (see Section 1B.4.2). The functions of PRV IE have been studied in some detail. Ihara *et al.* (1983) analysed RNA and protein synthesis during infection of cells with a PRV temperature-sensitive mutant *ts*G_1, which has a lesion in the IE polypeptide (Ben-Porat *et al.*, 1982). During infection of cells at the NPT only IE RNA was transcribed. Therefore, as with HSV-1, transcription of

later classes of PRV genes requires functional IE polypeptide, as suggested by earlier studies (Rakusanova *et al.*, 1971; Jean *et al.*, 1974; Feldman *et al.*, 1979). The IE polypeptide also represses transcription of its own RNA. Following infection at the NPT, productive infection occurred following shift-down to the PT at 3h p.i., but not at 5h p.i., when an abortive infection ensued. This aspect of the *ts*G_1 phenotype differs from that of HSV-1 mutants such as *ts*K, and suggests that overexpression of PRV IE may have a deleterious effect on macromolecular synthesis (Ihara *et al.*, 1983). It is not known whether any of the PRV genes depend for their efficient expression on DNA synthesis .

The PRV IE polypeptide stimulates transcription *in vitro* in a cell-free system (Abmayr *et al.*, 1985), indicating a direct effect of IE on the host's transcription machinery (rather than an indirect effect by, for example, increasing the amount of a limiting transcription factor). Further characterization of this system has demonstrated that PRV IE apparently activates transcription by increasing the rate or extent of recruitment of the general transcription factor, TFIID, into a pre-initiation complex at the TATA box (Abmayr *et al.*, 1988). This is discussed in more detail in Section 1D.5. PRV IE has subsequently been shown to bind, with differing affinities, to a degenerate set of DNA sequences in the adenovirus major late promoter (used in the *in vitro* transcription assay) (Cromlish *et al.*, 1989). Moreover, binding to *cis*-elements appears to be required for *trans*-activation *in vitro* since oligonucleotides homologous to the high affinity sites inhibit transcription, and binding of the *ts* IE polypeptide was not observed at the NPT (when IE fails to activate transcription)(Cromlish *et al.*, 1989).

1B.6. EHV-1 GENE REGULATION

Caughman *et al.* (1985) analysed EHV-1 polypeptide expression during infection of tissue culture cells in the absence and in the presence of metabolic inhibitors. A

total of thirty polypeptides specific to infected cells were identified. Four of these were designated "immediate early" polypeptides since they were detected in a cycloheximide reversal experiment, but not during a normal infection. The synthesis of twelve of the polypeptides was reduced in cells infected in the presence of inhibitors of viral DNA synthesis. These polypeptides were thus classified as "late" polypeptides. These findings indicate that EHV-1 polypeptide synthesis is temporally regulated in a manner similar to that demonstrated for HSV-1.

Grundy *et al.* (1989) have since reported the sequence of an EHV-1 gene which encodes a polypeptide with extensive homology to Vmw175 and the other alphaherpesvirus homologues. This polypeptide is thus implicated in the regulation of EHV-1 gene expression.

1C. THE HSV-1 IE3 GENE AND Vmw175

This thesis is concerned with the functions and properties of the VZV 140k polypeptide which is homologous to the HSV-1 IE polypeptide, Vmw175. Vmw175 has been extensively studied, and its functions and properties are discussed in this section.

Vmw175 is encoded by the diploid IE3 gene, one copy of which is present in each of the repeat sequences (R_S) bounding the short unique region (U_S) of the genome (Rixon *et al.*, 1982). The Vmw175 open reading frame is 3894 nucleotides in length and encodes a 1298 amino acid polypeptide with a predicted unmodified molecular weight of 132,835. Translation is predicted to commence at the first ATG of the transcribed region, at position 131128, and continues to the termination codon TAA at position 127234 (McGeoch *et al.*, 1986, 1988). All subsequent references to the Vmw175 orf will assume that the first base of the orf is numbered 1, proceeding through to the last base, 3894.

A full description of the *cis*-acting sequences and *trans*-acting factors involved in IE gene 3 regulation can be found in Section 1B.3.1.

1C.1. Physical Properties

1.1. Modifications

Vmw175 is a nuclear-localized phosphoprotein (Pereira *et al.*, 1977) which can be purified as a homodimer (Metzler and Wilcox, 1985).

The Vmw175 orf encodes a polypeptide of 1298 amino acids with a predicted molecular weight of 133 kDa, although migration of the polypeptide in SDS-PAGE indicates an M_r of around 175 kDa (McGeoch *et al.*, 1986). Vmw175 is phosphorylated at both serine and threonine residues (Faber and Wilcox, 1986a), and can also be poly(ADP-ribosyl)ated (Preston and Notarianni, 1983). The conserved serine-rich tract in region 1 (see Section 1C.3.4) is likely to be a major site of phosphorylation, though other sites have been proposed (DeLuca and Schaffer, 1988). Pereira *et al.* (1977) identified three forms of Vmw175 with slightly different

mobilities in SDS-PAGE, and these probably represent differentially modified forms of the polypeptide. The relative amounts of these different forms is altered in cells infected with the HSV-1 mutant, *ts*K (which has a lesion in Vmw175) at the NPT (Preston, 1979a), and, in addition, the *ts*K polypeptide does not appear to become poly(ADP-ribosyl)ated at the NPT (Preston and Notarianni, 1983). Furthermore, there is evidence that phosphate cycles on and off two of these forms during a normal infection (Wilcox *et al.*, 1980), and that the different forms bind to consensus and non-consensus Vmw175 DNA binding sites (see below) with different affinities (Michael *et al.*, 1988). Interestingly, phosphorylation has been shown to regulate the DNA-binding activity of E4F, an inducible cellular factor implicated in adenovirus EIa mediated induction of the viral E4 promoter (Raychaudhuri *et al.*, 1989). Alkaline phosphatase treatment abolished DNA binding activity of E4F, which was restored upon incubation of the inactivated factor with extract from virus infected cells. It will be interesting to determine the precise effect of phosphorylation on the DNA binding properties of Vmw175 and its role, if any, in regulation of viral gene expression. In this regard, it is interesting that Wu and Wilcox (1990) have demonstrated that the unphosphorylated Vmw175 DNA binding domain is competent in sequence-specific DNA binding. Nevertheless, phosphorylation outwith this domain might conceivably regulate DNA binding.

1.2. Nuclear Localization

Within 2-3h p.i., Vmw175 is localized to the nucleus of the infected cell, and can be found associated with cellular chromatin (Pereira *et al.*, 1977; Hay and Hay, 1980; Cabral *et al.*, 1980). At the NPT, several *ts* mutant forms of Vmw175 fail to localize to the nucleus, or exhibit altered intranuclear distribution (Preston, 1979b; Cabral *et al.*, 1980; Knipe and Smith, 1986). Surprisingly, the nuclear localization of Vmw110 and the major DNA binding protein (DBP) is apparently blocked by the presence of some, but not all *ts* alleles of Vmw175 at the NPT (Preston,

1979b; Knipe and Smith, 1986).

Immunofluorescence studies have demonstrated that Vmw175 has a diffuse nuclear distribution until the onset of DNA replication (when it may be associated with cellular chromatin), following which it becomes compartmentalized into globular foci (Randall and Dinwoodie, 1986; Knipe *et al.*, 1987). Strikingly, both major DBP and DNA polymerase are co-localized with Vmw175 in these foci or "replication compartments" (Quinlan *et al.*, 1984; Randall and Dinwoodie, 1986; Knipe *et al.*, 1987). It appears, therefore, that Vmw175 is recruited into sites of active DNA replication, and it is an intriguing possibility that the distribution of the polypeptide at early and late times may be related to the mechanisms of E and L gene *trans*-activation.

1.3. DNA Binding

Vmw175, like other HSV-1 IE polypeptides, is a DNA binding protein (Powell and Purifoy, 1976; Hay and Hay, 1980). Early reports suggested that Vmw175 bound indirectly to DNA via a host polypeptide (Freeman and Powell, 1982). However, Michael *et al.* (1988) employed the technique of south-western blotting to demonstrate that Vmw175 can bind directly to DNA. This has since been confirmed using affinity purified Vmw175 (Kattar-Cooley and Wilcox, 1989).

Vmw175 is the only one of the HSV-1 IE polypeptides which has been shown to possess a sequence specific DNA binding activity. A Vmw175 DNA binding site of consensus, ATCGTCnnnnYCGRC (*n*=any base; Y=pyrimidine; R=purine), has been proposed (Faber and Wilcox, 1986b). Copies of this consensus element are distributed throughout the HSV-1 genome. Notably, the promoters of IE gene 3 and IE gene 1, both of which bind Vmw175 (Kristie and Roizman, 1986a,b; Muller, 1987; Faber and Wilcox, 1988; Roberts, 1988; Resnick *et al.*, 1989), contain clear homologues of this consensus. Conversely, the other IE promoters, which do not contain a related sequence, bind Vmw175 weakly or not at all (Faber and Wilcox, 1988). The stoichiometry of binding to the IE3 and IE1 sites has been investigated and it was

found that each consensus (ATCGTC) site bound two monomers of Vmw175 (Michael and Roizman, 1989). The relationship between IE gene regulation and binding of Vmw175 to these sites is discussed in Section 1C.2.4.

The precise DNA sequence requirements for Vmw175 consensus binding have been studied in some detail. DNAse footprinting and methylation interference assays have implicated nucleotides outwith the consensus ATCGTC motif in efficient binding (Muller, 1987; Faber and Wilcox, 1988). Indeed, the ATCGTC element is essential, but not of itself sufficient, for DNA binding (Roberts *et al.*, 1988). Extensive point mutagenesis of the IE3 consensus element and flanking nucleotides has been performed. The results demonstrate that the ATCGT nucleotides of the consensus (ATCGTC) are most important for binding, but that downstream nucleotides also contribute (Roger Everett, pers. comm.). This analysis defined a binding site, ATCGTnnnnncGG (where the upper case characters are those determined to be most important), which is very close to the original consensus of Faber and Wilcox (1986b).

Binding of Vmw175 to a disparate set of non-consensus sites has also been described (Kristie and Roizman, 1986b; Michael *et al.*, 1988; Sheperd *et al.*, 1989; Tedder *et al.*, 1989; Michael and Roizman, 1989; Imbalzano *et al.*, 1990).

1C.2. GENE REGULATION BY VMW175

2.1. Genetic Analyses of Vmw175

The isolation and characterization of a number of *ts* mutants in IE gene 3 (discussed in Section 1B.3.3 [d]) demonstrates that Vmw175 has a pivotal role in the HSV-1 transcriptional programme. Two classes of mutants have been defined and the sites of their DNA lesions mapped. This analysis suggests that Vmw175 may comprise distinct functional domains. At the NPT, the more stringent mutants (of which *tsK* is an example) fail to express E and L polypeptides, and overexpress IE polypeptides (Courtney *et al.*, 1976; Marsden *et al.*, 1976; Watson and Clements, 1978,

1980; Preston, 1979a; Dixon and Schaffer, 1980). The lesions in this class of mutants typically lie in the central region or toward the amino-terminus of the polypeptide, that of *ts*K arising from a single point mutation which changes amino acid 475 (Davison *et al.*, 1984). The second class of mutants exhibit reduced, but clearly observable E gene expression and undergo limited DNA replication, yet fail to express L polypeptides (DeLuca and Schaffer, 1984). The phenotype of these mutants suggests that Vmw175 may possess a distinct function required for L gene expression. Consistent with this view, the lesions in these E gene "permissive" mutants are located within the carboxy-third of the polypeptide (DeLuca and Schaffer, 1984; Paterson *et al.*, 1990).

2.2. *Trans*-Activation by Vmw175

Following genetic studies with mutant viruses which demonstrated that functional Vmw175 was absolutely required for induction of E and L gene expression, its role in viral gene regulation has been extensively studied. This section will not review all of the data on *trans*-activation by Vmw175, but rather will discuss the salient features of this work, particularly those pertaining to the possible mechanism of action.

In an extension of the original virus studies, induction of a plasmid-borne viral E promoter (linked to a suitable reporter gene) during virus infection was shown to require functional Vmw175 (Everett, 1983; O'Hare and Hayward, 1984). Subsequently, a variety of approaches have been employed in order to study activation by Vmw175 in the absence of other IE polypeptides. Persson *et al.* (1985) constructed a stable transformed cell line which constitutively expressed Vmw175. These cells were infected in the absence of *de novo* protein synthesis and the induction of viral genes resident in infecting viral genomes analysed. It was found that a number of E genes were efficiently induced, whereas the L VP5 gene was only induced to very low levels, and induction of the L gC gene

was not detectable. A related study attempted co-transformation of cells with Vmw175 coding sequences and certain L genes (Arsenakis *et al.*, 1988). The results obtained were somewhat complicated, and suggested that under some circumstances, Vmw175 could regulate L gene expression both positively and negatively.

Transient transfection assays have been widely exploited to study *trans*-activation using cloned copies of the IE genes, both alone and in combination. In this way, Vmw175 has been shown to *trans*-activate a number of viral promoters of both E and L kinetic classes, and in some cases, activity was substantially increased in the presence of Vmw110 (Everett, 1984b; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a,b; Gelman and Silverstein, 1985, 1986). There are discrepancies between the various studies regarding the actual promoters which are responsive to activation, and the response of particular promoters to Vmw175 and Vmw110 in combination. This doubtless reflects the different cell-types used, and variations in experimental detail, as described by Everett (1988). Importantly, cloned copies of genes encoding *ts* forms of Vmw175 have been shown to be temperature-sensitive in their ability to activate gene expression in transfection assays (DeLuca and Schaffer, 1985).

Activation of certain promoters by Vmw175 and Vmw110 in synergy results in up to 20-fold greater activation than occurs with either polypeptide alone. The underlying basis of this phenomenon is not known. It may reflect a physical interaction or formation of a complex between Vmw175 and Vmw110. Alternatively, the polypeptides might act at different stages of the transcription process. Whether the phenomenon of synergy in transfection assays reflects an important function during infection *in vivo* remains to be determined. Interestingly, viruses which lack functional Vmw110 exhibit poor growth during infection at low multiplicity, apparently as a consequence of reduced viral gene expression (Everett, 1989). This infers that Vmw110 augments virus growth at low moi by increasing viral

gene expression. Perhaps this involves synergistic activation in combination with Vmw175.

Finally, Vmw175 has been shown to stimulate transcription of several viral genes in an *in vitro* transcription system (Beard *et al.*, 1986; Pizer *et al.*, 1986).

2.3. A Role for DNA-Binding in *Trans*-Activation?

The studies discussed in Sections 1B.3.3.(a) and 1B.3.3.(b) indicate that activation of E and L genes by viral IE polypeptides does not involve virus-specific sequences. Nevertheless, it is interesting to consider the studies on *trans*-activation of the HSV-1 gD promoter, which contains a consensus Vmw175 DNA binding site located upstream of the TATA box. It was found that the consensus sequence was dispensable for *trans*-activation by Vmw175 in transfection assays, although the efficiency of *trans*-activation was increased in its presence (Tedder and Pizer, 1988). In addition, multimerization of binding sites upstream of the TATA box increased expression relative to the wt promoter (Tedder and Pizer, 1988). In an extension of these studies Tedder *et al.* (1989) identified three Vmw175 binding sites within the gD gene: Site II contains a consensus ATCGTC motif, site III a near consensus, while site I shares no significant homology to either site II or III. These sites were found to contribute to *trans*-activation in an *in vitro* transcription system (although low level activation was observed in their absence), and it was thus proposed that binding of Vmw175 to multiple, non-homologous sites can stimulate transcription (Tedder *et al.*, 1989).

The results of Imbalzano *et al.* (1990) should also be mentioned. They investigated the functional relevance of non-consensus Vmw175 DNA binding sites in the TK gene promoter region (which lacks consensus sites). A mutant Vmw175 polypeptide, which did not detectably bind to non-consensus sites *in vitro*, apparently activated TK transcription as efficiently as wild type Vmw175. This

suggests that specific DNA binding might not be absolutely necessary for activation of all HSV promoters. The activities and relative amounts of cellular factors, and the number and arrangement of their cognate binding sites in viral promoters, may well be important in this regard.

It should be stressed that the precise role of individual binding sites is difficult to assess in such assays since if one site is deleted, the effect might be compensated by other weaker Vmw175 binding sites within the promoter or the vector (Tedder *et al.*, 1989).

2.4. Repression of IE Gene Expression By Vmw175

Analysis of viral *ts* mutants and use of metabolic inhibitors has implicated Vmw175 in repression of IE polypeptide expression. However, as discussed in Section 1B.3.2, only IE3 RNA expression appears to be repressed during a normal virus infection (Harris-Hamilton and Bachenheimer, 1985; Weinheimer and McKnight, 1987). Thus Vmw175 may be the only IE polypeptide whose expression is repressed at early and late times in infection.

Repression of the IE3 promoter has been reproduced in transfection assays (O'Hare and Hayward, 1985b). A direct correlation between the presence of a functional ATCGTC motif at the IE3 capsite (capable of binding Vmw175) and repression of the IE3 promoter has since been demonstrated (Roberts *et al.*, 1988). In agreement with this finding, mutation of the IE3 consensus binding site in the viral genome increased expression of Vmw175 during infection (DeLuca and Schaffer, 1988). It is thought that binding of Vmw175 to this site represses transcription by directly impeding the formation or progress of a transcription complex.

The IE gene 1 promoter also contains a consensus ATCGTC motif located upstream of the TATA box. Resnick *et al.* (1989) have investigated the role of the this consensus site in IE gene 1 regulation. They determined the effect of a point mutation within the consensus motif (which abolished Vmw175 binding) on regulation of the IE1 promoter

by Vmw175 (alone or in combination with Vmw110 or Vmw65) during transfection assays. Their results are somewhat complex. In some cases the presence of an intact consensus binding site did not affect expression, and in others it correlated with reduced activation (rather than repression). Notably, mutations within the IE1 consensus site in the viral genome do not effect the kinetics or efficiency of Vmw110 expression during a normal virus infection (Everett and Orr, 1991).

It is likely that the ability of Vmw175 to repress gene expression by binding to consensus ATCGTC motifs is dependent on the spatial relationship between the binding site and TATA box. This might require that the binding site be located close to the TATA box and/or that stereo-alignment of the sites is correct. In this regard, it is interesting to consider the results of DiDonato and Muller (1989). These workers investigated the binding of affinity purified Vmw175 to consensus sites in the IE1, IE3 and gD promoters, by high resolution copper footprinting. Binding to the IE3 site, which is located approximately 2.5 helical turns downstream of the TATA box, resulted in hypersensitive cleavage in the TATA region, indicative of an alteration in the helical geometry of the DNA. Binding to the IE1 site, located approximately 3.5 helical turns upstream of the TATA box, also resulted in TATA hypersensitivity, though this was less pronounced. In contrast, binding to the site in the gD promoter, 8 helical turns upstream of the TATA box, did not produce TATA hypersensitivity. Likewise, increasing the separation between the IE3 site and the TATA box to 5 helical turns abolished hypersensitivity. There appears then, to be a close correlation between TATA hypersensitivity and the distance of the binding site from the TATA box.

These observations suggest a model to account for the consequences of Vmw175 binding to consensus sites in these promoter regions. The prediction of the model is that binding which produces alterations in the DNA structure in the TATA box region might disrupt interactions between

TFIID and the TATA box, thereby reducing (or precluding) formation of an initiation complex, and decreasing transcription initiation. This could account for repression of the IE3 promoter. The lack of repression of the IE1 promoter by Vmw175 correlates with reduced TATA hypersensitivity induced by binding of Vmw175 to the IE1 consensus sequence. In this situation, the alterations in TATA box structure only have a moderate effect on TFIID binding, and expression is reduced rather than repressed. Binding to a site further (upstream) from the TATA box (such as that in the gD promoter), which does not induce TATA hypersensitivity, presumably does not diminish TFIID binding to TATA, and thus does not repress expression.

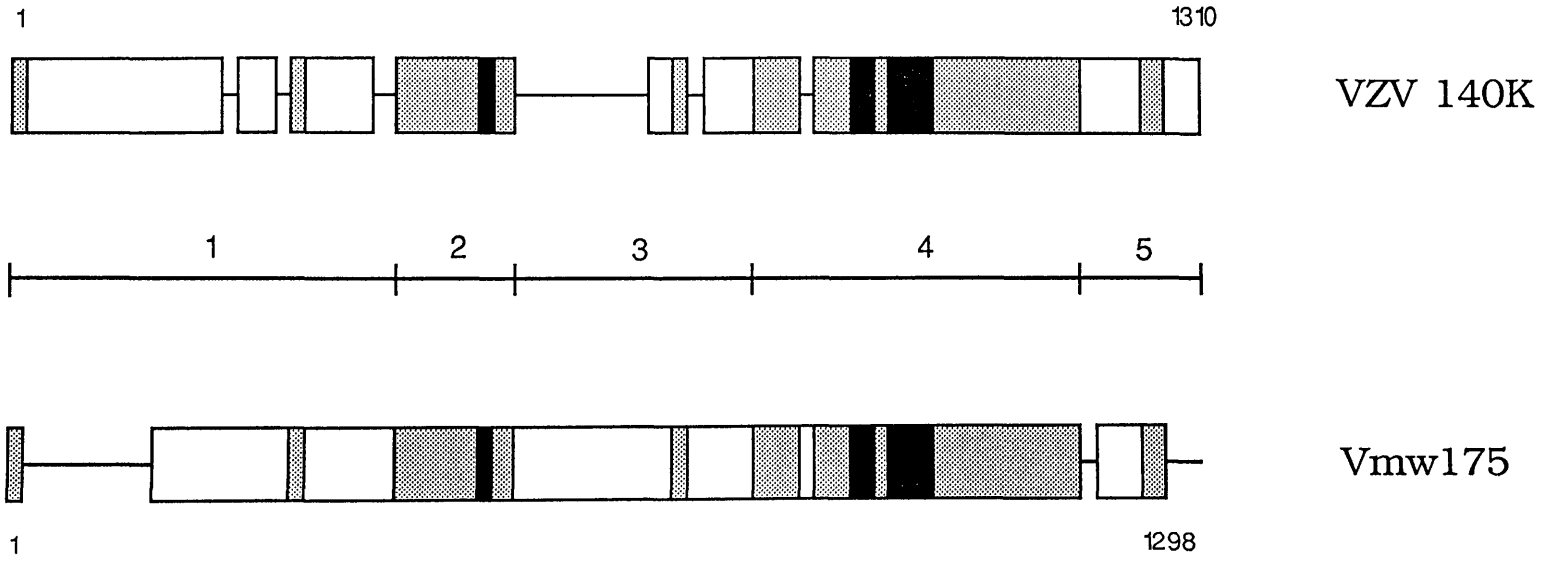
Alternatively, repression of the IE3 promoter by Vmw175 might result from direct disruption of the formation or passage of the preinitiation complex.

1C.3. Structure of Vmw175

As a consequence of the G+C content of the Vmw175 coding sequence, which is a remarkable 81.5%, alanine, proline, glycine, and arginine comprise 54.9% of all amino acids in the polypeptide (McGeoch *et al.*, 1986). The G+C rich codons specifying these amino acids tend to be located in regions of the polypeptide which are less conserved in the homologous IE polypeptides of other alphaherpesviruses, and hence which are presumably of less critical functional importance. It was suggested that such an extreme base composition has resulted from an evolutionary force acting directly on the DNA, rather than indirectly through amino acid sequence (and hence functional) requirements (McGeoch *et al.*, 1986).

The predicted amino acid sequence of Vmw175 was divided into five regions on the basis of homology with the corresponding sequence of VZV 140k (Figure 3; McGeoch *et al.*, 1986). Regions 2 and 4 contain the most highly conserved sequences, and within these are blocks of nearly-identical sequence. The sequences outwith regions 2 and 4 display marked divergence, and include insertions and

Figure 3. Sequence alignment of VZV 140k and HSV-1 Vmw175. The coding sequences of VZV 140k and Vmw175 are divided into five regions (1-5) on the basis of sequence homology (McGeoch *et al.*, 1986). Portions of the coding sequences are represented by blocks, the solid lines being spacers introduced to optimize the alignment. The extent of homology represented by the different shadings is indicated.


1
1310
VZV 140K
1
2
3
4
5
Vmw175
1
1298
LOW
HOMOLOGY
30-50%
HOMOLOGY
OVER 80%
HOMOLOGY

deletions relative to VZV 140k. The sequences comprising regions 2 and 4 are also highly conserved in the homologous PRV and EHV-1 IE polypeptides (Cheung, 1989; Grundy *et al.*, 1989; Vlcek *et al.*, 1989). Furthermore, it is noteworthy that of the Vmw175 *ts* mutants for which the precise DNA lesion has been determined, all alter residues (in region 2 or 4) conserved in all the known sequences (Paterson *et al.*, 1990). The functional importance of regions 2 and 4 suggested by the above sequence analyses has been confirmed by mutational analyses of Vmw175 (see below).

1C.4. Mutational Analysis of Vmw175

Vmw175 is a multifunctional polypeptide which possesses a DNA-binding activity, and functions during virus infection to *trans*-activate E and L gene expression, and in addition, probably represses the expression of IE gene 3. In order to determine which parts of the polypeptide are involved in the various functions, a number of groups have created defined mutations in plasmid-borne copies of the Vmw175 coding sequences. The activities of the mutant polypeptides have been determined by expression in transfection assays (DeLuca and Schaffer, 1987; Paterson and Everett, 1988a,b; Shepard *et al.*, 1989), and in some cases during viral infection following recombination of the mutations back into the normal Vmw175 loci of the HSV-1 genome (DeLuca and Schaffer, 1988; Shepard *et al.*, 1989; Paterson and Everett, 1990). Significantly, these analyses have shown that regions 2 and 4, which are highly conserved in the homologous IE polypeptides of other alphaherpesviruses (McGeoch *et al.*, 1986; Cheung, 1989; Grundy *et al.*, 1989; Vlcek *et al.*, 1989), are most critical for Vmw175 function. The functional properties of each of the 5 domains defined by McGeoch *et al.* (1986; see above) will be considered in turn.

Region 1 (Residues 1-314)

Insertion mutations within the majority of this region have little effect on Vmw175 function (Paterson and Everett, 1988a,b; Shepard *et al.*, 1989). The first 90

residues are dispensable for virus growth (DeLuca and Schaffer, 1987, 1988), but may contain sequences involved in dimerization of Vmw175 (Shepard and DeLuca, 1989). A striking run of serines centred on residue 193 (13 of 14 consecutive residues), which is highly conserved in both VZV and PRV, probably constitutes a phosphorylation site of Vmw175 (DeLuca and Schaffer, 1988). Surprisingly, deletion of this region from Vmw175 does not greatly affect its function in transfection assays or during lytic infection in tissue culture (Paterson and Everett, 1988a,b, 1990). Residues toward the carboxy end of region 1 appear to contribute to DNA binding, and this is discussed below.

Region 2 (Residues 315-484)

This region is clearly the most critical for Vmw175 function. Most of the insertion mutations within region 2 markedly debilitate DNA binding, *trans*-activation, and repression activities (Paterson and Everett, 1988a,b; Shepard *et al.*, 1989). Notably, the DNA lesion in *ts*K is located within this region (Davison *et al.*, 1984). The DNA binding function of this region has been analysed further. A *trpE* fusion protein containing residues 262-490 of Vmw175 bound specifically to a probe containing a consensus ATCGTC motif and, moreover, produced an identical footprint to intact Vmw175 (Wu and Wilcox, 1990). A fusion protein lacking residues 262-306 (from the distal portion of region 1) failed to bind DNA. An independent study revealed that proteinase K digestion of Vmw175 liberates a functional DNA binding domain (Everett *et al.*, 1990). Proteinase K treatment of mutant Vmw175 polypeptides mapped the amino-terminus of the DNA binding domain between residues 229 and 292 (i.e. within region 1), and the carboxy-terminus between residues 495 and 518. Several insertion mutants have intriguing DNA binding properties and suggest that further functional complexity exists within this domain (Shepard *et al.*, 1989). Thus residues 445 to 487, which contain a putative helix-turn-helix motif, were critical for DNA binding in general, whereas an insertion at residue 338 preferentially affected binding to

non-consensus sites.

Region 3 (Residues 485-796)

Region 3 exhibits considerable size heterogeneity between Vmw175 and its alphaherpesvirus homologues, and is one of the least conserved regions of the polypeptides. Region 3 contains a nuclear localization signal (DeLuca and Schaffer, 1987; Paterson and Everett, 1988a). This function lies between residues 640 and 773 and is probably attributable to the sequence pro-arg-glu-gly-arg-lys-arg-lys-ser-pro (underlined amino acids are conserved in VZV 140k) centred on amino acid 728, which is related to the nuclear localization signal of the SV40 large T antigen (Kalderon *et al.*, 1984). No other functions have been assigned to this region.

Regions 4 and 5 (Residues 797-1224 and 1225-1298)

The function of this part of the polypeptide is less well understood, but appears to be more important for *trans*-activation than repression. Intriguingly, small insertions into region 4 are apparently more deleterious than extensive deletions (Paterson and Everett, 1988a,b). Likewise, the amino-terminal 60% of Vmw175 (residues 1-774), which retains considerable activity in transfection studies, was more active than the amino-terminal 80% of the polypeptide (DeLuca and Schaffer, 1987). The shorter polypeptide, however, exhibited reduced *trans*-activation activity and was unable to support virus growth (DeLuca and Schaffer, 1988). There is evidence that some mutations in this region (including those of several *ts* mutants) affect the conformation of Vmw175, and it has been proposed that region 4 might modulate the activity of functions within other regions of the polypeptide (Everett *et al.*, 1990; Paterson *et al.*, 1990). Thus if regions 2 and 4 are closely apposed in the tertiary structure of Vmw175, such conformational alterations could conceivably alter the function of region 2. An extension of this hypothesis is that interaction with other viral or cellular factors might induce similar conformational changes in region 4, and modulate the activity of region 2 in an analogous way.

Since DNA binding is apparently required for *trans*-activation (and is a function of region 2), the primary consequence of these interactions might be to alter the specificity of DNA binding. In this way, the selectivity and efficiency of promoter activation might be modulated.

1D. CONTROL OF EUKARYOTIC GENE EXPRESSION

Control of eukaryotic gene expression is principally exerted at the level of transcription initiation. This section will describe the overall features of this process, and will go on to describe in detail those aspects most relevant to transcriptional control by viral IE polypeptides.

1D.1. RNA Polymerase II

Three distinct RNA polymerase enzymes, which are distinguished by their sensitivity to α-amanatin, have been isolated from eukaryotic cells. They are complex, multi-subunit enzymes with molecular weights in excess of 500,000. RNA polymerases I and III perform specialized roles: polymerase I synthesises the large ribosomal RNAs; polymerase III synthesises the small 5S RNA of the ribosome, and the transfer RNAs. Further discussion will be confined to RNA polymerase II (pol II) which synthesises all the mRNA precursors for protein biosynthesis.

Eukaryotic Pol II comprises two large, non-identical subunits (M_r in excess of 100,000 each), and 7 to 12 smaller polypeptides (M_r <50,000 each)(Lewis and Burgess, 1982). The two largest subunits share structural homology with the two largest subunits of prokaryotic RNA polymerase. However, the largest subunit of the eukaryotic enzyme has at its C-terminus a unique heptapeptide tandem repeat of consensus YSPTSPS; the exact sequence and number of these repeat units varies between species (yeast, 26 copies; Allison *et al.*, 1985; drosophila, 42 copies; Zehring *et al.*, 1988; mouse, 52 copies; Bartolomei *et al.*, 1988). Deletions which remove portions of this repeat element are functionally tolerated *in vivo*, though the minimal (functional) repeat length varies between species (Allison *et al.*, 1988; Bartolomei *et al.*, 1988; Zehring *et al.*, 1988). Additional *in vivo* studies have shown that the yeast repeat element can be replaced with that from hamster, whereas replacement with the drosophila element, which is more divergent, results in

a recessive lethal condition (Allison *et al.*, 1988).

The catalytically active form of pol II is phosphorylated, and there is good evidence that the site of phosphorylation is the C-terminal repeat element (Cadena and Dahmus, 1987). Interestingly, the repeat element is dispensable for accurate initiation of transcription in a minimal *in vitro* system, and it has been proposed that it may have a regulatory function *in vivo* (Zehring *et al.*, 1988). In this regard, it is interesting that mutations in GAL4 (a transcriptional regulatory factor of *Saccharomyces cerevisiae*) which reduce its intrinsic *trans*-activation activity can be enhanced or suppressed *in vivo* by mutations in pol II which decrease or increase (respectively) the length of the repeat regions (Allison *et al.*, 1989). Furthermore, GCN4 (also of *Saccharomyces cerevisiae*) and pol II have been shown to interact directly *in vitro* (Brandl and Struhl, 1989). Clearly such direct interactions between pol II and transcription factors bound to promoter sites might be important in regulation of transcription *in vivo*. Indirect interactions, mediated for example via TFIID bound to the TATA box, are also likely to be important (see Section 1D.5).

1D.2. Control of RNA Polymerase II Transcription Initiation

Pol II transcribes three subclasses of genes which are defined in terms of the characteristics of their expression and the nature of the sequences controlling their transcription. The majority of protein coding genes contain a TATA element; regulation of this class of genes has been extensively studied and is considered below. The two remaining subclasses do not possess TATA boxes (see Smale and Baltimore, 1989, for review). The so called "housekeeping" genes, which express proteins common to all cell types, have G+C rich promoters which exhibit low level constitutive activity, and frequently initiate transcription at sites spread over a wide region. The second class of TATA-less genes include those whose expression is regulated during mammalian

immunodifferentiation e.g. the terminal deoxynucleotidyltransferase gene (TdT; Landau *et al.*, 1984). Analysis of the TdT gene promoter identified an element, the initiator, that contains within itself the transcription start site (Smale and Baltimore, 1989). The initiator is the simplest functional promoter yet identified.

1D.2.1. Regulation of TATA Containing Class II Genes

The promoters of these genes share a common architecture which includes a TATA element and various upstream regulatory elements (for reviews see Dynan and Tjian, 1985; Maniatis *et al.*, 1987).

The TATA box (consensus TATAA)

The TATA element, which is located 25-30 nucleotides upstream of the transcription start site in mammalian genes, is the most highly conserved *cis*-acting sequence in class II promoters. The effects of mutations in this element have been studied *in vitro* and *in vivo*. These analyses reveal that the TATA element is primarily involved in controlling the fidelity of initiation, although in some cases dramatic reductions in transcription were observed (Grosschedl and Birnstiel, 1980; Wasylyk *et al.*, 1980; Benoist and Chambon, 1981; Grosveld *et al.*, 1981; Mathis and Chambon, 1981; Grosveld *et al.*, 1982). The TATA box binds the transcription factor, TFIID (see Section 1D.3.3).

Upstream Promoter Elements (UPEs)

Many promoters contain one or more UPE within approximately 100 nucleotides of the TATA box. These elements are 10-12 bp in size and interact with cellular DNA-binding proteins (transcription factors). They function in either orientation, but exhibit reduced activity when moved further upstream of TATA. Significantly, alterations in spacing which displace an element (relative to TATA) by half a helical turn are more deleterious than those involving a full helical turn. These observations are consistent with the idea that factors bound to UPEs influence events at the TATA box, probably by promoting

formation of an initiation complex containing pol II and other factors (see Section 1D.5). Some UPEs bind generalized transcription factors which contribute to the constitutive activity of a promoter. Other UPEs bind factors which are regulated by specific stimuli such as heat shock, and exposure to heavy metals or steroids; in the absence of the appropriate stimuli, these UPEs are not occupied by their cognate transcription factors.

Enhancers

The activity of a number of promoters is modulated by a *cis*-linked enhancer. The characteristic features which define enhancers are their ability to function over large distances (1000 bp or more from TATA), both upstream and downstream of the TATA box. The activity of enhancers often displays cell-type specificity. Enhancers are larger and more complex than UPEs, comprising sometimes hundreds of bases and containing multiple sequence elements. These elements, like UPEs, bind transcription factors. Indeed, some elements (e.g. the GC box which binds Sp1) function both as UPEs and as components of enhancers. Hence it is likely that enhancers and UPEs stimulate transcription by similar, if not identical mechanisms. The characteristic properties of enhancers probably reflect the number and arrangement of binding sites for cellular transcription factors.

Many of the transcription factors which bind to UPEs and enhancer elements have been identified. Since these factors are not directly relevant to the content of this thesis, it would be neither worthwhile, nor indeed possible, to review them here. Subsequent sections will instead focus specifically on events at the TATA box which have been the focus of much attention in recent years. Notably, the TATA box has been shown to be the target for *trans*-activation by a number of eukaryotic transcription factors, including GAL4 (Horikoshi *et al.*, 1988a) and ATF (Hai *et al.*, 1988; Horikoshi *et al.*, 1988b). Furthermore, the TATA box is thought to be a target for a number of

viral IE *trans*-activators (Abmayr *et al.*, 1985, 1988; Coen *et al.*, 1986; Wu *et al*, 1987; Simon *et al.*, 1988; Wu and Berk, 1988).

1D.3. General Factor Requirements for Pol II Initiation

3.1. An Overview

Purified pol II is not competent for transcription initiation *in vitro*. Fractionation of mammalian cell extracts combined with reconstitution experiments has identified five factors, TFIIA, IIB, IID, IIE and IIF, all of which are required in addition to polII for accurate transcription of a DNA template *in vitro* (Matsui *et al.*, 1980; Samuels *et al.*, 1982; Sawadogo and Roeder, 1985; Reinberg and Roeder, 1987; Reinberg *et al.*, 1987). The variable requirement for TFIIA in some early experiments doubtless reflects contamination of other fractions with this factor. Kinetic experiments have established that the various factors are involved at different stages of the initiation process. In particular, TFIID and TFIIA were shown to generate the first stable complex in initiation. It was found that preincubation of template DNA with TFIID and TFIIA generated a preinitiation complex resistant to subsequent challenge with inhibitors of *in vitro* pol II transcription (poly(dI.dC) or sarkosyl; Fire *et al.*, 1984; Reinberg *et al.*, 1987). This preinitiation complex is said to be "committed" to transcription. Increasingly pure preparations of these factors have been obtained, and in some cases the genes encoding them cloned, thus permitting a more detailed biochemical analysis of their properties. This is described in Section 1D.3.3.

3.2. Conservation of the Transcription Machinery Between Yeast and Mammals

There is a high degree of conservation between yeast and mammalian transcription systems. Thus yeast GAL4 *trans*-activates transcription in a Hela cell-derived *in vitro* transcription system (Lin *et al.*, 1988) and in mammalian cells (Kakidani and Ptashne, 1988), and fos-LexA

fusion proteins (in which the LexA DNA binding domain is fused to c-fos or v-fos) activate yeast promoters containing LexA binding sites *in vivo* (Lech *et al.*, 1988). The general transcription factors, TFIID and TFIIA, have since been shown to be fully interchangeable between the systems (Buratowski *et al.*, 1988; Hahn *et al.*, 1989a).

The purification of mammalian transcription factors for biochemical analysis has proved to be an arduous task. In this regard, the conservation between yeast and mammalian systems has proved to be particularly important. Significant advances in our understanding of mammalian transcription have stemmed from our ability to readily purify the corresponding yeast factors. For example, partial amino acid sequencing of yeast TFIID led to several groups simultaneously isolating the gene encoding this important factor (Cavallini *et al.*, 1989; Hahn *et al.*, 1989b; Horikoshi *et al.*, 1989; Schmidt *et al.*, 1989). The availability of the yeast TFIID gene sequence then quickly led to the cloning of the human gene (Kao *et al.*, 1990; Peterson *et al.*, 1990). The power of yeast genetics will doubtless lead to the cloning of other yeast genes encoding general transcription factors (and hence the corresponding mammalian genes) in the near future and, furthermore, permit analysis of their function *in vivo*.

3.3. Functions and Biochemical Properties of the General Transcription Factors

TFIID

TFIID binds directly to the TATA element (Nakajima *et al.*, 1988). This is the first step in formation of a preinitiation complex (Davison *et al*, 1983; Fire *et al.*, 1984).

Genes encoding TFIID have now been isolated from *Saccharomyces cerevisiae* (Cavallini *et al.*, 1989; Hahn *et al.*, 1989b; Horikoshi *et al.*, 1989; Schmidt *et al.*, 1989), *Schizosaccharomyces pombe* (Hoffman *et al.*, 1990), *Drosophila melanogaster* (Hoey *et al.*, 1990), and humans (Kao *et al.*, 1990; Peterson *et al.*, 1990). The existence of

a spectrum of TATA elements inferred that TFIID might comprise a family of proteins with different sequence specificity. That a number of groups have isolated the same yeast TFIID gene (and two groups the same human gene) argues against this. It seems likely, therefore, that a single TFIID protein interacts differently with distinct TATA sequences (Nakajima *et al.*, 1988).

The yeast TFIID genes encode proteins of predicted M_r 27,000, whereas the predicted human and drosophila products are significantly larger at 38,000. Comparison of the various predicted amino acid sequences reveals a number of interesting features. The C-terminal 180 amino acids are highly conserved in all three species; there is 90% identity between the human and drosophila sequences, both of which share 80% identity with the yeast sequence. This C-terminal domain contains a region with similarity to the bacterial sigma factor (see Section 1D.3.4), which is required for accurate initiation by bacterial RNA polymerase (Horikoshi *et al.*, 1989). In contrast, the N-termini are considerably divergent and account for the size heterogeneity between the various proteins. It has been proposed that the N-termini may participate in species specific regulatory interactions. Results from mutational analyses of the yeast protein are consistent with this idea. Thus a truncated protein consisting solely of the conserved C-terminal sequences is sufficient for promoter recognition and basal level transcription (Horikoshi *et al.*, 1990; Peterson *et al.*, 1990), whereas activation of transcription by factors bound at UPEs appears to involve N-terminal sequences (Peterson *et al.*, 1990). Interestingly, Van Dyke and Sawadogo (1990) reported that purified TFIID contains a protease-resistant core which binds TATA elements and supports basal level transcription. Hence it is likely that this core contains C-terminal sequences.

Finally, analysis of yeast strains with mutations in the TFIID gene have demonstrated that TFIID function is essential for growth and, moreover, that alterations in

TFIID can alter transcription initiation *in vivo* (Eisenmann *et al.*, 1989).

TFIIA

The availability of more highly purified preparations of general transcription factors has confirmed that TFIIA is essential for accurate initiation of transcription *in vitro*. Kinetic experiments indicate that TFIIA, like TFIID, acts at an early stage in transcription initiation (Fire *et al.*, 1984). TFIIA does not itself bind DNA, but forms a complex with DNA-bound TFIID, resulting in an extension of the TFIID DNAse I footprint (Buratowski *et al.*, 1989; Hahn *et al.*, 1989a). This led Hahn *et al.* to speculate that TFIIA functions by altering the conformation of TFIID (in such a way as to promote transcription initiation) and/or by serving as a bridge between TFIID and pol II or other general transcription factors.

TFIIB and TFIIE

Little is known of the function of these factors, except that they each bind pol II in solution, and that TFIIE appears to be associated with a DNA-dependent ATPase activity (Reinberg and Roeder, 1987; Zheng *et al.*, 1987).

TFIIF

Burton *et al.* (1986) reported that the transcription initiation activity of a HeLa cell extract could be abolished by passing it down a column containing immobilized pol II. A factor which was eluted from the column by 0.5M KCl was sufficient to reconstitute activity. This factor, called RAP30/74 (RAP: RNA polymerase Associated Protein), comprises two subunits of apparent M_r 30,000 and 74,000 (Burton *et al.*, 1988; Flores *et al.*, 1989). Gel filtration analysis established that native RAP30/74 has an M_r of 220 kDa, and is thus probably a dimer of the RAP30/74 heterodimer (Flores *et al.*, 1990). RAP30/74 is believed to be identical to TFIIF since the addition of purified RAP30 and RAP74 subunits to TFIIF depleted extracts restores transcription activity (Flores *et al.*, 1988, 1990). TFIIF functions at a stage following template commitment and possibly pol II binding (Flores *et*

al., 1989).

The gene encoding the RAP30 subunit has been cloned (Sopta *et al.*, 1989). A portion of RAP30 exhibits homology to a part of the bacterial sigma factor which is conserved among all sigma factors, and is believed to function in binding to RNA polymerase. The RAP30/74 complex also has an associated DNA helicase activity, probably attributable to RAP74 (Sopta *et al.*, 1989). Taken together, these results suggest that RAP30 may be involved in the correct positioning (relative to pol II) of the helicase activity (RAP74) in the initiation complex. Once appropriately located, the helicase would melt the DNA at the start site to facilitate initiation.

3.4. Two Factors Related to Bacterial Sigma?

The predicted amino acid sequences of TFIID and RAP30 both contain regions which are homologous to the bacterial sigma factor (Horikoshi *et al.*, 1989; Sopta *et al.*, 1989). Sigma possesses a spectrum of activities including RNA polymerase binding, promoter recognition, and DNA melting. Strikingly, the homology between TFIID and sigma seems to be restricted to a region of sigma implicated in binding to the TATA-like sequence in bacterial promoters (Horikoshi *et al.*, 1989). Moreover, the region of sigma which shares homology with the RAP30 subunit of TFIIF is believed to be responsible for RNA polymerase binding (Sopta *et al.*, 1989). It is conceivable, therefore, that the activities of sigma are distributed among a number of the eukaryotic factors. This would clearly increase the number of points at which transcription could be regulated in eukaryotes (relative to prokaryotes). It will be interesting to see whether the sequence of RAP74 (the gene for which has yet to be cloned) is homologous to the region of sigma proposed to melt DNA during initiation.

1D.4. A Model For Initiation Complex Assembly

Buratowski *et al.* (1989) analysed complexes formed on the adenovirus major late promoter by general transcription factors and pol II. Using a native gel retardation DNA binding assay, they were able to determine the order with which the components were recruited onto the template. They identified a minimum of five intermediate complexes leading to a functional initiation complex. Furthermore, DNAse I footprint analysis was employed to ascertain the relative positions of each of the factors in the various complexes. It is apparent from this and other studies (Van Dyke *et al.*, 1988) that a preinitiation complex is formed following the orderly assembly of general transcription factors and pol II onto the DNA template, and that this involves protein:protein and protein:DNA interactions. These results are described below (see also Figure 4).

Complex 1: The initial step in preinitiation complex formation was recognition and binding of the TATA element by TFIID. This event was a prerequisite for formation of all other complexes. Binding of TFIID alone protected residues -37 to -17 from DNAse I digestion, but did not produce a complex which was detectable in gel retardation assays, indicating that it may be an unstable interaction.

Complex 2: The addition of TFIIA to complex 1 resulted in formation of complex 2 with a concomitant increase in DNAse 1 protection to between -42 and -17, indicating that TFIIA is located upstream of TFIID. It was proposed that TFIIA functions by increasing the affinity of TFIID for DNA, or perhaps by stabilizing TFIID:DNA interactions. However, more recent evidence suggests that this is not the case (Hahn *et al.*, 1989a). Rather, the increased DNAse I footprint may be indicative of a conformational change induced in TFIID by TFIIA, possibly reflecting transition of TFIID from an "inactive" to an "active" state. Alternatively, TFIIA may stabilize interactions between TFIID and other factors. In this case the increased footprint might represent a weak interaction between TFIIA

and sequences upstream of TFIID, or may simply be due to steric effects. This complex probably corresponds to the "committed" complex defined in kinetic studies (Davison, B.L. *et al.*, 1983; Fire *et al.*, 1984; Reinberg *et al.*, 1987).

Complex 3: The next factor to associate with the DNA template, TFIIB, induced partial protection from DNAse I digestion at some sites between -10 and +10. Intriguingly, this appeared to be specific for the coding strand. Hence TFIIB may associate with the coding strand, and extend from the TFIID:TFIIA complex to beyond the initiation site. Recruitment of pol II to the preinitiation complex was dependent upon the presence of TFIIB, suggesting that TFIIB stabilizes interactions between pol II and TFIID (N.B. TFIIB binds to pol II in solution; Reinberg and Roeder, 1987).

Complex 4: Following the addition of TFIIB, Pol II became stably associated with the preinitiation complex. DNAse I protection in complex 4 was extensive; the upstream boundary was located at approximately -47, and the downstream boundary at around +20. This infers that part of pol II is in close proximity to transcription factors bound to UPEs. Recall that some factors appear to interact directly with a domain of pol II implicated in regulation of transcription (see Section 1D.1). Thus, when present in a preinitiation complex, pol II may be directly "activated" by interactions with transcription factors bound to UPEs.

Complex 5: The model proposes that TFIIE now binds to complete formation of the preinitiation complex. However, Flores *et al.* (1989) have since demonstrated that partially purified preparations of TFIIE (as used in this study) actually contain two factors, TFIIE and TFIIF, thus complicating the interpretation of the results. Formation of complex 5 correlated with the appearance of a novel DNAse I protected region between +20 and +30, suggesting that one or both factors bind downstream of pol II. It should be borne in mind that *in vivo* TFIIE and/or TFIIF may associate with pol II in solution to form a heteromeric

complex, which then associates with the developing preinitiation complex.

TFIIF is associated with a helicase activity and presumably interacts with pol II (see Section 1D.3.3). Earlier results suggested that TFIIE possesses a DNA-dependent ATPase activity (Reinberg and Roeder, 1987). Whether this was associated with contaminating TFIIF, or does in fact reflect a *bona fide* function of TFIIE is not clear. It is possible that the activities are due to separate factors which become associated in the final preinitiation complex. As noted above (Section 1D.3.4), such a scheme would enable more subtle regulation than would be possible with a single, multifunctional polypeptide. Irrespective of the precise details, it is clear that the helicase and ATPase activities are recruited into a committed complex, whereupon hydrolysis of ATP (or dATP) presumably results in unwinding of the DNA, making the template strand accessible to pol II.

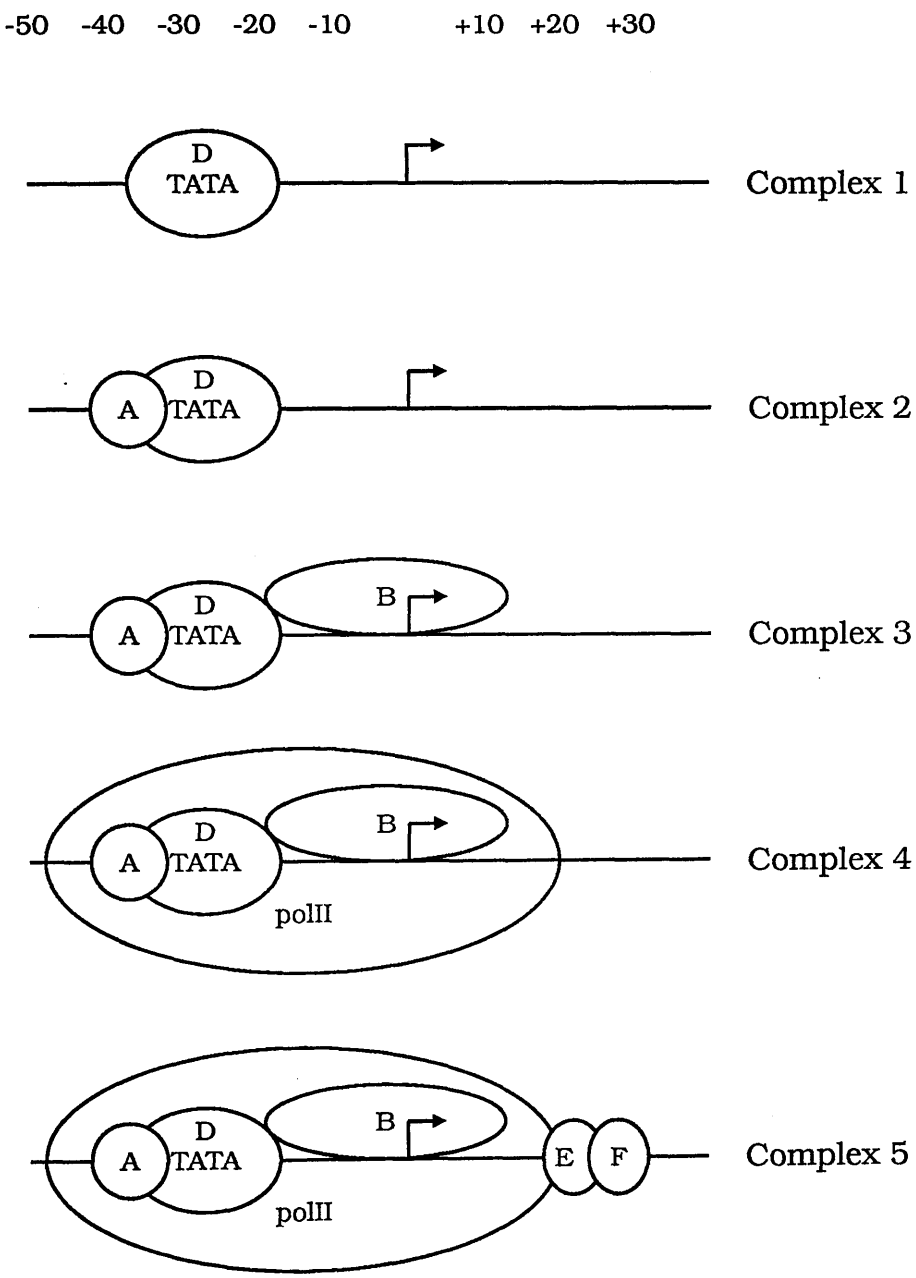
Finally, it was found that addition of NTPs resulted in formation of novel complexes containing accurately initiated transcripts. These were of increased mobility relative to complex 5, and it was suggested that TFIIE (and TFIIF?) might dissociate from the active complex following initiation.

1D.5. TFIID is a Target for *Trans*-Activation

There is now compelling evidence that GAL4, ATF and the PRV-IE polypeptide *trans*-activate transcription through interactions with TFIID.

Horikoshi *et al.* (1988a) reported that GAL4 binding to synthetic GAL4 binding sites in the adenovirus E4 promoter qualitatively altered the interaction of TFIID with the TATA box region. Similar alterations were detected following ATF binding to the E4 promoter (Horikoshi *et al.*, 1988b). Moreover, the ATF-induced alterations in TFIID:TATA interactions were shown to facilitate the subsequent binding of pol II, TFIIB and TFIIE. In an accompanying paper, Hai *et al.* (1988) reported the results of

Figure 4. A model for preinitiation complex assembly. RNA polymerase II (polII) and the general transcription factors TFIID (D), TFIIA (A), TFIIB (B), TFIIE (E), and TFIIF (F), are assembled into a preinitiation complex on the DNA template. Buratowski *et al.* (1989) identified a minimum of five intermediate complexes leading to a functional preinitiation complex *in vitro*. The order with which the components were recruited onto the template and their relative positions within each complex were determined; this information is summarized opposite and discussed in Section 1D.4. The transcription start site (+1) is marked by the arrow, the numbering at the top of the figure refers to nucleotide positions relative to this. This figure is adapted from an original figure in Buratowski *et al.* (1989).


-50 -40 -30 -20 -10 +10 +20 +30
D
TATA
Complex 1
A
Complex 2
B
Complex 3
polII
Complex 4
E
F
Complex 5

experiments which investigated *trans*-activation by ATF during *in vitro* transcription assays. Their results, together with those of Horikoshi *et al.* (1988b), are consistent with a model whereby ATF functions transiently during the template commitment stage of preinitiation complex formation. It is proposed that ATF, through direct interaction with TFIID, triggers multiple cooperative interactions between general transcription factors leading to formation of a functional preinitiation complex.

TFIID is apparently also the target for *trans*-activation by the PRV-IE polypeptide. However, in contrast to GAL4 and ATF, PRV-IE quantitatively alters TFIID:promoter interactions. During *in vitro* transcription assays, maximal activation by PRV-IE is observed at low template concentrations, when the rate or extent of association of TFIID with template DNA is limiting for transcription. PRV-IE is thought to function by increasing the rate or extent of association of TFIID with promoter sequences, thereby stimulating preinitiation complex formation (Abmayr *et al.*, 1988). Workman *et al.* (1988) investigated *trans*-activation by PRV-IE during *in vitro* nucleosome assembly. It was found that when placed in direct competition with nucleosome assembly, the rate or extent of TFIID binding was insufficient to overcome nucleosome-mediated repression of transcription. Under these conditions, PRV-IE apparently stimulated the rate or stability of TFIID binding, and transcription was activated rather than repressed.

1D.6. Activation by Sp1: Evidence for Transcriptional Adaptors?

Sp1 stimulates transcription from TATA-containing promoters in the presence of general transcription factors. Semi-purified TFIID fractions from human or drosophila cells are able to support Sp1 stimulation, whereas purified, cloned drosophila or yeast TFIID proteins are not (Hoey *et al.*, 1990; Pugh *et al.*, 1990). This suggests that interaction between *trans*-activators and the general

transcription machinery is mediated via an adaptor protein, and that partially purified preparations of TFIID contain this moiety. Several other groups have reported indirect evidence which supports this hypothesis (Berger *et al.*, 1990; Kelleher III *et al.*, 1990). Pugh *et al.* (1990) also present evidence of species specificity in activator-adaptor-TFIID interactions. Thus the drosophila adaptor functions with the homologous drosophila TFIID protein, but not with heterologous yeast TFIID. Likewise, the adaptor function from human cells does not interact with drosophila TFIID. Furthermore, they suggest that different types of activation domain may each function through distinct adaptor proteins, that is, specific adaptors exist for acidic activation domains and so on. This would provide a mechanism whereby the various types of activation domain could interact with a single species of TFIID. Confirmation of the existence of transcriptional adaptor proteins awaits their purification and characterization.

1D.7. Gene Regulation by Viral IE Polypeptides

As discussed in Section 1B.3.3.(c), a number of viral IE *trans*-activators activate transcription through the TATA box. We have seen that formation of an initiation complex at the TATA box involves the ordered assembly of general transcription factors on to the DNA template. In theory, any event in this process which is rate limiting for transcription is a potential target for *trans*-activators. At least one viral activator, the PRV-IE polypeptide, appears to stimulate association of TFIID with the TATA box. Given the extensive conservation between the IE polypeptides of alphaherpesviruses, it is likely that VZV 140k and HSV-1 Vmw175 function by a similar mechanism. This probably accounts for activation of a majority of promoters.

7.1. A Model For *Trans*-Activation and Repression by Vmw175

The studies discussed in Section 1C indicate that *trans*-activation of most (though possibly not all) promoters involves DNA binding by Vmw175, as does repression of the IE3 promoter. Since Vmw175 binds to consensus and non-consensus sites in viral DNA, the potential role of each of these activities will be considered in turn.

Although some viral promoters (such as that of the gD gene) contain consensus ATCGTC binding sites, the majority do not. Thus let us first consider the potential role of non-consensus DNA binding in *trans*-activation. It is proposed that Vmw175 binds to disparate sites in viral promoters and brings about *trans*-activation by directing TFIID to bind at the TATA box. This is the rate limiting step in pre-initiation complex formation during *in vitro* transcription at low template concentrations (which is presumably analogous to the situation at the outset of viral infections *in vivo*) (Abmayr *et al.*, 1988). The requirement for Vmw175 is probably transient, since once TFIID is bound at the TATA box, binding of additional factors stabilizes TFIID:TATA interactions.

Binding of Vmw175 to the consensus sequence, ATCGTC, is implicated in both repression and *trans*-activation of transcription. (Thus far, non-consensus binding has not been implicated in repression.) As discussed in Section 1C.2.4., repression probably requires a particular spatial relationship between the binding site and TATA box (proximity of the binding site to the TATA box is probably the key factor). In promoters which satisfy these requirements (such as the IE gene 3 promoter), binding of Vmw175 reduces binding of TFIID to the TATA box and/or preinitiation complex formation, thereby repressing transcription. However, the relationship between the binding site and TATA box in the majority of promoters (such as gD) probably does not meet these requirements, and binding of Vmw175 thus causes *trans*-activation, presumably as described above for non-consensus binding.

Indirect mechanisms might also operate. Thus an activator could function by increasing the level of a limiting transcription factor in the infected cell. Indeed, this may be important in the SV40 replication cycle. During infection of tissue culture cells by SV40, there is a 10-fold increase in Sp1 mRNA levels (attributable to an early viral function), which is accompanied by a corresponding increase in levels of Sp1 protein (Saffer *et al.*, 1990). Expression of a number of viral and cellular genes is stimulated following this increase in Sp1 levels. The adenovirus E1a protein, in addition to interacting with TFIID, probably functions through alternative mechanisms. For example, the transcription factor E2F is complexed with various cellular factors in uninfected cells. During adenovirus infection, E1a apparently dissociates these complexes liberating free E2F, which then forms a complex with the adenovirus E4 protein. This new complex has a novel promoter specificity, which includes binding to, and subsequent activation of the E2F promoter (Bagchi *et al.*, 1990).

1E. Aims of the Work Presented in This Thesis

There is an accumulating body of evidence which suggests that VZV 140k is the functional equivalent of the HSV-1 immediate early polypeptide, Vmw175, and it was thus of interest to investigate its properties and functions in more detail. A number of approaches were taken. Firstly, a recombinant virus, HSV-140, was constructed in which VZV 140k coding sequences replace those of Vmw175 in the short region repeats of the HSV-1 genome. The properties of this virus were compared to HSV-1 in order to assess the degree to which VZV 140k is able to complement for loss of Vmw175 function in HSV-1. Secondly, the *trans*-activation and *trans*-repression properties of wild-type and mutant forms of the VZV 140k polypeptide were studied in transient transfection assays. Finally, the DNA binding properties of VZV 140k were investigated. These studies have demonstrated that while VZV 140k and Vmw175 share many

properties, they are not indistinguishable. The results of these studies are presented.

2. MATERIALS AND METHODS

2A. MATERIALS

1. Cells

(i) BHK-21 clone 13 (Macpherson and Stoker, 1962), a fibroblastic cell line derived from baby hamster kidney cells, was generally used for growth of virus stocks and viral gene expression experiments. They were also used for transient transfection assays which investigated *trans*-repression, and in nuclear extract experiments.

(ii) W.S.HeLa cells (obtained from Dr. W. Schaffner, Zurich) were mainly used for transient transfection assays which investigated *trans*-activation.

(iii) Flow HeLa cells (from the ATCC via Flow Laboratories) were used for nuclear extract experiments. These cells differed significantly in morphology, growth conditions and behaviour compared to W.S.HeLa cells.

(iv) Vero cells (also obtained from the ATCC via Flow Laboratories) were used for transient transfection assays.

(v) HFL cells (Flow Laboratories) were obtained from Dr. T.A. McKee and used for growth of VZV (and propagation of virus as infected cells). They were also used for nuclear extract experiments and HSV-140 viral gene expression experiments.

(vi) Monkey CV1 cells (obtained from Dr. A. Davison) were also used for growth and propagation of VZV and in nuclear extract experiments.

2. Tissue Culture Media

(i) BHK cells were grown in Glasgow Modified Eagle's Medium (GMEM) supplemented with 100 units/ml penicillin, 100µg/ml streptomycin, 0.2µg/ml amphotericin, 0.02% phenol red and 10% newborn calf serum. (=EC10)

(ii) W.S.HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (supplied by Gibco) supplemented with 100 units/ml penicillin, 100µg/ml streptomycin, 0.2µg/ml amphotericin, 2.5% foetal calf serum and 2.5% newborn calf

serum.

(iii) Flow HeLa cells were grown in supplemented GMEM as for BHK cells but with 10% foetal calf serum replacing the newborn calf serum. (=EF10)

(iv) Vero cells were grown in EF10.

(v) HFL cells were also grown in EF10.

(vi) CV1 cells were grown in DMEM supplemented as for W.S.HeLas but with 5% foetal calf serum and no newborn calf serum

(vi) HSV-1 viral titrations were overlaid with GMEM supplemented as above and with 5% human serum (EH5).

3. Viruses

The VZV strain used by Dumas *et al.* (1981) was used in all experiments. Stocks of VZV-infected HFL and CV1 cells were obtained from Dr. T.A. McKee and Dr. A. Davison respectively.

All other viruses used in this study were derivatives of the HSV-1 Glasgow strain 17 syn$^+$ (Brown *et al.*, 1973). Derivative D30EBA (Paterson and Everett, 1990) contains a large deletion within IE gene 3 which removes codons 83 to 1236.

4. Bacteria

Plasmids based on M13 vectors were propagated in the *Escherichia coli* strain JM101 (*supE delta(lac-proAB); F', traD36 proAB$^+$ lacI^q lacZ*deltaM15; Messing, 1979). All other plasmids used in this study were propagated in *Escherichia coli*. K12 strain HB101 (F$^-$, *ramC1, proA2, galK2, strA, recA*; Boyer and Roulland-Dussoix, 1969).

5. Bacterial Culture Media

JM101 strains were grown on 2YT-broth (5g/l NaCl, 16g/l Difco Bactopeptone, 10g/l yeast extract). HB101 strains were grown on L-broth (177mM Nacl, 10g/l Difco Bactopeptone, 5g/l yeast extract). Agar plates contained 1.5% (w/v) agar in L-broth and where appropriate the medium

was supplemented with 100µg/ml ampicillin.

6. Plasmids

The following plasmids were kindly provided by the acknowledged authors.

p140 (McKee *et al.*, 1990) was provided by Dr. R.D. Everett. VZV gene 62 is cloned on a fragment from the *Cla*I site at -1146 to the genome terminal site of pVZVSstf (Everett, 1984) between the *Acc*I and *Pst*I sites of a pUC9 plasmid from which the *EcoRI* had been removed by cutting and filling in.

p175 (Everett, 1987). The HSV-1 IE-3 gene is cloned on a *Bam*HI-*Sst*I fragment (HSV-1 coordinates 125.1-133.0) under the control of the SV40 early promoter and enhancer (SV40 coordinates 5171-346).

pI13 and pI38 (Paterson and Everett, 1988a). Derivatives of p175 created by insertion of a 12bp *Eco*RI linker into *Sma*I and *Nae*I sites respectively within the Vmw175 coding sequences.

p111 (Everett, 1987). The HSV-1 IE-1 gene is cloned on a *Hpa*I-*Sst*I fragment (HSV-1 coordinates 120.4-125.1).

pIE3CAT (Stow *et al.*, 1986). The promoter of the HSV-1 IE-3 gene is cloned on a *Hind*III-*Bam*HI fragment (-331 to +26) fused to the CAT reporter gene in a vector derived from pBLW2 (Gaffney *et al.*, 1985).

pgDCAT (Everett, 1986). The promoter of the gene encoding glycoprotein D (gD) (US6) is cloned on a *Sst*I-*Hind*III fragment (-392 to +11) fused to the CAT gene in a vector derived from pBLW2 (Gaffney *et al.*, 1985).

pSVEB (Everett, unpublished). The SV40 promoter and enhancer (coordinates 5171-346) cloned into pBR322.

pGX161 (Preston and Fisher, 1984) was provided by Dr. C.M. Preston. The HSV-1 IE gene 3 promoter is cloned on a *Hind*III-*Bam*HI fragment (-331 to +26) into a derivative of pAT153.

pTM14CAT (Dr. T.A. McKee, Ph.D thesis, University of Glasgow, 1990; see Section 3D.1). Sequences upstream of VZV gene 62 from *Dde*I (-781) to *Sca*I (-22) are fused to the CAT

gene in a derivative of pFJ3 (Rixon and McLauclan, 1990) in which the CAT reporter gene coding sequences replace those of the β-gal gene.

pTM15CAT (Dr. T.A. McKee, Ph.D thesis, University of Glasgow, 1990; see Section 3D.1). A double stranded oligonucleotide encoding VZV gene 62 sequences from the *Afl*III site (-33) to +20 was inserted between the *Afl*III (-33) and *Bam*HI sites of pTM14CAT (see above) to create pTM15CAT (the *Bam*HI site of pTM14 CAT is within polylinker sequences located between the VZV insert and the CAT gene).

pMC1 (Campbell *et al*, 1984) was provided by Dr T.A. McKee. Plasmid pMC1 contains the HSV-1 UL48 gene encoding Vmw65.

7. Reagents

Oligonucleotides were synthesised in this Institute by Dr. John McLauchlan using a Biosearch 8600 DNA synthesizer.

In general analytical grade chemicals were purchased from BDH Chemicals UK Ltd. or Sigma Chemical Co. Ltd.; TEMED (N,N,N',N'tetramethylethylenediamine) and ammonium persulphate were obtained from BIO-RAD Laboratories; caesium chloride, acrylamide, sodium hydroxide and boric acid were obtained from Koch-Light Laboratories; nitrocellulose paper from Schleicher and Schuell Inc.; 0.25mm silica gel thin-layer chromatography (TLC) plates were obtained from Camlab; En^{3}hance autoradiography enhancer was supplied by Du Pont UK Ltd.; Ampicillin (penbritin) was obtained from Beecham Research Labs. Ltd.; tris-[tris(hydroxymethyl-aminomethane)] was supplied by Boehringer Mannheim; double-stranded polydI:polydC was purchased from Pharmacia; Formamide from Fluka; Actinomycin D was from Merck Sharp and Dohme and cycloheximide was from Sigma; 2'-deoxyribonucleoside 5'-triphosphates, unlabelled dNTPs and ultrapure dNTP set, ribonucleoside 5'triphosphates, universal sequencing primer, and 7-deaza-dGTP were purchased from Pharmacia LKB Ltd.

8. Radiochemicals

Radiolabelled compounds were supplied by Amersham International plc at the following specific activities:

5'[α-^{32}P] dNTPs, 3000 Ci/mmol (10µCi/µl)

5'[gamma-^{32}P] ATP, 5000Ci/mmol (10 µCi/µl)

L-^{35}S-Methionine, around 800mCi/mmol

^{14}C Chloramphenicol, 45 mCi/mmol (from NEN DuPont)

9. Enzymes

Restriction enzymes were purchased from Bethesda Research Labs. or New England Biolabs., except for *Afl*III, which was obtained from Northumbria Biologicals Ltd. Proteinase K, Klenow fragment DNA polymerase (LFP), DNA polymerase holoenzyme, T4 polynucleotide kinase, and T4 DNA ligase were from Boehringer Mannheim. Bacterial alkaline phosphatase (BAP) was obtained from Worthington and purified by M. Dunlop. RNAse and lysozyme were supplied by Sigma.

10. Solutions

Bradford's Reagent: 0.01% Coomassie brilliant blue G, 0.003% SDS, 4.75% (v/v) ethanol, 8.5% (v/v) phosphoric acid

CLB (cell lysis buffer): 0.5% SDS, 20mM Tris-HCL (pH 7.5), 2mM EDTA

DC buffer: 5% glycerol, 1mM β-mercaptoethanol, (50mM-2M NaCl) in 1x TE

50x Denhardt's: 1% polyvinylpyrrolidone, 1% BSA, 1% Ficoll

20x dNTPs: 1mM dATP, dCTP, dGTP and dTTP

5x gel loading buffer: 1xTBE, 1% SDS, 50% glycerol plus bromophenol blue

Gel soak I: 200mM NaOH, 600mM NaCl

Gel soak II: 1M Tris.HCl (pH 7.5), 600mM NaCl

Gel Elution Buffer: 0.5M Ammonium Acetate, 20mM Tris.HCl (pH 7.5), 2mM EDTA

Giemsa stain: 1.5% suspension of Giemsa in glycerol,

heated at 56°C for 2h and diluted with an equal volume of methanol

2x HBS: 280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4 pH to 7.12

Hybridization mix: 6x SSC, 5x Denhardt's, 0.1% SDS

HZ stop: 0.3M NaAcetate (pH 5.5), 0.1mM EDTA, 25µg/ml tRNA

5x Kinase buffer: 350mM Tris.HCL (pH 7.5), 50mM $MgCl_2$, 25mM DTT

5x Ligase buffer: 100mM Tris.HCl (pH 7.5), 50mM $MgCl_2$, 50mM DTT, 3mM ATP

NE buffer A: 10mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5% NonidetP40

NE buffer C (0.45): 20mM HEPES (pH 7.9), 25% glycerol, 0.45M NaCl, 1.5mM $MgCl_2$, 0.5mM PMSF, 0.5mM DTT

NE buffer C (0.15): As C (0.45) but containing 0.15M NaCl

NT Stop: TE plus 0.1% SDS

PBS-A: 170mM NaCl, 3.4mM KCl, Na_2HPO_4, 2mM KH_2PO_4 (pH 7.2)

PBSc: PBS-A plus $CaCl_2.H_2O$ and $MgCl_2.6H_2O$ at 1g/l

PEG/NaCl: 20% polyethylene glycol in 2.5M NaCl

BRL REact™ Buffers (1x concentration):

REact 1: 50mM Tris.HCl (pH 8), 10mM $MgCl_2$

REact 2: 50mM Tris.HCl (pH 8), 10mM $MgCl_2$, 50mM NaCl

REact 3: 50mM Tris.HCl (pH 8), 10mM $MgCl_2$, 100mM NaCl

REact 4: 20mM Tris.HCl (pH 8), 5mM $MgCl_2$, 50mM KCl

REact 10: 100mM Tris.HCl (pH 8), 10mM $MgCl_2$, 150mM NaCl

RGB (SDS-PAGE resolving gel buffer): 181.5g Tris, 4g SDS, 1l H_2O pH to 8.9 at RT with (HCL)

SDS boiling mix: 1ml SGB, 0.8ml 25% SDS, 0.5ml

2-mercaptoethanol, bromophenol blue

SDS tank buffer: 6.32g Tris, 4g glycine, 1g SDS, 1l H_2O

SGB (SDS-PAGE stacking gel buffer): 59g Tris, 4g SDS, 1l H_2O pH to 6.7 at RT

20x SSC: 174g/l NaCl, 88.2g/l TriSodium Citrate

STET: 8% Sucrose, 5% Triton X-100, 50mM EDTA (pH 8), 60mM Tris.HCl (pH 8)

5x SUPER buffer: 50mM Tris.HCl, 5mM EDTA, 1.5M NaCl, 0.5% NP40 pH to 7.5

TBE: 125mM Tris, 40mM boric acid, 2.7mM EDTA, not pH'd

TBS: 25mM Tris.HCl (pH 7.5), 137mM NaCl, 5mM KCl, 0.7mM $CaCl_2$, 0.5mM $MgCl_2$, 0.6mM Na_2HPO_4, pH to 7.4 with HCl

TE: 10mM Tris.HCl, 1mM EDTA (pH 8)

TE/5: 2mM Tris, 0.02 mM EDTA (pH 8)

TEN: 150mM NaCl, 40mM Tris.HCL, 1mM EDTA pH 7.5

TEP: 1mM Tris.HCl, 0.1mM EDTA pH 7.5

T4 Pol buffer: 33mM Tris Acetate (pH 7.9), 66mM Na Acetate, 10mM MgAcetate, 100mg/ml BSA 0.5mM DTT

Trypsin: 0.25% (w/v) trypsin dissolved in Tris-saline

Versene: 0.6mM EDTA in PBS-A plus 0.002% (w/v) phenol red

2B. METHODS

1. Tissue Culture

Baby hamster kidney BHK-21 (C13) cells were routinely grown in EC10 medium at 37°C, in 175cm² (Nunclon) flasks, in an atmosphere of 95% air, 5% CO_2. Confluent monolayers were harvested in 10ml EC10 after two Versene washes and brief trypsinization with trypsin:versene (1:4). Cells were resuspended by pipetting and remained viable for at least

five days stored at 4°C. This suspension was used to seed further monolayers.

HeLa, Vero, HFL and CV1 cells were routinely passaged in 175cm² (Nunclon) flasks in appropriate medium and harvested as described above. Cells did not remain viable at 4°C and for long term storage harvested cells were pelleted and resuspended in medium plus 5% glycerol and 15% foetal calf serum, aliquoted and frozen slowly to -140°C. Cells were recovered by rapid thawing and resuspension in growth medium.

2. Preparation of Stocks of Infectious Virus

2.1. HSV-1 and HSV-140

An 80% confluent monolayer of BHK cells in an 850cm² roller bottle was seeded with HSV-1 at a moi of 0.003pfu/cell or HSV-140 at a moi of 5 particles/cell (ppc), and incubated for 2-4 days until the cells exhibited obvious cytopathic effect (cpe). The cells were shaken into the medium and pelleted at 2K rpm/ 5 min/ 4°C. Cell associated virus (CAV) was prepared by sonicating the pellet in 1.5ml EC10. Cell released virus (CRV) was pelleted from the supernatant at 12K rpm/ 2 hours/ 4°C and gently resuspended in 2ml supernatant and sonicated. Sterility checks were performed by streaking virus preparations on blood agar plates and incubating at 31°C for 5 days. CAV and CRV virus stocks were stored at -70°C.

2.2. VZV

Stocks of VZV-infected HFL cells were produced by infecting subconfluent HFL monolayers (in a 175cm² flask) with VZV-infected HFL cells. These were incubated until a cpe of 60-80% had developed (normally 4-5 days). Infected cells were harvested using trypsin and versene as described above (Section 2B.1). Infection was continued in another flask of subconfluent cells (as required) by addition of 1/6 of the product of a 175cm² flask. The remaining cells were pelleted and stored at -140°C as described above (Section 2B.1). Stocks of VZV-infected CV1 cells were produced in an identical manner.

3. Titration of HSV-1 Virus Stocks

BHK cells were seeded at 10^6 cells per 35mm plate in 2.5ml medium. The following day cells were infected with serial 10-fold virus dilutions in 0.3ml medium; after 1h absorption time (gently mixing every 10 minutes) plates were overlaid with 2ml EH5, to prevent secondary plaque formation, and incubated for 2-3 days prior to fixing with cidex and staining with Giemsa stain. The plates were washed after 15 minutes and plaques counted using a dissection microscope.

4. Preparation of Total Infected Cell DNA

10mm linbro wells were seeded with around $4x10^5$ BHK cells and infected with HSV-1 or HSV-140 the following day. For preparation of total cellular DNA during construction and purification of HSV-140 (for analysis by Southern blotting), infections utilized serial dilutions of small scale virus preparations as described in the text. In the case of viral DNA replication experiments, cells were infected with 10pfu/cell HSV-1 or 100ppc HSV-140. When cpe became apparent, the supernatant was removed and stored as a CRV stock. 0.2ml of cell lysis buffer (CLB) containing 0.25mg/ml proteinase K was added to the cell layer and incubated at 37°C for 3-5 hours. The glutinous extract was transferred to a 1.5ml Eppendorf tube and 15µl of 5M NaCl added. Serial 30 minute phenol then chloroform extractions were performed, with gentle inversion. DNA was precipitated with 2.5 volumes of ethanol, washed with 70% ethanol, dessicated briefly, and resuspended in 50ul of TE overnight at 4°C.

5. Analysis of Virus Induced Polypeptides

5.1. Immediate-early polypeptides

IE extracts were made from BHK cells infected at 38.5°C. The best results were obtained when monolayers of cells at 50-70% confluence were infected. Thus 10mm linbro wells were seeded at $3-4x10^5$ cells and incubated at 37°C. The following day cells were pretreated with 100µg/ml

cycloheximide in growth medium (EC10/CHX) for 15-30min prior to infection with 20pfu/cell HSV-1 or 100ppc HSV-140, also in EC10/CHX (in a total volume of 100µl). After a 1h absorption (with frequent mixing) cells were washed and overlaid with 0.5ml EC10/CHX. At 5h p.a. cells were washed 4x with prewarmed PBSc, with the final wash containing 2.5ug/µl Actinomycin D. 200µl of PBSc containing 2.5 ug/ml Actinomycin D and 50µCi [^{35}S]methionine was added to each well and cells incubated at 38.5°C. Finally, after a 1h labelling period, cells were washed 3x with PBSc and 100µl of SDS boiling mix added. Samples were transferred to Eppendorf tubes and stored at -20°C.

5.2. Pulse Labelling of Polypeptides

10mm linbro wells were seeded with around 5x10^5 BHK or HFL cells and infected the following day with HSV-1 or HSV-140 at the required multiplicity. Following a 1h absorption period (mixing every 10 minutes) medium was replaced and the infection allowed to proceed at 37°C. At the appropriate time p.a. (as specified in the text), medium was removed and cells washed with prewarmed PBSc, and 200µl of PBSc containing 50µCi [^{35}S]methionine added to each well and cells incubated for a further 2h at 37°C. Finally, cells were washed and harvested as described above.

5.3. SDS Polyacrylamide Gel Electrophoresis

Based on the procedure of Marsden *et al.*, (1978). [^{35}S]methionine labelled polypeptides were resolved on 0.75 mm thick 7.5% single concentration polyacrylamide gels using a Bio-Rad Mini Protean™ II electrophoresis kit, following the manufacturer's instructions. Gel plates were carefully washed with Decon then ethanol, and allowed to dry. The resolving and stacking gels were made up as follows:

For two gels:

	Resolving gel	Stacking gel
30% (a+b) stock (2.5% bis acrylamide)	2.5ml	0.4ml
RGB	2.5ml	
SGB		0.6ml
distilled water	5ml	1.4ml
10% APS	80µl	20µl
TEMED	8µl	3µl

The resolving gel was overlaid with butan-2-ol and allowed to set. After washing, the resolving gel was overlaid with the stacking gel, and wells created with a teflon comb.

Protein samples were boiled for 2min prior to loading around $2x10^5$ cell equivalents per cm of gel. Gels were run at a constant voltage of 200V in freshly prepared SDS tank buffer until the dye front was approximately 0.5cm from the bottom of the gel. Gels were fixed for 10 minutes in ethanol/ acetic acid/ H_2O (1:4:35) before drying down under vacuum at 80°C onto a sheet of Whatman 3MM paper and exposing to Kodak X-Omat S XS-1 film for 18-72h.

6. Transfection of Cells With DNA

6.1. Transfection of HeLa, Vero and BHK Cells for CAT Assays

Cells were seeded at a density of 10^6 cells per 50mm petri dish and the following day the rapidly dividing, subconfluent monolayers were transfected by the calcium phosphate precipitation method (Corsalo and Pearson, 1981). The amounts of CAT reporter plasmids and test plasmids used in experiments are given in the relevant Sections of the Results. The amount of DNA in each of the transfection mixes within an experiment was equalized by the addition of pUC9 where appropriate. TEP buffer was added to the plasmid DNA to a final volume of 140ul, then 20µl of 2M $CaCl_2$ added to the solution whilst vortexing. This was then added dropwise to 160ul of 2xHBS (vortexing), and the resulting mixture left to stand at RT for 30 minutes. The

precipitate thus formed was added dropwise to the media in the culture dishes, mixed thoroughly to ensure an even distribution, and the cells incubated at 38.5°C. The cells were washed 2x with TBS after 24h and fresh medium replaced, and after a further 24h at 37°C extracts were prepared by washing the cells in PBS-A then scraping into 3ml TEN. The cells were pelleted at 2K rpm/ 4°C/ 1min, then resuspended in 0.25M Tris pH 7.8 and sonicated. The sonicates were cleared by a 2min microfuge spin and stored at -20°C.

6.2. Transfection of F.HeLa Cells for Protein Extracts

Transfections were carried out as described above (6.1) but the volumes increased 3-fold for 90mm plates and 7-fold for 135mm plates. 30 or 60µg of plasmid p140 were used, with 15 or 30µg pUC9 as carrier.

6.3. Transfection of Cells with Viral DNA for Marker Rescue

BHK cells seeded at a density of $2x10^6$ cells per 50mm dish were transfected by the method of Stow and Wilkie, (1976). A mixture of approximately 1ug of linearized pGD140, 3µg of intact D30EBA viral DNA (kindly provided by Dr. T. Paterson) and 5µg of calf thymus carrier DNA were precipitated as described above (6.1). After 30 minutes medium was removed from the cells and the precipitate added, the cells were then incubated at 38.5°C for 4h, being overlaid with 2ml medium after 40 min. The cells were then washed once with growth medium and overlaid with 25% DMSO in HBS for 4min. The cells were then washed a final time and overlaid with growth medium. After 2-4 days at 37°C the monolayers developed extensive cpe, and the cells were scraped into the medium and sonicated to release infectious virus for further analysis.

7. CAT Assays

Cell sonicates prepared as described above (6.1) were analysed for chloramphenicol acetyl transferase (CAT) activity essentially as described by Gorman *et al.* (1982).

Assay mixtures contained 1µl 50mM acetyl-CoA, 0.5ul of stock [^{14}C]chloramphenicol and 14µl distilled H_2O together with 25ul neat or diluted sonicate. After incubation for 30 minutes at 37°C the products were extracted with 200µl ethyl acetate, dried down and resuspended in 20µl ethyl acetate to spot onto thin layer chromatography (TLC) plates. TLCs were run in 95% chloroform/ 5% methanol (A/R), air dried and autoradiographed at room temperature. The percentage conversion was determined by cutting out non-acetylated substrate and 3'-monoacetylated product from the TLC plate and quantitating radioactivity in the resulting strips of TLC plate by scintillation counting in 2ml Ecoscint. The protein concentration of the extract was determined by the method of Bradford (1976; see Section 8 below) and the percentage conversion from substrate to product per ug protein calculated.

8. Determination of Protein Concentrations in Cell Extracts

The protein concentration of CAT extracts, whole cell extracts and nuclear protein extracts was determined by the method of Bradford *et al.* (1976). 5-10µl of extract was made up to 100ul in 0.25M Tris.HCl pH 7.8 and 1ml of Bradford's reagent added and mixed. After a 15min incubation the OD at 595nm was measured in disposable plastic cuvettes by spectrophotometry. The protein concentration of extracts were determined by constructing a standard curve using standard concentrations of BSA (5-80µg) assayed as above, and OD plotted against concentration mg/ml.

9. Preparation of Whole Cell and Nuclear Extracts for DNA Binding Studies and Purification

9.1. Standard Protocol

Nuclear extracts were prepared from infected or transfected cells by a procedure modified from that of Dignam *et al.* (1983). Cells scraped from 90mm or 135mm culture dishes were washed in PBSc, pelleted and resuspended in 2 volumes NE buffer A without NP40. NP40 was

added to a final concentration of 0.5% and the cells lysed on ice for 10 minutes. Nuclei were then pelleted by successive 2K/10min and 12K/20min spins in the Sorval/SS34. Nuclei were resuspended in 1.5 volumes NE buffer C (0.45) and nuclear proteins eluted by incubating for 30 minutes on ice before clearing with a 30 minute 15K rpm spin (Sorval/SS34). The supernatants were flash frozen and stored at -140°C.

Whole cell extracts containing both nuclear and cytoplasmic proteins were prepared by lysing cells directly in NE buffer C containing 0.5% NP40 then proceeding as above.

9.2. Modified Protocol

Cells were lysed in NE buffer A and nuclei isolated as above. Nuclei were resuspended in 1.5 volumes NE buffer C (0.15) (rather than buffer C (0.45)) and incubated on ice for 15 minutes prior to pelleting nuclei with a 15K/15min spin. This procedure was repeated twice more and nuclei then resuspended in NE buffer C (0.45) and a high salt extract isolated and stored as above.

10. Gel Retardation Assay

Probes for gel retardation assays were prepared by end labelling appropriate DNA fragments (see Section 3E) by filling in (see Section 18.2) and purification on 5-8% polyacrylamide gels and a Sephadex G-50 "nick" column (Pharmacia)(Section 2B.15). Standard binding reactions were performed at 0°C or 20°C for 20 minutes in a 20-30µl mix containing 1-5µg poly(dI):poly(dC), approximately 0.1ng probe (2000cpm) and 1-4µl nuclear extract (2-16µg protein). Samples were resolved on 4% native polyacrylamide gels (3.3% cross linker) run in 0.5x TBE, and dried down before autoradiography. The specific details of alterations to this protocol are given in the text.

11. Large Scale Plasmid Preparation

Isolated single bacterial colonies on plates selective for a plasmid-borne resistance marker (generally amp^r) were inoculated into 5ml L-broth and grown overnight. These cultures were added to 300ml L-broth in a 2l flask and grown shaking at 37°C until the cells reached an OD_{650} of approximately 0.8. Then chloramphenicol was added to a final concentration of 100µg/ml and the cultures left shaking overnight to amplify the plasmids.

Plasmid DNA was prepared by the "maxi-boiling" technique of Holmes and Quigley (1981). Cells were pelleted at 5K rpm/ 6min (Sorvall GS3 rotor) and resuspended in 17ml STET per 300ml culture. 2ml of 10mg/ml lysozyme in STET was added for 1 minute prior to bringing the suspension to the boil for 40 seconds. The lysate was cleared with a 45min/ 18K rpm/ 4°C spin (Sorvall SS34 rotor) and DNA precipitated from the supernatant by addition of 0.9vol isopropanol. The DNA was pelleted at 3K rpm/ 4min/ RT (MSE Coolspin) and the pellets drained well before resuspending in TE and adding CsCl to give a final density of 1.6g/ml and EtBr to 0.5mg/ml. The solution was incubated on ice for 15 minutes and then cleared with a 3K rpm/ 10min/ 4°C spin (MSE Coolspin). The DNA was banded by spinning at 40K rpm/ 15°C/ 16 hours in the vertical TV865 rotor. The DNA was visualized by daylight or long-wave UV illumination and the lower, supercoiled plasmid DNA band collected with a large bore needle and syringe (after removal of the upper, chromosomal DNA band if necessary). EtBr was extracted from the DNA with 2x 2.5 volumes of Butan-1-ol (TE saturated), the DNA dialysed against TE at RT for 2h, treated with 10µg/ml RNAse (1h/65°C), then proteinase K (1ug/ml) plus 0.1% SDS (1h/37°C), before extraction with phenol/chloroform then chloroform and precipitation with ethanol. The pellet was resuspended in 400ul 0.3M NaAcetate and DNA again ethanol precipitated, washed with 70% ethanol, lyophilized and resuspended in 300µl TE overnight. The concentration of the DNA was determined by spectrophotometry (OD_{260} 1.0=50µg DNA/ml).

12. Miniprep Plasmid DNA Preparation

Single plasmid-transformed bacterial colonies were grown overnight in 3ml L-broth at 37°C with shaking. 1.5ml stationary phase culture was aliquoted into a 1.5ml Eppendorf tube and cells pelleted at 12K rpm/ 15sec (benchtop minicentrifuge). Cells were resuspended in 200ul STET, 5µl of lysozyme (10mg/ml) added, and the suspension immediately placed in a 100°C water bath for 45 seconds, then centrifuged at 12K rpm/ 10min. The glutinous pellet was removed and DNA precipitated from the supernatant by addition of 0.9vol isopropanol and a 12K rpm/ 5min spin. The pellet was washed with 70% ethanol, dried and resuspended in 20µl TE. Miniprep DNA was stored at -20 or 4°C and 2-5µl used for restriction enzyme analysis (0.2µl 1mg/ml RNAse was added to digestions of miniprep DNA).

13. Restriction Enzyme Digests

Restriction enzyme (RE) digests were generally carried out in the recommended BRL REact™ buffer, using 0.5µg DNA and 1 unit of enzyme for 1h at the appropriate temperature. Preparative rather than diagnostic RE digestions used increased amounts of DNA and RE under similar conditions, in 20-50ul volumes.

14. Separation and Preparation of Purified DNA Restriction Fragments

14.1. Non-denaturing agarose gels

Slab gels of 0.5-2.0% agarose in 1xTBE (or 1x L-buffer for southern transfers) were run submerged at up to 12v/cm. Gels were run for 1-3h until the dye was around 0.5cm from the end, and then stained for 10min in 1ug/ml EtBr and examined and photographed with short-wave UV illumination (long-wave UV was used to reduce DNA damage when preparative gels were being analysed).

Following resolution, DNA fragments were isolated as follows. A strip of DE-81 paper (presoaked in 2.5M NaCl overnight, then stored in 1mM EDTA at 4°C) was inserted in front of the band of interest, the gel reassembled and the

fragment electrophoresed onto the paper (15-30mins). The paper was then pulverized and the fragment eluted by shaking overnight in 1.5M NaCl/ 1x TE, following which the solution was filtered through siliconized glass wool, clarified and the fragment ethanol precipitated.

14.2. Non-denaturing polyacrylamide gels

Fragments of 40-400bp in size were resolved in 4-8% polyacrylamide (acrylamide:bis-acrylamide, 19:1) gels run in 1x TBE. Gels were prepared in 1.5mm thick vertical glass plate sandwiches, and cross-linked with 0.001 vol TEMED catalysed with 0.01vol 10% APS. Samples were loaded and electrophoresed at a maximum voltage of 16V/cm, and DNA visualized by autoradiography or EtBr staining. ^{32}P-labelled DNA fragments were cut out of the gel after detection by autoradiography and purified in one of two ways. Fragments for sequencing were recovered from polyacrylamide strips by extrusion through a pierced 0.4ml vial into a 1.5ml tube (benchtop minicentrifuge/ 12K rpm/ 5mins). The DNA was eluted by shaking overnight in TE at 37°C, the slurry filtered through siliconized glass wool and DNA precipitated. Probes for gel retardation assays were eluted into gel elution buffer (2x 3h/45°C incubations) after dicing the polyacrylamide strips. The supernatant was then purified on a Sephadex G-50 column (Section 2B.15).

14.3. Denaturing polyacrylamide gels

The products of DNA sequencing reactions were resolved in 0.35mm thick gels prepared to a final concentration of 8-20% acrylamide (acrylamide:bis-acrylamide, 19:1) in 1x TBE containing 7M urea. The acrylamide was filtered through a 0.45 micron filter and degassed prior to polymerization, and the completed gel pre-run at 40W for 30min. Sequencing reaction samples were denatured at 90°C for 2min, and loaded onto the gel and run at 40W for 2-5h. Gels were covered with cling-film and autoradiographed at -70°C with screens.

15. Sephadex G-50 Columns (gel exclusion chromatography)

Sephadex G-50 "nick" columns obtained from Pharmacia were used to purify ^{32}P-labelled fragments for use as probes in gel retardation assays, and also to purify nick-translated plasmid probes used in southern blotting. Columns for purifying gel retardation probes were run in distilled H_2O, whereas all other columns were run in TE plus 0.1% SDS. 100-800ul of sample were loaded onto the column and the fastest eluting fractions (detected by scintillation counting) taken as purified DNA. Nick-translated probes were used directly. Gel retardation probe fractions were pooled, ethanol precipitated in the presence of 1ug of poly(dI):poly(dC), and resuspended in a suitable volume (usually 100µl) and stored at -20°C prior to use.

16. DNA Ligations

DNA ligations were performed in 10µl 1x ligase buffer (which contains 0.6mM ATP) with 50-500ng DNA and 1 unit of T4 DNA ligase, for 1-2h at RT or overnight at 16°C.

17. Preparation and Transformation of Competent *E.coli* strain HB101

A sample of overnight culture of bacteria was streaked out onto a nutrient agar plate, and incubated overnight at 37°C. The following day a single colony was diluted into fresh L-broth plus 10mM $MgCl_2$ / 10mM $MgSO_4$ and and aerated vigorously at 37°C until reaching an OD_{540} of 0.3. Using sterile, ice cold procedures throughout, cells were pelleted (5K rpm/ 2°C/ 10min), resuspended in 1/4 vol 0.1M $MgCl_2$ and immediately pelleted again (5K rpm/ 2°C/ 10min). Cells were resuspended in 1/20 vol 0.1M $CaCl_2$ and left on ice for 45min before again pelleting cells (5K/ 2°C/ 10min. Cells were resuspended in the same volume of 0.1M MOPS/ 50mM $CaCl_2$/20% glycerol and left on ice for 20 min prior to dispensing 50-200µl aliquots into sterile Eppendorf reaction vials, snap freezing and storing at -70°C.

Transformations generally utilized stored, frozen competent cells. 100-200µl of competent cells (as required) were thawed on ice, 100-500ng of plasmid DNA (either ligation mix or purified plasmid) added, and the mixture left on ice for 20 minutes. Then cells were heat-shocked at 37°C for 1min, 500µl of prewarmed L-broth added and the mixture incubated at 37°C for 30mins prior to plating the mixture out onto antibiotic (usually ampicillin) containing nutrient agar plates. Plates were incubated at 37°C overnight following which they could be stored for several months at 4°C.

18. Radioactive Labelling of DNA

18.1. 5'-end labelling

This approach was used to label DNA fragments from insertion mutant plasmids prior to chemical sequencing. In order to label DNA fragments at the 5'-end the 5'-phosphate was first removed by treatment with bacterial alkaline phosphatase (BAP) for 1h at 65°C in TE pH 8.0 (or in react 1, 2 or 3). The sample was diluted to 100µl and extensively extracted with phenol then chloroform to remove BAP, and ethanol precipitated to concentrate. The precipitated DNA was resuspended in 10-20µl 1x kinase buffer containing 10-50µCi [gamma-^{32}P]dATP and 1 unit of T4 polynucleotide kinase (37°C/1h). The labelled DNA was purified from unincorporated label by gel electrophoresis, ethanol precipitation in the presence of 1ug carrier tRNA, or by running on a Sephadex G-50 column.

Oligonucleotides were phosphorylated in a similar fashion. For use as probes in plaque hybridization assays, oligonucleotides were phosphorylated by addition of oligonucleotide, 5x kinase buffer and enzyme direct to [gamma-^{32}P]dATP to ensure labelling to high specific activity. 0.5mM cold ATP replaced label in the reaction mixture when oligonucleotides were to be phosphorylated for cloning purposes. The reaction was stopped at 70°C.

18.2. 3'-end labelling

3'-ends were labelled by filling in 5'-overhangs with either klenow polymerase or T4 DNA polymerase. Digests of 1-2µg of plasmid DNA were filled in after heat-inactivating the restriction enzymes at 65°C/10min. Klenow reactions were carried out in REact™ 2 buffer, and used 1 unit of enzyme incubated at RT for 20min with appropriate hot 5'-[α-^{32}P]- and cold dNTPs followed by a 20min chase with all four cold dNTPs. T4 DNA polymerase reactions differed in that they were performed in T4 DNA polymerase buffer at 37°C for 1h. Both enzymes were inactivated at 65°C/10min prior to second enzyme digestions. The labelled DNA fragments were finally purified by gel electrophoresis.

18.3. Internal labelling by nick translation

Plasmid DNA was radiolabelled by the method of Rigby *et al.* (1977). Labelling reactions were performed in 1x REact™ 1 buffer and included 100ng of plasmid DNA, 10µCi of all 5'-[α-^{32}P]dNTPs, 1 unit *E.coli* DNA polymerase holoenzyme and 5x10^{-7} µg/ml DNAse I in a final volume of 20µl. Following incubation for 1h at RT the reaction was stopped by addition of 100µl of NT stop and labelled DNA purified on a Sephadex G-50 column (Section 2B,15).

19. Chemical Sequencing of DNA

The nucleotide sequence of 5' or 3'-end labelled DNA fragments derived from insertion mutant plasmids (pVI1-pVI8) was determined by the chemical methodology of Maxam and Gilbert (1977, 1980). Uniquely end-labelled DNA restriction fragments were prepared by filling in or phosphorylating a cut RE site (see Sections 18.1 and 18.2 above) then cutting with a second enzyme to yield fragments 50-500bp in length labelled solely on one strand. These were then purified as described (Section 14.2) and 20-50,000 cpm aliquots reacted to modify either purines or cytosine bases. For the purine reaction the DNA was resuspended in 2% Diphenylamine/ 1mM EDTA/ 66% formic acid

for 2min at 25°C. The DNA was then ether extracted and lyophilized. Cytosine residues were modified by treatment with hydrazine in the presence of 1.5M NaCl (2min/25°C), stopped by addition of 0.3M NaCl and 0.1M EDTA and ethanol precipitated. The partially modified DNA was then resuspended in fresh 1M piperidine at 90°C for 30min to cleave the DNA at the modified bases. The products were lyophilized three times, reconstituted in 5µl of formamide dyes mix, and 2µl resolved on a denaturing polyacrylamide gel of appropriate concentration (Section 14.3).

20. Transformation of *E.coli* Strain JM101

Ligation mix containing 50-500ng of M13 replicative form (RF) DNA was added to 100ul of competent *E.coli* JM101 cells and the mixture left on ice for 20min before heat-shock at 42°C/ 40 seconds. Half of this transformation mix was added to a prewarmed tube (42°C) containing 3ml top agar, 50µl of a fresh overnight culture of JM101, 25µl of X-gal and 10µl of IPTG. The contents were mixed and poured onto an L-broth agar plate which was gently swirled to ensure an even distribution of bacteria and top agar. The top agar was left to set for 10min, the plate inverted and incubated at 37°C overnight. Blue plaques and clear plaques developed. The clear plaques harboured bacteriophage containing insertions into the vector cloning sites within *lacZ* coding sequences.

21. Small-Scale Preparation of Replicative-form and Template DNAs from JM101 Bacteria Transformed with M13 Vectors

20µl of a fresh overnight culture of *E.coli* JM101 bacteria was added to 100ml of 2YT broth. Single, isolated plaques, freshly picked with a sterile pasteur pipette, were added to 1.5ml aliquots of this suspension in sterile universals. The cultures were incubated with shaking at 37°C/5-6h. 1ml of the final infected culture was transferred to a 1.5ml Eppendorf vial and centrifuged twice at 12K rpm/2min, turning the tube through 180° between

spins. (The residual culture was retained as a stock of bacteriophage.) Single-stranded template DNA and replicative form (RF) DNA were then prepared as follows:

Template DNA

800µl of the supernatant were transferred to a fresh tube containing 200µl of 20% PEG/ 2.5M NaCl, mixed and left for 30min at RT. Precipitated bacteriophage were recovered by centrifugation at 12K rpm/ 10min in a minicentrifuge. All of the supernatant was carefully removed using a sterile disposable pipette tip and the pellet of bacteriophage resuspended in 100ul of TE. 100µl of phenol (equilibrated with TE) was added, the contents of the tube mixed well and then centrifuged at 12K rpm/ 10min. The upper aqueous phase was transferred to a fresh tube, 10µl of 4M NaAcetate and 250µl of ethanol added, the mixture vortexed and again centrifuged at 12K rpm/ 10min. The pellet of single-stranded bacteriophage DNA was washed with 70% ethanol, dried and resuspended in 50µl TE/5.

RF DNA

The pellet of infected bacteria from above was resuspended in 200µl STET/ lysozyme and RF DNA prepared by the method described in Section 12.

22. Dideoxy Sequencing of DNA

The products of site-directed mutagenesis experiments were sequenced by the dideoxynucleotide chain termination method of Sanger *et al.* (1977). Single-stranded template DNA was prepared from a 1.5ml culture in 2YT broth (as described in 21 above) and dissolved in 50µl TE/5. 2µl of this template DNA was annealed to 2.5ng of the M13 single-stranded universal primer in a final volume of 10µl 1x M13 sequencing buffer (10mM Tris.HCl pH 8.5, 10mM $MgCl_2$) at 37°C/ 30min. 2 units of Klenow DNA polymerase were added to the annealed DNA and 2µl aliquots of the mixture dispensed into four corresponding T, C, G and A wells of a U-bottomed 96-well microwell plate (Nunclon). To each well

was added 2µl of the appropriate sequencing mix containing dNTPs and specific ddNTPs (see Table below), 0.09µm dATP and 4µCi [α-^{32}P]dATP. The tray was spun briefly and the reaction allowed to proceed for 15min at 31°C. 2µl of chase mix (4x cold dNTPs each at 0.25mM) were added to each well and the incubation continued for a further 30min at 31°C. The reaction was stopped by addition of 2µl formyl dyes mixture (0.1% bromophenol blue (w/v), 0.1% xylene cyanol (w/v) in deionised formamide). The plate was heated at 100°C for 1min and 2-3µl of each reaction resolved on a denaturing polyacrylamide sequencing gel (Section 14.3).

Sequencing Solutions

	dA-0	dT-0	dC-0	dG-0
5mM dTTP	20	1	20	20
5mM dCTP	20	20	1	20
5mM 7-deaza dGTP	20	20	20	1
10x TE(seq)	50	50	50	50
H_2O	540	370	370	370

TE(seq): 10mM Tris-HCl pH8, 0.1mM EDTA

Nucleotide Concentrations in Sequencing Mixes

	dN-0 mix	ddNTP	H_2O
T seq	500	500 (600µMddTTP)	0
C seq	500	105 (140µMddCTP)	395
G seq	500	155 (200µMddGTP)	345
A seq	500	250 (140µMddATP)	250

(ddNTPs sold in solution at 5mM)

23. Oligonucleotide-Mediated M13 Site Directed Mutagenesis

Mutagenesis of VZV gene 62 DNA fragments was performed by the standard double-primer method (Norris *et al.*, 1983; Zoller and Smith, 1987). A suitable fragment containing the region to be mutagenised was cloned into M13mp18 (Norrander *et al.*, 1983). Ligation mix was transformed into competent *E.coli* JM101 (Section 20), recombinant bacteriophage plaques isolated and single-stranded template DNA prepared (Section 21). In order to check the specificity of hybridization of the mutagenic oligonucleotide to the DNA template, a set of DNA sequencing reactions were carried out in which the mutagenic oligonucleotide replaced the M13 universal

primer. Provided this generated the expected sequence the oligonucleotide was used in the mutagenesis protocol described below.

Hybridization of oligonucleotides to template DNA and primer extension

The M13 universal primer and mutagenic oligonucleotides were phosphorylated as described in Section 18.1. 4µl of template DNA and 2µl of each phosphorylated oligonucleotide were diluted to a final volume of 20µl in 1x M13 sequencing buffer (10mM Tris.HCl pH 8.5, 10mM $MgCl_2$). The mixture was incubated at 37°C for 30min to allow the oligonucleotides to anneal to the template. Then 1µl each of T4 DNA ligase, Klenow DNA polymerase, 20x dNTPs and 10mM ATP were added and primer extension and ligation allowed to proceed at 15°C overnight, resulting in formation of double-stranded heteroduplex DNA.

Transformation of JM101 and screening for mutants

2µl of the above material were transformed into competent *E.coli* JM101 as described (Section 20). (In some cases it was necessary to dilute the ligation mix prior to transformation in order to obtain well separated plaques.) The plaques obtained were screened in a plaque hybridization assay (Section 24) using ^{32}P-labelled mutagenic oligonucleotide as a probe. Plaques identified as harbouring recombinant bacteriophage containing the desired mutation probably contained a mixture of mutant and wild type phage at this stage. Thus in order to purify the desired phage, candidate plaques were picked and plated out as follows. Plaques of interest were picked into 100ul of 2YT broth, vortexed and a ten-fold dilution series prepared in 2YT. 50µl of each dilution were then added to prewarmed (42°C) tubes containing 3ml top agar and 50µl of a fresh overnight culture of JM101 and plated onto LB-agar plates. Following incubation at 37°C/ overnight, a plate containing 30-100 plaques was selected for further analysis.

Confirmation of the presence of the mutation

Plaques from above were picked and RF and template DNAs prepared as described in Section 21. RF DNAs were analysed by RE digestion for the presence of a novel restriction site or a deletion in order to identify phage containing the desired mutation. The presence of the mutation was confirmed by dideoxy sequencing (Section 22) of the corresponding template.

Large scale preparation of RF DNA containing the mutation

Residual culture corresponding to the RF and template DNA preparations which contained the mutation (see above) was used as a stock of bacteriophage to infect cultures of JM101 for large scale preparation of mutant RF DNA. 5µl of residual culture was inoculated into 200ml 2YT broth containing 100ul of a fresh overnight culture of JM101. Following incubation at 37°C for 5h with vigorous aeration, infected cells were isolated and by centrifugation at 5K rpm/ 6min (GS3/Sorvall), resuspended in 17ml STET and RF DNA prepared by the method described in Section 11.

24. M13 Plaque Hybridization Assay

Following the primer extension/ligation reaction an aliquot of the resulting double-stranded DNA was transformed into JM101 and plated out as described (Section 23). Plates were incubated at 37°C overnight and then placed at 4°C for 15min. A nitrocellulose filter was then carefully placed onto the surface of the plate and the orientation marked. The filter was removed after 5min and air dried for 5min/RT before baking at 80°C in a vacuum oven for 2h. Filters were prehybridized at 37°C/1h in prehybridization buffer (6x SSC/ 0.2% SDS/ 0.5% dried skimmed milk). Following prehybridization, mutagenic oligonucleotide which had been labelled by phosphorylation with [gamma-^{32}P]dATP and T4 kinase (Section 18.1) was diluted to 100µl with TE and added to the prehybridization mixture in the bag. Hybridization was performed overnight

under the same conditions as prehybridization. The following day the filter was washed twice in 6x SSC/ 5min/ RT and then once in 6x SSC/ 5min/ 37°C, and autoradiographed at -70°C with a screen (30-60min). The filter was then washed at increasing temperatures in 6x SSC/ 15min, beginning 10 degrees below the calculated T_M (T_M= 4x[G+C] + 2x[A+T]) for the oligonucleotide and increasing in 2 degree intervals near the T_M. The filter was monitored at each stage with a Geiger counter to determine the point at which counts decreased. At this stage the oligonucleotide was hybridizing selectively to DNA from plaques harbouring mutant phage, and the filter was again autoradiographed at -70°C to allow location of plaques containing mutant phage on the plate.

25. Southern Transfer of DNA to Nitrocellulose

Restricted DNAs for Southern transfer (Southern, 1975) were resolved on agarose gels of the appropriate concentration run in L-buffer. Following electrophoresis the gel was washed in Gel Soak I for 45min then in Gel Soak II for 45min. The DNA was blotted overnight onto presoaked Nitrocellulose paper using 10x SSC, a wick of Whatman 3MM paper and a weighted capillary stack of absorbent paper towelling. The filter was air dried and baked at 80°C for 2h in a vacuum oven prior to hybridization.

26. Immobilization of DNA on Nitrocellulose by Slot-Blot

A Schleicher and Schuell slot-blot apparatus was used to immobilize equivalent quantities of viral infected total cell DNA onto nitrocellulose. DNA was extracted as described above (Section 4) and digested with RNAse. DNA was denatured by addition of 1 vol 1M NaOH (10min/RT) then neutralized with 2 vols of 2M ammonium acetate. The denatured sample was then applied to a presoaked (2x SSC) membrane under vacuum using the slot-blot apparatus, and washed through with 1M ammonium acetate. The filter was then air dried and baked at 80°C in a vacuum oven prior to hybridization.

27. DNA-DNA Hybridization

Nitrocellulose filters were prehybridized in fresh hybridization mix for 2-4h at 65-70°C. 10^7-10^8 cpm of nick translated plasmid probe DNA was denatured by addition of 0.04vol 5M NaOH then neutralized with 5M HCl prior to adding to the filter bag and hybridizing overnight under the same conditions used for prehybridization. The filter was then washed twice at RT in 2x SSC for 10min and twice at the hybridization temperature in 2x SSC plus 0.1% SDS for 1h each before autoradiography at -70°C with screens.

28. FPLC Gel Filtration Chromatography

Gel filtration chromatography was performed on an automated Pharmacia FPLC system using a Superose 12 column under the conditions outlined in Section 3F.

29. Computing

Computer analyses of DNA and protein sequences were performed on the MicroVAX 11 computer in this department using the University of Wisconsin Genetics Computer Group software package.

3. RESULTS

3A. VZV GENE 62 AND PLASMID p140

Much of the work described in this thesis has utilized either plasmid p140 (which contains VZV gene 62 sequences) or derivatives of p140. Therefore, before entering the main results section I will describe in detail the structure of VZV gene 62 and plasmid p140.

3A.1. Gene and Transcript Structure

The VZV 140k polypeptide is encoded by a diploid gene (designated genes 62 and 71), a copy of which is located within each of the repeat sequences bounding the U_S region of the genome (Davison and Scott, 1986). A 4.3kb transcript has been mapped to within these repeats (Ostrove *et al.*, 1985; Reinhold *et al.*, 1988), and its capsite determined to lie 73 nucleotides upstream of the ATG codon (McKee *et al.*, 1990). Gene 62 is located within the internal repeat and is transcribed from the capsite at 109206 towards U_L and the polyadenylation signal at 105147. Translation of the polypeptide is predicted to begin at the ATG codon at position 109133 and to continue to the termination codon at 105204 (Davison and Scott, 1986). The corresponding gene 71 is located within the terminal repeat and is transcribed from the capsite at 120691 towards the genome terminus and polyadenylation signal at 124750. The open reading frame begins at 120764 and terminates at 124693 (Davison and Scott, 1986). The 1310 amino acid residue polypeptide encoded by these open reading frames has a predicted unmodified molecular weight of 140,000 (140k).

3A.2. Plasmid p140

Within this thesis gene 62 nucleotide sequences will be numbered according to the following conventions. Nucleotides within the reading frame will be numbered sequentially from the first base, 1, through to the last base, 3930. Sequences upstream of the reading frame will be

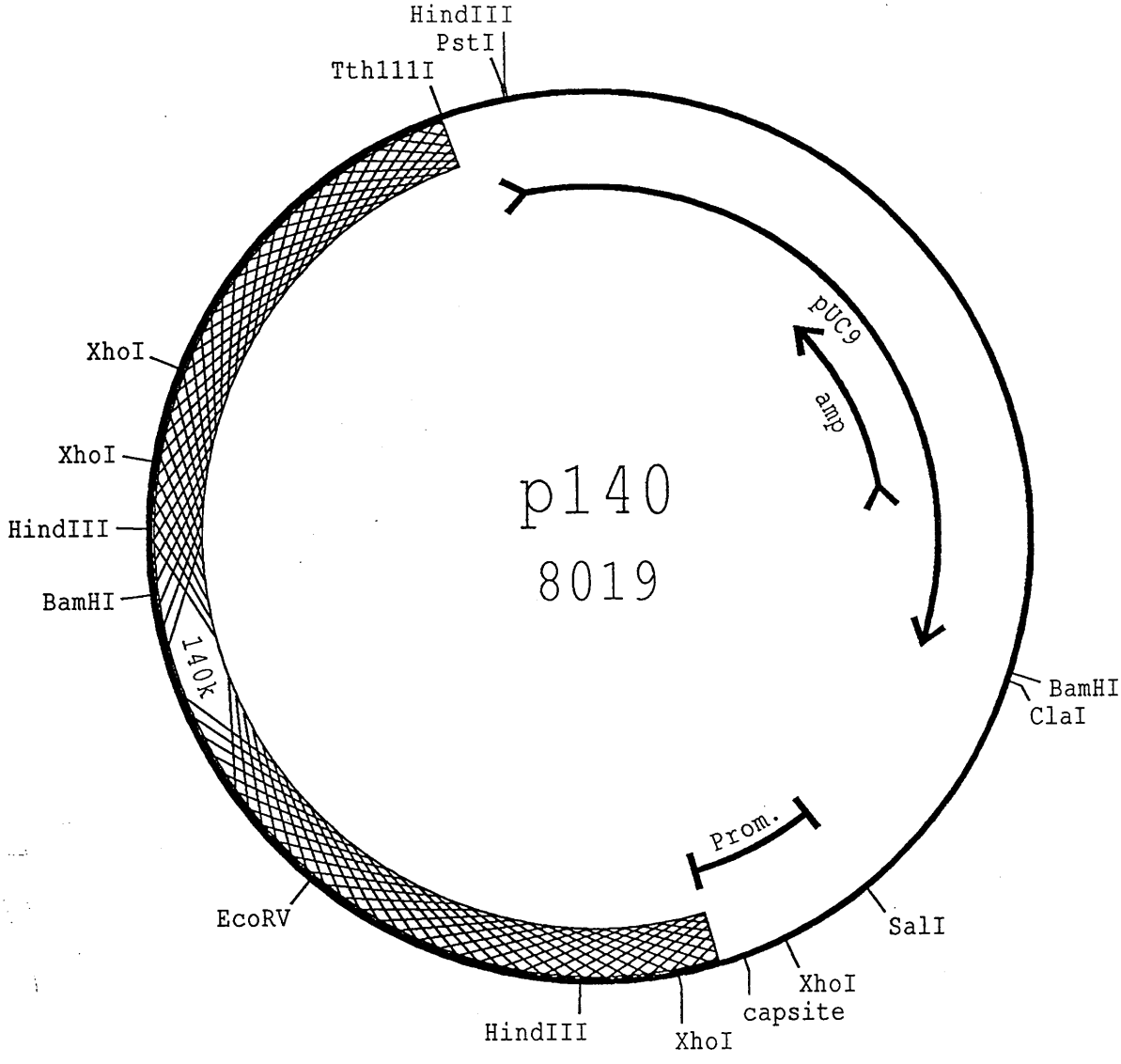
numbered relative to the capsite, which is designated +1, positions upstream of the capsite having negative values, while the positions of nucleotides located between the capsite and the reading frame have positive values.

Plasmid p140 contains VZV gene 62* in a fragment from the *Cla*I site at -1146 to the genome terminal *Pst*I site of pVZVSstf (Everett, 1984) inserted between the *Acc*I and *Pst*I sites of a pUC9 plasmid from which the *Eco*RI site had been removed by cutting and filling in (McKee et al., 1990; see figure 5). Gene 62 sequences in this plasmid are expressed from the authentic gene 62 promoter which has been determined to lie within the region -410 to +57 (McKee *et al.*, 1990). Within p140 there are two *Bam*HI sites which will frequently be referred to when describing cloning procedures in later sections. The first of these is located in the pUC9 polylinker sequences, 5' to the VZV insert, the second being located within the gene 62 coding sequences at position 2198.

* N.B. Gene 71 sequences (which are assumed to be identical to those of gene 62) were actually cloned.

Figure 5. Structure of Plasmid p140. Plasmid p140 (8019 bp) contains VZV gene 62 on a *Cla*I-*Pst*I fragment (-1146 to the genome terminus) derived from pVZVSstf (Everett, 1984) cloned between the *Acc*I and *Pst*I sites of a pUC9 plasmid from which the *Eco*RI site had been removed (McKee *et al.*, 1990). VZV 140k coding sequences are represented by the hatched region. The sequences controlling expression of VZV 140k (which are located between *Sal*I (-410) and the orf) are indicated. The pUC9 vector sequences are marked by the arrow, and the location of the ampicillin resistance gene (amp) within the vector sequences is shown. The locations of some restriction enzyme recognition sites are marked. Plasmid p140 contains two *Bam*HI sites, one of which is located in pUC9 polylinker sequences immediately adjacent (upstream) to the VZV insert, the other within the VZV 140k orf. The *Tth*111I site is located just 2 bp beyond the 3' end of the VZV 140k orf.

A loose copy of this Figure can be found inside the rear cover


HindIII
PstI
Tth111I
XhoI
XhoI
HindIII
BamHI
140k
EcoRV
HindIII
XhoI
capsite
XhoI
SalI
Prom.
BamHI
ClaI
pUC9
amp
p140
8019

3B. CONSTRUCTION AND CHARACTERIZATION OF THE RECOMBINANT VIRUS HSV-140

The overall aim of the work presented in this thesis was to investigate the biochemical and functional properties of the VZV 140k polypeptide. This section describes the construction and characterization of an HSV-1 recombinant (HSV-140) in which VZV 140K coding sequences replace both copies of the Vmw175 coding sequences in the repeat sequences bounding the short unique region of the HSV-1 genome.

The primary aim of this work was to characterize the phenotype of the recombinant virus in order to determine the extent to which 140k and Vmw175 are functionally interchangeable. Additionally, it was envisaged that HSV-140 might be a useful vector with which to express 140k in tissue culture cells, thus permitting a biochemical analysis of the polypeptide. Analysis of DNA replication and the expression of viral polypeptides during HSV-140 infection of tissue culture cells (in the absence and presence of metabolic inhibitors) has provided more detailed information on the functional relationship between 140k and Vmw175 than was afforded by the earlier complementation studies of Felser *et al.* (1987, 1988). The results demonstrate that 140k can perform many of the functions of Vmw175 during growth of HSV-1, but that slight differences in the detail of gene expression might lead to less efficient virus growth.

3B.1. CONSTRUCTION

1.1. Strategy

The objective was to introduce VZV gene 62 coding sequences into the normal Vmw175 loci in the HSV-1 genome by the process of homologous recombination. To this end plasmid pGD140 was constructed in which gene 62 coding sequences are contained in an IE gene 3 transcription unit comprising both 5' promoter sequences and 3' transcription termination signals, and also the HSV-1 "a" sequence. The parent virus for the recombination was D30EBA in which the

majority of the Vmw175 coding sequences have been deleted from both copies of the IE3 gene. Like other HSV-1 viruses lacking Vmw175 function, D30EBA is unable to grow on normal cell lines. Since D30EBA lacks endogenous Vmw175 coding sequences, its use minimises problems which might be encountered due to intragenic recombination between homologous regions of Vmw175 and VZV 140k. In addition, its use provides a powerful selection for progeny virus which, unlike D30EBA, should be competent for growth on normal cell lines. Thus plasmid pGD140 was linearized at a unique *PstI* site (within vector sequences) and cotransfected into BHK cells with D30EBA viral DNA, progeny virus isolated, and screened for the presence of the desired recombinant.

1.2. Virus D30EBA

D30EBA (Paterson and Everett, 1990) is a mutant of HSV-1 in which nucleotides 248-3806 of the Vmw175 orf (amino acid residues 83-1269) are deleted from both copies of the IE3 gene (a description of the location and structure of the IE3 gene can be found in Section 1C). D30EBA was constructed from the HSV-1 strain *in*1411 (Russell *et al.*, 1987) which has an oligonucleotide encoding a translation termination codon introduced into the *Pvu*II site at position 248 of the IE3 reading frame, and thus expresses a truncated form of Vmw175. A marker rescue experiment was performed in which linearized pdelI11 DNA was cotransfected into M64A cells (see below) with *in*1411 DNA; D30EBA was isolated and purified from progeny virus by plaque picking on M64A cells. Plasmid pdelI11 carries a copy of the Vmw175 coding sequences from which nucleotides 248-3806 have been deleted. Both D30EBA and *in*1411 are unable to grow on normal cell lines and were propagated on M64A cells which express functional Vmw175. M64A cells (which are equivalent to M65 cells as described by Davidson and Stow, 1985) are a biochemically transformed cell line derived by transfection of the *Xho*I-*Hin*dIII fragment spanning IR_S in HSV-1 (and containing the tk gene

and the linked IE3 gene) into tk- BHK cells.

1.3. Construction of Plasmid pGD140

The construction of plasmid pGD140, which contains the VZV 140k coding sequences in an HSV-1 IE-3 transcription unit (Figure 6), first required construction of a plasmid (p140BTE) from which the intact VZV 140k coding sequences (lacking flanking 5' and 3' VZV sequences) could be conveniently isolated. Before describing the construction of pGD140 in detail, the construction of p140BTE will be outlined.

Plasmid p140BTE is a derivative of p140 (see Section 3A.2 and Figure 5) which has a unique *Bgl*II site at +57 (relative to the transcription start site at +1), 16 nucleotides upstream of the ATG initiation codon, and a unique *Eco*RI site 4 nucleotides beyond the 3' end of the reading frame. The first step was the production of a plasmid containing the *Bgl*II site (p140BT). M13 oligonucleotide directed mutagenesis was employed to mutate nucleotide +57 of gene 62 from A to G, changing the sequence from AAATTC to GAATTC. To facilitate mutagenesis, a *Bam*HI-*Eco*RI fragment representing VZV gene 62 sequences from -1146 (Cla*I*) to nucleotide 1412 of the reading frame was isolated from pVI8 (a derivative of p140 created by insertion of an 8 base *Eco*RI linker into the *Sau*3A site at nucleotide 1412; see section 3C.1.1), and inserted between the *Bam*HI and *Eco*RI sites of M13 mp18. Following mutagenesis, miniprep DNAs were screened for the presence of a new *Eco*RI site, a clone (pM13E13) selected, and its DNA sequence in the region of the mutation confirmed by dideoxy sequencing. A *Bam*HI-*Eco*RV fragment representing sequences from -1146 to nucleotide 1245 (*Eco*RV), and containing the single A to G mutation, was isolated from M13E13 and used to replace the wild type *Bam*HI-*Eco*RV fragment in p140 to create p140ET. Plasmid p140ET was cleaved at the unique *Eco*RI site, the 5' overhangs filled in with Klenow polymerase, and ligated directly to the 8 base *Bgl*II oligonucleotide linker ^{5}'GAGATCTC^{3}' to create

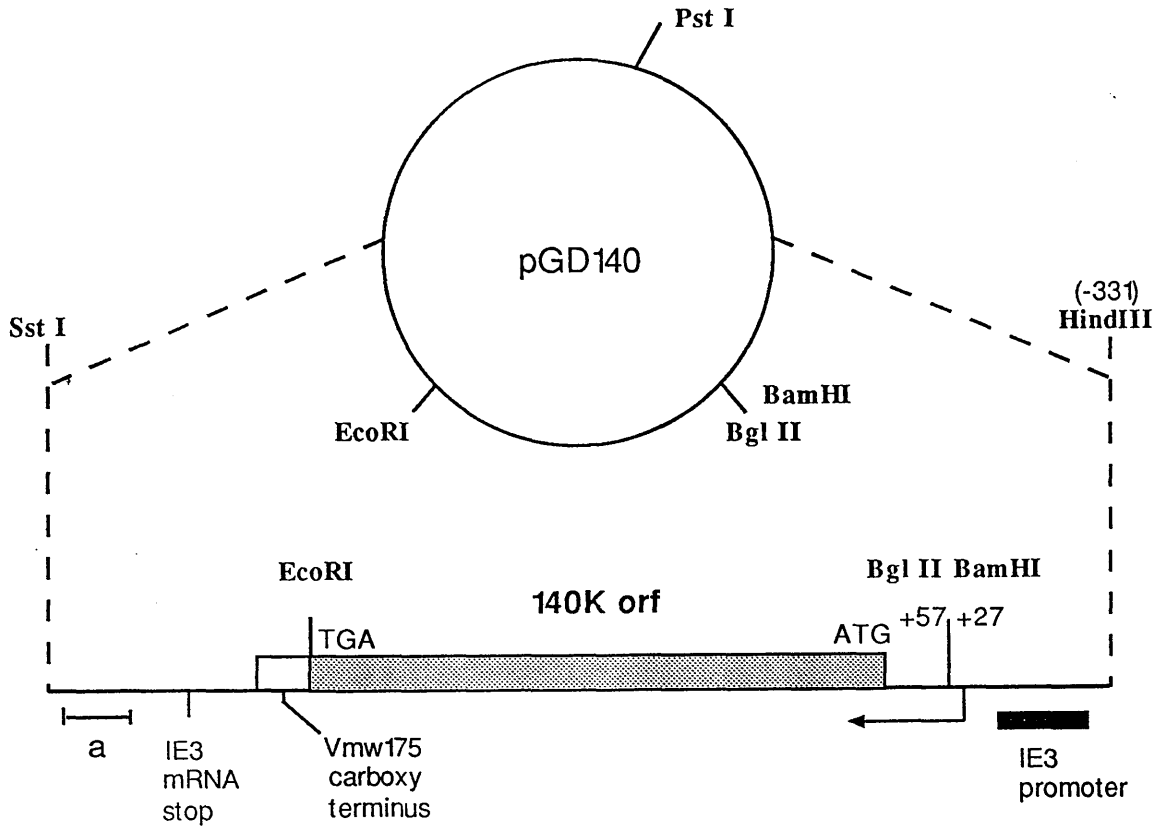


Figure 6. The structure of plasmid pGD140. Plasmid pGD140 contains VZV gene 62 coding sequences flanked by HSV-1 IE3 transcriptional control sequences. The vector sequences extend from the *Pvu*II site of pBR322 (which had been altered to an *Sst*I site as indicated), through the ampicillin resistance gene (with its *Pst*I site) to the *Hind*III site. The *Hind*III site is located at the junction of HSV-1 and pAT153 sequences and is joined to the *Sma*I site at position -331 of the IE3 promoter. The VZV gene 62 sequences from position +57 to an introduced *Eco*RI site just after the VZV 140K stop codon are shown, with the coding sequence in a stippled box. Sequences downstream of this include the 3' portion of IE3, the "a" sequence and part of TR_L as far as the *Sst*I site as shown (not to scale). The plasmid was constructed by ligation of the *Pst*I-*Bam*HI fragment of pGX161, the *Bgl*II-*Eco*RI fragment of p140BTE and the *Eco*RI-*Sst*I fragment of pI38 as detailed in the text. Ligation of complementary ends produced by enzymes *Bgl*II and *Bam*HI results in a sequence (marked *Bgl*II *Bam*HI) which is cut by neither enzyme.

p140BT. p140BT was then linearised by digestion with *Tth*111I, which cuts 2 nucleotides beyond the 3' end of the gene 62 reading frame, and EcoRI linkers inserted (after making the ends blunt) to yield p140BTE.

Plasmid pGD140 was constructed from fragments derived from pGX161, pI38, and p140BTE. Plasmid pGX161 (kindly provided by Dr C M Preston) has been described previously (Preston and Fisher, 1984) and contains the promoter and upstream regulatory sequences of the HSV-1 IE3 gene (nucleotides -331 to +27) between the *Hin*dIII and *Bam*HI sites of pAT153. Plasmid pI38 is a derivative of p175 (Perry et al, 1986) created by insertion of a 12 base *Eco*RI linker oligonucleotide into a *Nae*I site within the Vmw175 coding sequences, resulting in an in-frame insertion of four amino acids into the proline codon at position 1236 (Paterson and Everett, 1988a). Plasmid pGD140 was constructed in a tri-partite ligation using the *Bgl*II-*Eco*RI fragment from p140BTE containing the VZV 140K coding sequences, the smaller *Pst*I-*Bam*HI fragment of pGX161 containing the IE3 promoter (and part of the vector sequences), and the smaller *Pst*I-*Eco*RI fragment of pI38 which provides the rest of the vector sequences, the C-terminal 62 codons of Vmw175, the 3' end of the IE3 transcription unit and subsequent HSV-1 DNA through to the *Sst*I site in TR_L (figure 6). The C-terminal portion of Vmw175 in this construct follows the TGA stop codon of VZV gene 62.

1.4. Marker Rescue of D30EBA

A mixture containing approximately 1ug of plasmid pGD140 DNA (linearised with *Pst*I), 3ug of D30EBA viral DNA and 5ug of calf thymus DNA (as carrier) was transfected into $2x10^6$ BHK cells by the method of Stow and Wilkie (1976). Cells were also transfected with viral or plasmid DNA alone as a control. After four days cpe was observed in the cells co-transfected with viral and plasmid DNA, whereas no cpe was observed in the control transfection with viral DNA alone. Cells and medium combined from both

the control and co-transfected plates were sonicated and retained as virus stocks. These stocks were used to infect further BHK cells and total infected cell DNA prepared. This was initially analysed by DNA slot blots using nick translated p140 (McKee et al, 1990) as a probe, which showed that only DNA prepared from the co-transfected cells hybridised to VZV sequences (results not shown).

The recombinant viral DNA was further analysed by Southern blotting (Figure 7). The controls show that neither mock nor wt HSV-1 infected cell DNA samples hybridized to the p140 probe. The *Bam*HI restriction map for the expected recombinant virus (Figure 8) predicts that the probe will hybridize to the following fragments on the blot: *Bam*HI [k'] (6.0kb) and *Bam*HI [y'+n] (7.1kb) derived from IR_s; *Bam*HI [x+y'] (4.1kb) and *Bam*HI [q'] (3.0kb) derived from TR_s. Fragments corresponding in size to the three larger predicted *Bam*HI restriction fragments are clearly observed on the blot. However, the *Bam*HI [q'] fragment, which represents sequences from the genome terminus, was not observed in this experiment. A weakly hybridizing fragment of 3kb was once observed during the isolation and purification of HSV-140, which probably indicates that viral DNA prepared from infected cells is highly concatamerised with consequent under-representation of the terminal fragment. The simplest interpretation of these results is that an intact copy of VZV gene 62 is present in both IE3 gene loci in an HSV-1 genome in our primary isolate of the recombinant virus, named HSV-140.

The initial isolate was amplified and purification by plaqueing under agar attempted, but despite several attempts no plaques were observed (see below). Virus HSV-140 was subsequently purified by limiting dilution in linbro wells, such that cpe was observed in 30% or less of wells. Medium was retained from wells that displayed cpe, and used to infect cells for total cellular DNA preparation. Southern blot analysis of the resulting DNAs showed all eight stocks of virus to be of the predicted structure with no evidence of contamination. Recombinant

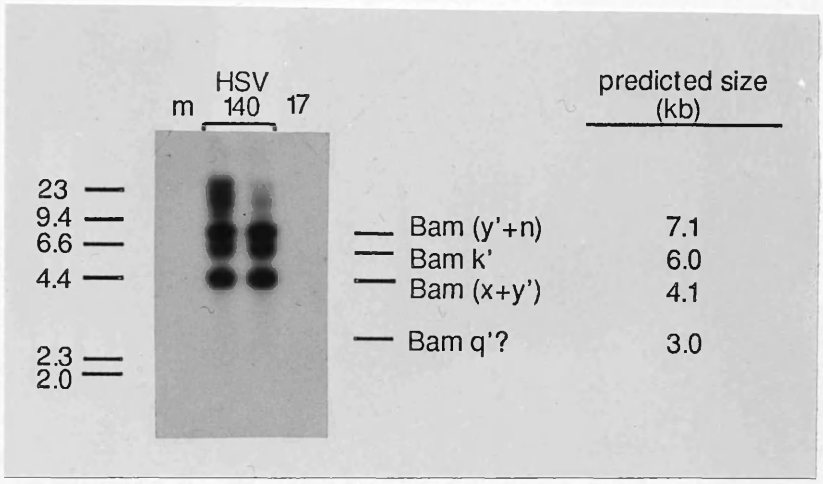
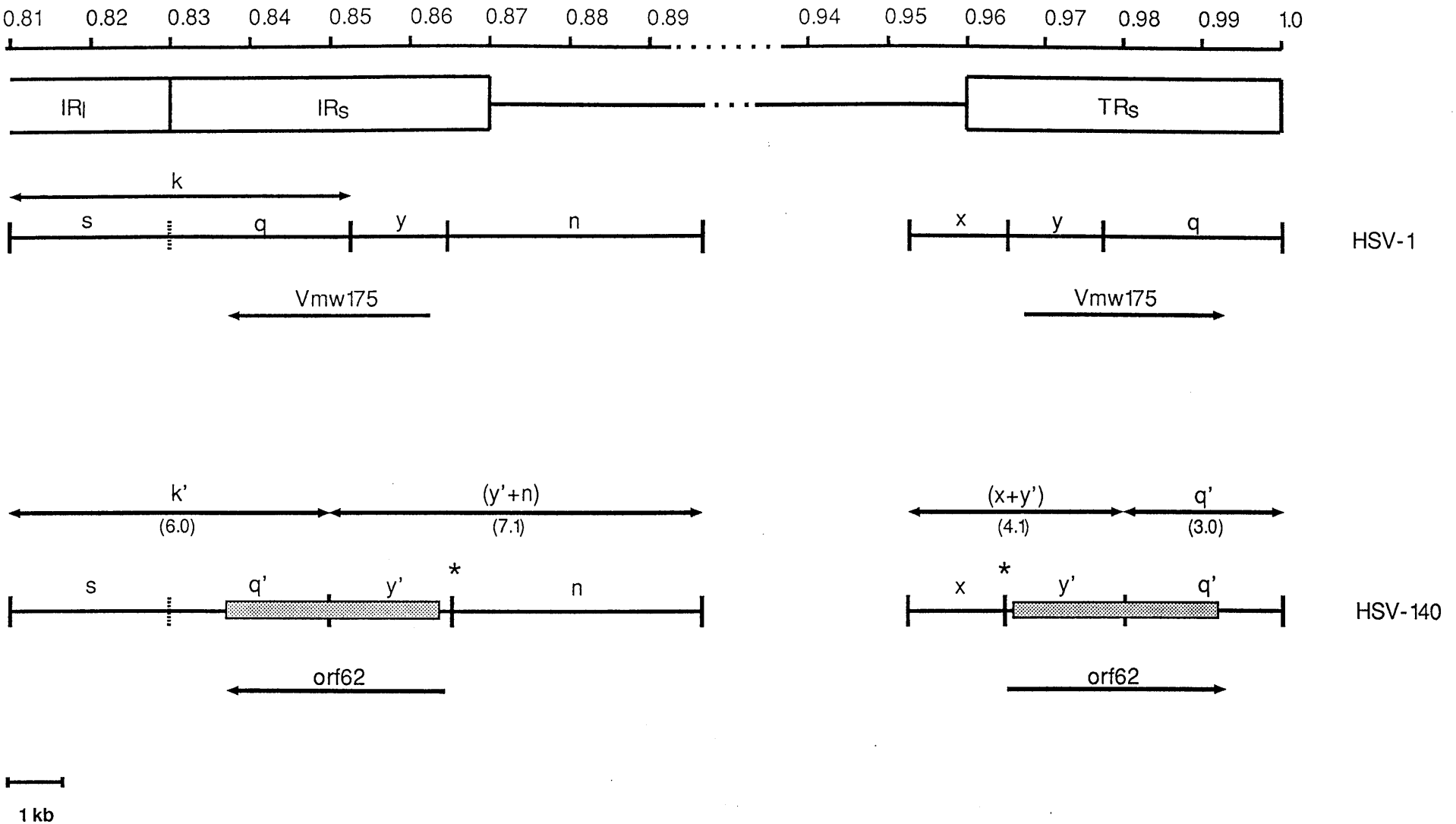


Figure 7. Southern blot analysis of DNA prepared from HSV-140 infected cells. DNA was prepared from infected cells when extensive cpe was apparent. The DNA was then digested with *Bam*HI and subjected to Southern blot analysis using a nick translated plasmid p140 probe which contains VZV gene 62 but no HSV-1 sequences. Track 1: mock infected cell DNA; tracks 2 and 3: HSV-140 infected cell DNA using two different virus stocks; track 4: HSV-1 infected cell DNA. The positions of Lambda *Hind*III size markers are shown on the left, and the identities of fragments hybridizing to the probe were assigned by comparison of their estimated sizes to those predicted from the maps in Figure 8. The larger fragments in lane 2 represent partial digestion products.

Figure 8. Predicted *Bam*HI restriction maps for the short regions of the wild type HSV-1 and HSV-140 genomes. The HSV-1 and HSV-140 genomes are shown. VZV gene 62 coding sequences replace both copies of Vmw175 in the short region repeats of the HSV-1 genome in HSV-140; VZV sequences are represented by the stippled box, and the limits of the open reading frames shown by arrows. The Vmw175 coding sequences in HSV-1 are contained within *Bam*HI q and *Bam*HI y, their replacement by the corresponding gene 62 sequences in HSV-140 results in restriction fragments designated *Bam*HI q' and *Bam*HI y'. *Bam*HI k' represents (q'+ s) derived from the IR_l/IR_s junction. The *Bam*HI site near the capsite of the IE-3 gene (present at map units 0.865 and 0.965 approximately in HSV-1) was destroyed during the construction of pGD140 (marked * in HSV-140, see Figure 6). Therefore *Bam*HI digestion of HSV-140 DNA produces fragments (y'+ n) and (x+y'). The sizes (kb) of these four novel fragments containing portions of the VZV gene 62 sequence are shown.


0.81
0.82
0.83
0.84
0.85
0.86
0.87
0.88
0.89
0.94
0.95
0.96
0.97
0.98
0.99
1.0
IRl
IRs
TRs
k
s
q
y
n
x
y
q
HSV-1
Vmw175
Vmw175
k'
(6.0)
(y'+n)
(7.1)
(x+y')
(4.1)
q'
(3.0)
s
q'
y'
*
n
x
*
y'
q'
HSV-140
orf62
orf62
1 kb

viruses carrying only one copy of gene 62 were not detected at any stage, suggesting that HSV-140, with two copies of gene 62, has a strong growth advantage. One of the small scale stocks was used to infect $2X10^8$ BHK cells for preparation of a large scale virus stock which was again checked by Southern blotting (result not shown). The isolation of HSV-140 on normal BHK cells shows that VZV-140K can functionally replace Vmw175.

3B.2. CHARACTERIZATION OF HSV-140

HSV-140 was initially compared to wt virus in a standard plaque assay in BHK cells to assess the extent to which VZV 140K can complement for loss of Vmw175 function. Cells were infected with increasing dilutions of each virus and fixed and stained after two days. At lower dilutions both viruses produced extensive cpe, although the appearance of cpe during HSV-140 infections was delayed approximately 12h compared to wt virus. At increasing dilutions single plaques were obtained with wt virus as normal, but no obvious plaques were produced by HSV-140. The assay was repeated several times with independent stocks of HSV-140 and in no case were distinct plaques formed. Similar results were obtained using HFL cells. The results suggest that while HSV-140 is able to grow in the absence of Vmw175 expression, plaque formation is impaired, at least in the cell types used in these studies.

The numbers of virus particles in the large scale stocks of cell-associated and supernatant HSV-140 virus were determined. HSV-140 cell-associated stocks were routinely around $3x10^{10}$ particles/ml, which was equivalent to the corresponding particle counts of stocks of wt virus produced in parallel. The supernatant virus particle count of HSV-140 was reduced by a factor of ten compared to the cell-associated virus (and to the wt supernatant virus stock also). If this reflects a slower rate of virus assembly or release then it may in part explain the lack of plaque formation by HSV-140.

2.1. Titration of HSV-140

As plaque forming unit titres could not be determined for HSV-140 virus stocks, viral polypeptide synthesis in HSV-140 and wt virus infected cells were compared using 10pfu/cell of wt virus, and increasing dilutions of HSV-140. Cells were labelled with [^{35}S]-methionine from 3-6 hours post-absorption (pa) and proteins analysed by SDS-PAGE. Infection with dilutions of HSV-140 corresponding to multiplicities of 2500, 500, and 100 particles per cell (ppc) resulted in levels of viral gene expression that were equivalent to the wt virus infection (results not shown). Since an amount of VZV inoculum corresponding to 100 ppc gave viral polypeptide expression similar to 10pfu wt virus per cell in this single experiment, this amount of HSV-140 was used in a subsequent time-course experiment.

2.2. Time-Course of HSV-140 Viral Polypeptide Synthesis

Cells were mock-infected or infected with wt and HSV-140 viruses and labelled between 0-2, 2-4, 4-6, 6-8, 8-10, 10-12, 16-18, and 22-24 hours pa. These results are shown in Figure 9. The protein expression profiles of the two viruses were similar, but a significant difference was the kinetics of expression of a high molecular weight protein in HSV-140 infected cells. On the basis of its mobility, expression by HSV-140 and Western blotting using a polyclonal rabbit antiserum directed against a fusion protein including part of VZV 140K (data not shown; D. Stevenson and A. Davison, personal communication) the most likely interpretation is that this high molecular weight band is the VZV 140K polypeptide. It is notable that the kinetics of expression of VZV 140K in HSV-140 infected cells is very different compared to Vmw175 in wt virus infected cells. Strikingly, VZV 140K was expressed as an early protein and was abundant at late times in infection. Thus VZV 140K is apparently not subject to autoregulation in HSV-140, which suggests that the (apparent) normal repression of IE gene expression may be defective in this

virus. It is not possible to discern from this data whether Vmw110, Vmw68, or Vmw63 are also overexpressed during HSV-140 infection.

Most other clearly visible viral proteins are expressed in HSV-140 and wt virus infected cells in similar amounts and with similar kinetics. However, the expression of certain late gene products (for example, Vmw65 and the major capsid protein) appears to be lower in HSV-140 infected cells; this may be the result of less efficient replication of viral DNA during HSV-140 infection (see below).

2.3. VZV 140k expressed during HSV-140 infection is localized to the nucleus

In order to determine the cellular location (i.e. nucleus or cytoplasm) of VZV 140k during HSV-140 infection, BHK cells were infected with 100ppc of virus, and labelled with [^{35}S]-methionine from 12-14h p.a. Labelled infected cells were isolated, and cytoplasmic and nuclear fractions prepared (as described in Section 2B.9.1). SDS-PAGE analysis of these fractions (Section 3F: Figure 42) revealed that 140k is exclusively localized to the nucleus during HSV-140 infection.

2.4. The Kinetics of 140K Protein Expression

The expression of VZV 140K in HSV-140 is under the control of the HSV-1 IE3 promoter, and therefore should be expressed as an IE protein. However the time-course experiment (Figure 9) indicated that VZV 140K was expressed in increasing amounts as infection progressed. To investigate this further, the expression of viral proteins under IE conditions was examined in a cycloheximide reversal experiment. BHK cells were infected with HSV-140 and wt virus in the presence of cycloheximide. At 5h pa the cycloheximide was removed, and [^{35}S]-methionine labelling carried out in the presence of actinomycin D. The labelled proteins were analysed by SDS gel electrophoresis (Figure 10). In the wt virus infection,

the IE proteins Vmw63, Vmw68, Vmw110, and Vmw175 were prominently labelled as expected. In contrast, in the HSV-140 infection Vmw63, Vmw68, and Vmw110 were synthesised in similar amounts to the wt virus infection, but the VZV 140K protein was absent. Despite the fact that VZV 140K is expressed from an authentic HSV-1 IE promoter in HSV-140, the protein was poorly expressed under IE conditions. These results also indicate that HSV-140 is much less effective in the shutoff of host cell protein synthesis compared to wt virus (see also Section 2.7).

2.5. Comparison of Viral DNA Replication During Wild-Type and HSV-140 Infections

BHK cells in linbro wells were infected with HSV-140 and wt virus and total cellular DNA prepared at 4, 6, 8, 10, and 16 hours pa. The DNA samples were treated with RNase and analysed in a slot blot experiment to compare the amounts of viral DNA present in the two infections. Nick translated p111 (which contains HSV-1 IE gene 1 sequences; Everett, 1987) was used as a probe as it contains sequences common to both viruses. In cells infected with wt virus, DNA accumulation began prior to the earliest time point of 4h pa, and continued through to 16h pa (Figure 11). The onset of HSV-140 DNA replication was delayed 2-4h relative to wt virus and the amounts of viral DNA produced were generally reduced; no accumulation of DNA was seen at 4h pa, and only very low levels were seen at 6h pa (Figure 11).

2.6. Viral Gene Expression During Infection With HSV-140 is Multiplicity Dependent

Several mutants of HSV-1 containing mutations in regulatory functions have been shown to have defects in viral gene expression at low multiplicities in certain cell types (Stow and Stow, 1986; Sacks and Schaffer, 1987; Ace et al, 1989; Everett, 1989). We decided to investigate the synthesis of viral polypeptides during high and low multiplicity infections of BHK and HFL cells by HSV-140.

In an attempt to minimize experimental variation, the virus stocks used in this study were all prepared in parallel from the same batch of cells. BHK or HFL cells in linbro wells were infected at 50, 30, 10, and 3 ppc with HSV-140 or wt virus and labelled with [^{35}S]-methionine at 10-12h pa (Figure 12). In agreement with the time-course experiment (Figure 9) at higher multiplicities both viruses expressed similar levels of most viral polypeptides, although the expression of certain late proteins (for example Vmw65 and the major capsid protein) was again reduced in HSV-140 infections. At the lower multiplicities of 10 and 3 ppc, HSV-140 expressed reduced amounts of all viral polypeptides compared to wt virus in both BHK and HFL cells. This finding was repeated with several different stocks of HSV-140. Therefore HSV-140 exhibits a multiplicity dependent gene expression defect which, in the cases examined, is not cell type dependent.

2.7. HSV-140 is Defective in Virus-Induced Shut-Off of Host Protein Synthesis

The results in Figures 10 and 12, while suggesting that the ability of HSV-140 to shutoff host protein synthesis is significantly impaired, cannot be considered conclusive. The cycloheximide reversal experiment (Figure 10) was not performed with equal ppc multiplicities of wt and HSV-140 viruses, and in the particle titration experiment (Figure 12) labelling at late times means that secondary infection by the faster growing wt virus could contribute to the observed host shutoff. In order to assess whether HSV-140 is defective in shutoff, an experiment was performed in which cells were infected with 1000 particles per cell each of wt virus, HSV-140, and HSV-2 strain G (which has a strong shut-off phenotype; Fenwick & Clark, 1983) in the presence of actinomycin D (2.5 ug/ul), and polypeptides labelled with ^{35}S-methionine following a 2h infection period. The results show that while wt virus and HSV-2 strain G shut-off host protein synthesis with a similar efficiency in this experiment

(strain G is normally more efficient), there is clearly no detectable shut-off following HSV-140 infection (Figure 13). This could be due to inefficient presentation by HSV-140 of the virion host shutoff function encoded by gene UL41 (Kwong et al, 1988; McGeoch et al, 1988). This might simply reflect a reduced amount of the UL41 product in virions, although an alternative interpretation might be that slight alterations in the composition or structure of HSV-140 virus particles reduces their efficiency of cellular absorption or penetration.

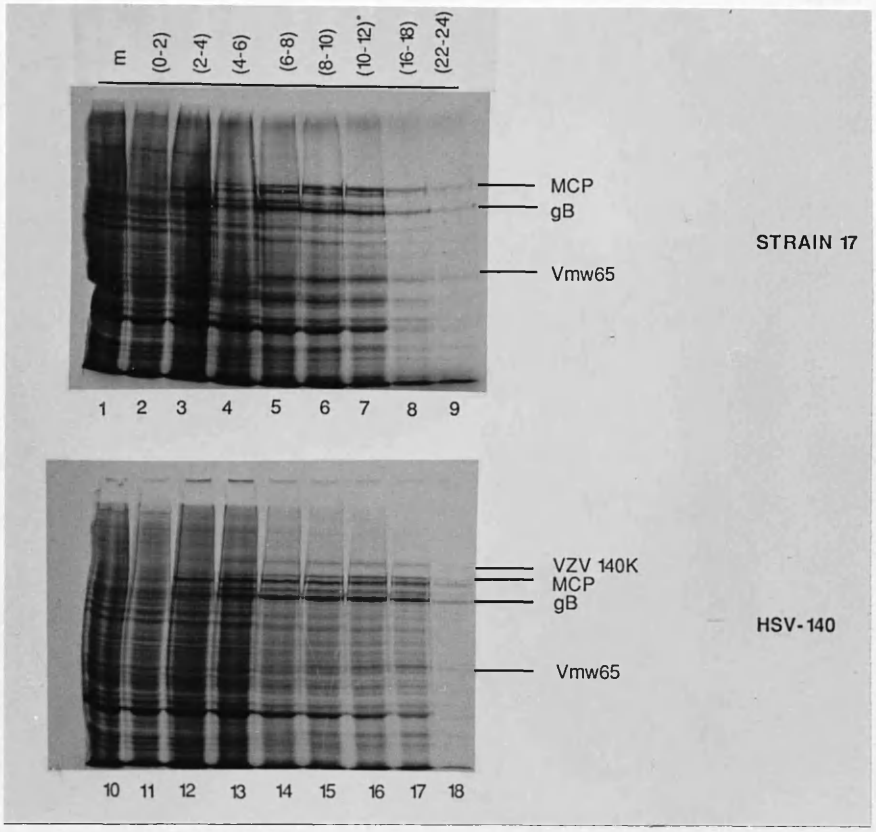


Figure 9. The time-course of viral protein expression in BHK cells infected with HSV-140 and wt virus. Linbro wells containing $4x10^5$ cells were infected with 10 pfu/cell wt HSV-1 (strain 17) or 100 ppc HSV-140. Proteins were labelled with [^{35}S]-methionine at the indicated times (hours) post-adsorption and analysed by SDS-PAGE. The samples in lanes 1 and 10 are from mock infected cells. Some of the major viral proteins are indicated: major capsid protein, MCP; glycoprotein B, gB; Vmw65; and VZV 140K (HSV-140 infected cells only). Less intense labelling in the later time point samples is due to loss of terminally infected cells.

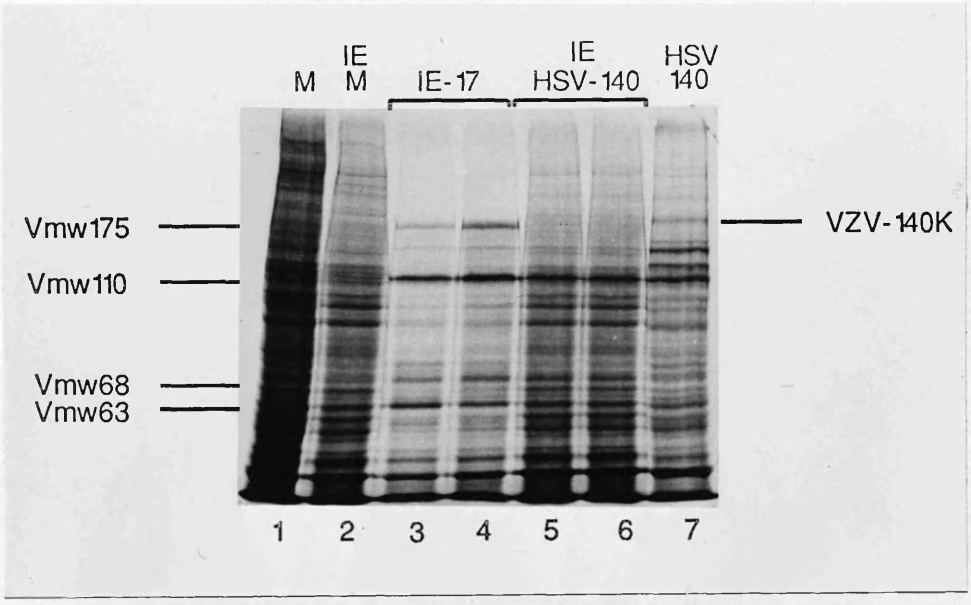


Figure 10. HSV-140 expresses VZV 140K poorly in cycloheximide reversal experiments. Subconfluent BHK cells were infected in the presence of cycloheximide. At 5h pa, cycloheximide was removed to allow protein labelling with [^{35}S]-methionine (in the presence of actinomycin D) and their analysis by SDS-PAGE. Tracks 3 and 4 contain the labelled proteins from wt virus infected cells, and tracks 5 and 6 the results using HSV-140. Track 2 is a mock infected cell extract made under IE conditions, and tracks 1 and 7 show mock and HSV-140 infected cell extracts made under normal conditions. The positions of IE polypeptides are indicated.

There is a very slight amount of VZV 140k expressed under these conditions (compare lanes 5 and 6 with lane 2).

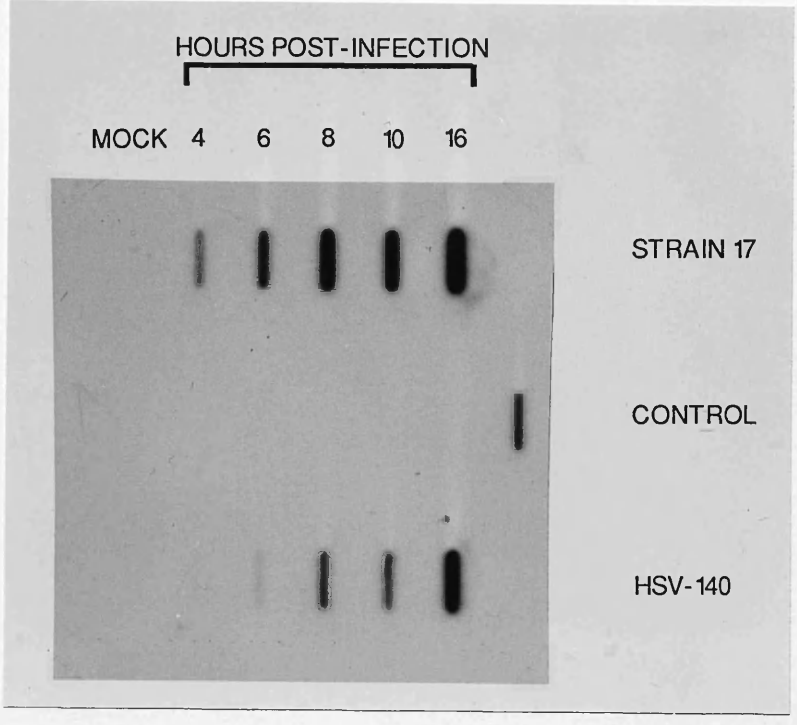
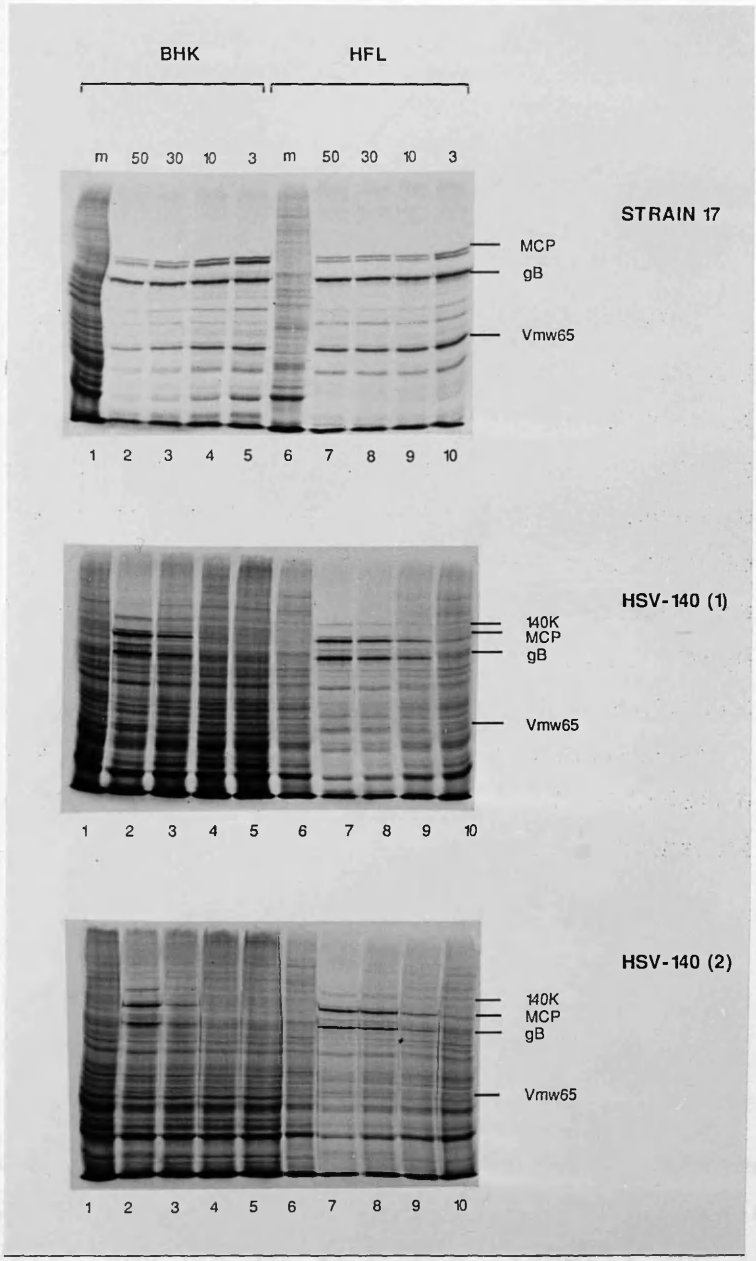


Figure 11. Comparison of viral DNA replication in BHK cells infected with HSV-140 and wt virus. Cells were infected as described in Figure 9, and harvested at 4, 6, 8, 10, and 16 hours post-absorption for total cellular DNA preparation. DNA was also isolated from mock infected cells. The samples were treated with RNase prior to slot blot analysis using a nick translated p111 probe which contains HSV-1 IE gene 1 sequences common to HSV-140 and wt HSV-1. The control contains 10ng of p111.

Figure 12. HSV-140 viral gene expression is impaired at lower multiplicities of infection. BHK or HFL cells in linbro wells were infected with HSV-140 or wt virus at multiplicities corresponding to 50, 30, 10 and 3 ppc. Cells were labelled with [^{35}S]-methionine from 10-12 hours pa and proteins analysed by SDS-PAGE. Tracks 1 to 5 of each gel are samples prepared from BHK cells, tracks 6 to 10 are samples from HFL cells. Tracks 1 and 6 of each gel are mock infected samples. Some major viral proteins are indicated, as described in the legend to Figure 9. HSV-140(1) and HSV-140(2) are two separate stocks of virus prepared in parallel.


BHK
HFL
m 50 30 10 3 m 50 30 10 3
STRAIN 17
MCP
gB
Vmw65
1 2 3 4 5 6 7 8 9 10
HSV-140 (1)
140K
MCP
gB
Vmw65
1 2 3 4 5 6 7 8 9 10
HSV-140 (2)
140K
MCP
gB
Vmw65
1 2 3 4 5 6 7 8 9 10

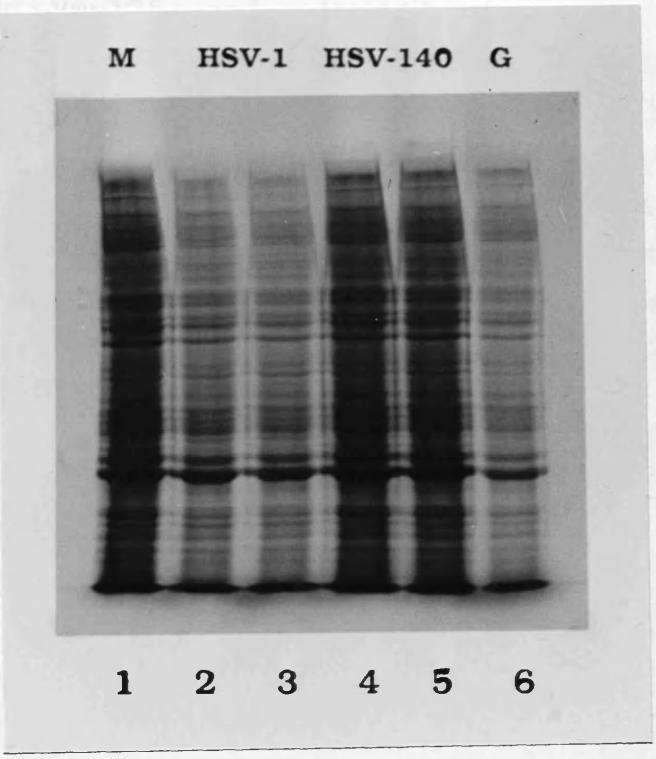


Figure 13. HSV-140 is defective in virus-induced shut-off of host protein synthesis. BHK cells in linbro wells were infected with 1000 ppc of wt virus (lanes 2 and 3), HSV-140 (lanes 4 and 5), or HSV-2 strain G (lane 6) in the presence of actinomycin D. Following a 2h infection period polypeptides were labelled with [^{35}S]methionine and analysed by SDS-PAGE. The sample in lane 1 is from mock infected cells.

3C. INVESTIGATION OF THE *trans*-ACTIVATION PROPERTIES OF 140k

The *trans*-activation properties of VZV 140k and Vmw175 of HSV-1 are markedly different in transfection assays. Thus 140k is a much more powerful *trans*-activator of the HSV-1 glycoprotein D (gD) promoter than Vmw175 (see Section 3C.1.2). Furthermore, whereas activation of the gD promoter by Vmw175 and Vmw110 exhibits synergy (i.e. activation by the polypeptides in combination is far greater than by either polypeptide alone), 140k and Vmw110 do not appear to function in synergy (Section 3C.2.3). The primary sequence characteristics of 140k responsible for these striking differences were investigated. The effects of insertion and deletion mutagenesis of sequences in the N-terminal third of the protein will be described, as will the construction and characterization of plasmids expressing chimeric 140k/Vmw175 polypeptides.

3C.1. INSERTION MUTAGENESIS OF VZV 140k

1.1. Construction of insertion mutants

N.B. The construction of the insertion mutants and preliminary mapping of their sites of insertion was performed by Bridget Harris.

Plasmid p140 (10µg) was digested with 6u of *Sau*3A for 30 minutes in the presence of 20, 40, 60 or 80µg/ml ethidium bromide in a final volume of 50µl. Digestion in the presence of ethidium bromide maximizes the production of singly cut linear molecules. The DNA was loaded onto a 5-20% sucrose density gradient and centrifuged for 16h at 28,500 rpm and 15°C in the Sorvall TST41 rotor. Linear DNA (which sedimented more slowly than circular forms and was easily separated) was isolated, ethanol precipitated overnight, and resuspended in a volume of 20µl. 5µl of this material was filled in with Klenow polymerase in a volume of 20µl. Finally, this blunt ended DNA was ligated to phosphorylated 8bp *Eco*RI oligonucleotide linkers ($^{5'}$GGAATTCC$^{3'}$). Plasmids with linker inserts were identified by screening small-scale plasmid preparations.

The site of insertion was initially determined by restriction enzyme analysis and their location and integrity confirmed by DNA sequencing. The number of linkers inserted was determined by restriction analysis and, in the case of multiple insertions, reduced to a single copy by digestion with *Eco*RI, followed by religation.

The locations of the eight insertion mutants obtained is summarized in Table 3. Although there are *Sau*3A sites distributed along the length of the 140k coding sequences, all the insertions isolated were located within the N-terminal third of the protein. Upon sequencing the first of the mutants (pVI1), one nucleotide was found to have been deleted from within the filled in *Sau*3A site sequences, 3' to the *Eco*RI linker, altering the translational reading frame downstream of the insert. Of the remaining plasmids, pVI2-pVI7 have insertions within homology region 1, whereas the insertion in pVI8 is located within region 2.

1.2. *Trans*-Activation of the HSV-1 gD Promoter by 140k and the Insertion Mutants

WS-HeLa cells were seeded at a density of 10^6 cells per 50mm petri dish and transfected by calcium phosphate precipitation as described in Section 2B.6.1. Transfections included 4µg of the CAT reporter plasmid, pgDCAT, and 4µg of p140 or pVI plasmid. A negative control transfection was performed in which 4µg of pUC9 replaced p140 (or pVI derivative). Cells were washed after 24h and replaced in fresh medium, and CAT extracts prepared after a further 24h (Section 2B.6.1). The CAT activity and protein concentrations of the extracts were determined (Sections 2B.7 & 2B.8), and the percentage conversion from substrate to product per microgram of protein calculated.

Cotransfection of cells with pgDCAT and p140 resulted in CAT activity being increased around 100-fold above that obtained when cells were transfected with pgDCAT alone, demonstrating the powerful intrinsic

trans-activation activity of VZV 140k. This increase was used as the assay to test the phenotype of the insertion mutants.

The activity of each of the seven in-frame insertion mutants (pVI2-pVI8) was determined and expressed as a percentage of the activity of p140. These data are shown in Table 3. The activities of pVI4-pVI6 were 80-100% of that obtained with p140, and these mutations can be considered as having no effect upon activity. Plasmids pVI2, pVI3, and pVI7 had activities in the range 30-50% and, although reduced relative to p140, are still strong activators. In a similar analysis of Vmw175 it was likewise found that many of the insertion mutations decreased activity to 40-50% of the wild-type polypeptides activity, indicating that the polypeptide was sensitive to small insertions in many regions (Paterson & Everett, 1988a). Thus none of the insertions between residues 48 and 418 (and including the majority of region 1) greatly affected the *trans*-activation function of 140k. In contrast, the insertion in pVI8 clearly has a much more deleterious effect on activity, reducing it to less than 1% of that observed with p140. Significantly, this insertion lies within homology region 2 which, in the case of Vmw175, was found to be the most critical for *trans*-activation (Paterson and Everett, 1988a; DeLuca and Schaffer, 1988). Although only a single region 2 insertion mutant has been isolated and characterized in 140k, it seems likely, particularly given the extensive sequence homology between region 2 of 140k and Vmw175, that region 2 sequences of the two polypeptides fulfil similar roles in *trans*-activation.

Table 3. A summary of the positions and activities of the insertion mutants. The insertions in plasmids pVI1 to pVI8 are all into *Sau*3A restriction sites within the VZV 140k coding sequences of plasmid p140. The coordinate given for each mutant is the position of the first base in the *Sau*3A site at which the insertion occurs, numbering from the first base of the reading frame. The insertion in pVI2 lies between two codons, whereas the remaining insertions all disrupt a codon. The amino acids inserted into or between, and their positions in the orf are shown. The *trans*-activation activity of the mutants was determined in transfection assays using pgDCAT, which contains the HSV-1 glycoprotein D (gD) promoter linked to the coding sequences of the CAT reporter gene. The activities shown are expressed as a percentage of that obtained with p140 in parallel experiments. These results are from a single experiment and were consistent with other independent experiments. (N.D.= no data.)

Plasmid	Insertion Site		*Trans*-Activation Activity (%)
	Nucleotide	Amino Acid	
pVI1	142	Leu (48)	N.D.
pVI2	189	Ile/Val (63/64)	55
pVI3	292	Leu (98)	27
pVI4	538	Gln (180)	100
pVI5	989	Ser (130)	85
pVI6	1088	Ser (363)	83
pVI7	1253	Ser (418)	63
pVI8	1415	Ser (472)	0.7

3C.2. CONSTRUCTION OF PLASMIDS EXPRESSING CHIMERIC POLYPEPTIDES COMPOSED OF SEQUENCES DERIVED FROM VZV 140K AND Vmw 175

The aim of this work was to construct a series of plasmids expressing chimeric 140k/Vmw175 polypeptides. It was hoped that analysis of such polypeptides might identify regions of 140k that contribute to its potent *trans*-activation phenotype (relative to Vmw175) and, furthermore, that such sequences might confer this phenotype upon portions of Vmw175. Likewise, Vmw175 derived sequences might confer upon portions of 140k the ability to activate gene expression in synergy with Vmw110. Analysis of intertypic recombinant viruses generated between HSV-1 and HSV-2 has demonstrated that chimeric proteins composed of sequences from the type 1 and type 2 Vmw175 proteins (which are closely homologous) are fully functional during virus infection (Smith and Schaffer, 1987a,b), indicating that chimeric 140k/Vmw175 polypeptides generated by this approach are likely to be functional.*

2.1. Strategy

A panel of 39 plasmids (pI1 to pI39) harbouring a single insertion of 12 nucleotides (i.e. in-frame) within the Vmw175 orf have been created by *Eco*RI oligonucleotide linker insertion mutagenesis of plasmid p175, which contains a single copy of the HSV-1 IE3 gene (Paterson and Everett, 1988a).

Eight analogous plasmids (pVI1 to pVI8) were derived by insertional mutagenesis of p140 (Section 3C.1). Seven of the insertions isolated were within region 1, and one in region 2.

It was proposed to utilize the unique *Eco*RI sites within pairs of insertion mutant plasmids (pVI and pI series) to join portions of the VZV 140k and Vmw175 coding sequences.

* The results of other "domain swap" experiments are consistent with this view (see, for example, Ptashne, 1988).

2.2. Rationale for Selecting Pairs of Plasmids

Pairs of pI and pVI series plasmids were selected according to the following criteria:

i) The function of the polypeptides expressed by the plasmids should not be significantly affected by the insertion mutations (i.e. the insertion should not lie within an important functional domain).

ii) The insertions should lie within the same translational reading frame such that sequences downstream of the 140k/Vmw175 junction in the resulting chimeric construct are correctly translated.

iii) The insertions in the pair of plasmids should have approximately equivalent locations within the reading frames.

2.3. Transient Transfection Assay For Characterization of Chimeric Plasmids

All of the chimeric plasmids which will be described were tested for their ability to *trans*-activate expression from the HSV-1 gD promoter, both alone and in synergy with p111 (which expresses Vmw110). Transfections contained 4µg of plasmid pgDCAT, 4µg of either p140, p175, or chimeric plasmid, and 4µg of plasmid p111 where appropriate. The total amount of plasmid DNA in each transfection was equalized to 12µg with pUC9. The procedure for transfection and determination of CAT activities is described in Sections 2B.6.1 and 2B.7.

In a typical assay (Figure 15 and Table 4) cotransfection with either plasmid p175 or p111 (which express the HSV-1 transcriptional regulators Vmw175 and Vmw110) resulted in CAT activity being increased 3- to 5-fold above that obtained when cells were transfected with pgDCAT alone. However, when both p175 and p111 were present in transfections CAT activity was increased 30-100-fold (reflecting synergistic activation), as

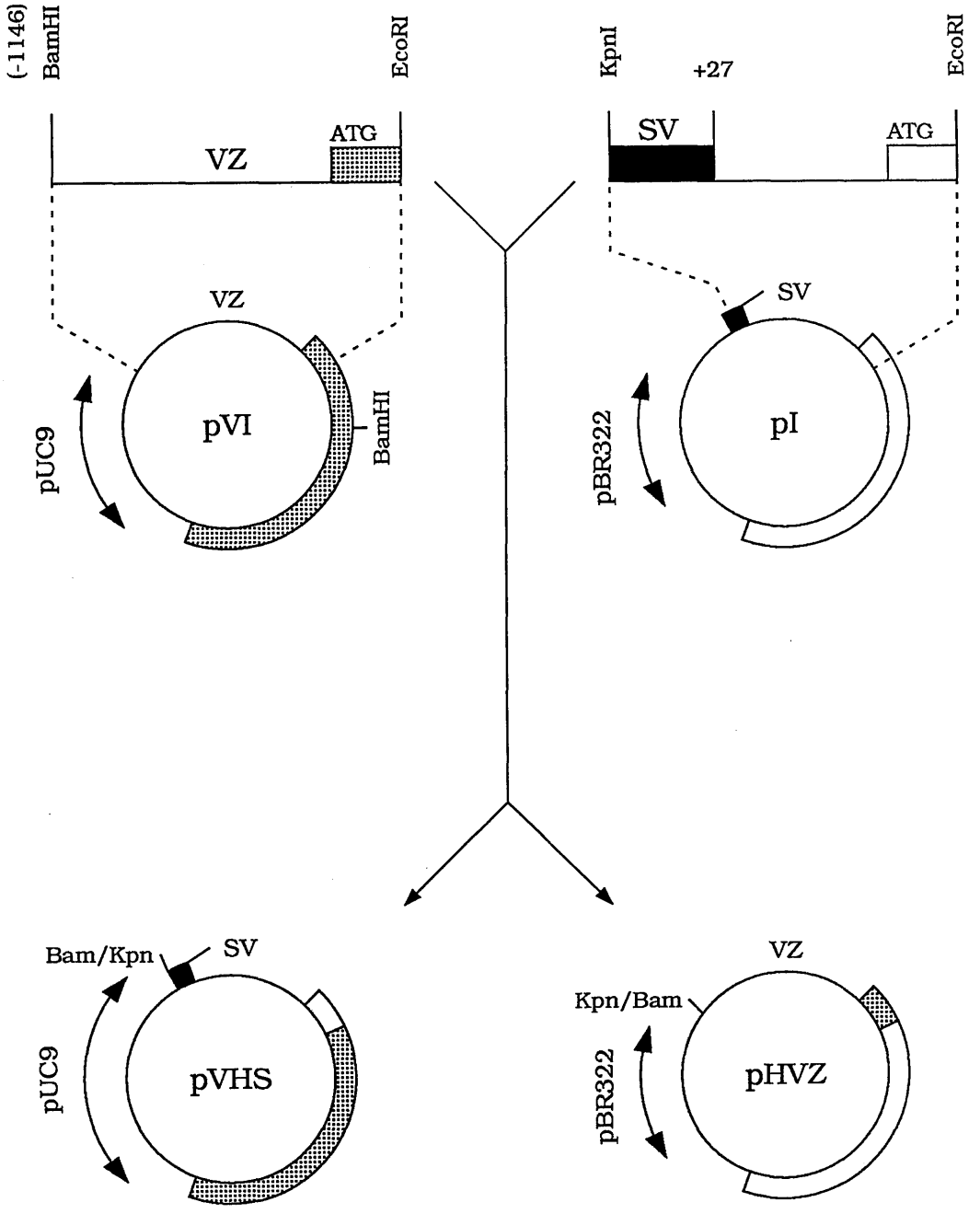
described previously (Everett, 1987). In contrast, cotransfection with p140 alone increased CAT activity by around 100-fold, and inclusion of p111 in the transfection failed to increase CAT activity further.

2.4. Construction of Plasmids pVHS1 and pHVZ1

Plasmid p140 *trans*-activates the gD promoter approximately 20- to 30-fold more efficiently than p175, and it was of interest to determine the molecular basis for this striking difference. One possibility was the strength of the promoter sequences expressing 140k and Vmw175 (in p140 and p175), which may not be equivalent; 140k is expressed from the VZV gene 62 promoter, whereas Vmw175 is expressed from the SV40 early promoter/enhancer region. (SV40 sequences are linked to the IE3 gene at nucleotide +27.) Thus if the gene 62 promoter is significantly stronger than the SV40 regulatory sequences (thereby increasing the expression of 140k relative to Vmw175 in transfection assays), it could account, at least in part, for the efficiency with which p140 activates gene expression. The first pair of chimeric plasmids, pVHS1 and pHVZ1, were constructed with this in mind.

The insertion mutant plasmids chosen for construction of this pair of chimeric plasmids were pI1, which has an insertion at nucleotide 33 of the Vmw175 orf, and pVI3, which has an insertion at nucleotide 292 of the 140k orf. Fragments encoding promoter-leader sequences in their entirety (and a limited amount of linked N-terminal coding sequence) were exchanged between pVI3 and pI1 to create the chimeric plasmids pVHS1 and pHVZ1, as illustrated in Figure 14. Plasmid pVI3, like p140 and other pVI plasmids, contains two *Bam*HI sites (see Section 3A.2 and Figure 5). The cloning procedure required that the *Bam*HI-*Eco*RI fragment representing gene 62 sequences from -1146 to nucleotide 292 of the orf was excised, whilst leaving the residual coding sequences intact (i.e. avoiding cleavage by *Bam*HI at 2198). Thus pVI3 was partially digested with *Bam*HI, followed by extensive digestion with

Figure 14. A generalized sheme for construction of the pVHS and pHVZ series of chimeric plasmids. The chimeric plasmids were constructed using fragments derived from pairs of pVI and pI series plasmids. The pVI plasmids each contain a single in-frame *Eco*RI linker insertion within VZV 140k coding sequences (represented by the stippled section). VZV 140k coding sequences are expressed from the authentic VZV gene 62 promoter which is marked VZ. The two *Bam*HI sites in pVI plasmids, one of which is located immediately 5' to the VZV insert (-1146), the other within the orf, are marked. The analogous pI plasmids contain *Eco*RI linker insertions within Vmw175 coding sequences (represented by the open section). Vmw175 coding sequences are expressed from the SV40 early promoter-enhancer region which is represented by the solid section and marked SV. The details of the cloning procedure are described fully in Section 3C.2.4. Essentially, *Bam*HI-*Eco*RI and *Kpn*I-*Eco*RI fragments (representing promoter sequences linked to N-terminal coding sequences) were excised from pVI and pI plasmids respectively as shown. The excised pVI *Bam*HI-*Eco*RI fragment was ligated between the *Kpn*I and *Eco*RI sites of the pI plasmid to generate a pHVZ plasmid. Likewise, ligation of the excised *Kpn*I-*Eco*RI pI fragment between the *Bam*HI and *Eco*RI sites of the pVI plasmid yielded a pVHS plasmid. Thus portions of N-terminal coding sequences and linked promoter sequences were exchanged.


(-1146)
BamHI
EcoRI
VZ
ATG
VZ
pVI
pUC9
BamHI
KpnI
+27
EcoRI
SV
ATG
SV
pI
pBR322
Bam/Kpn
SV
pVHS
pUC9
VZ
Kpn/Bam
pHVZ
pBR322

*Eco*RI, and both the excised *Bam*HI-*Eco*RI fragment (-1146 to 292), and the appropriate partial digestion product isolated. The *Kpn*I-*Eco*RI fragment representing the SV40 promoter-enhancer region linked to IE3 sequences (extending to nucleotide 33 of the orf) was excised from pI1 (and isolated), and replaced with the corresponding *Bam*HI-*Eco*RI fragment (-1146 to 292) excised from pVI3, resulting in fusion of nucleotides 1-292 of the gene 62 orf to nucleotides 34-3894 of the IE3 orf (by linker sequences), creating pHVZ1. The chimeric polypeptide encoded by pHVZ1, which consists of predominantly Vmw175 sequences, is thus expressed from gene 62 promoter sequences. In the reciprocal ligation, the *Kpn*I-*Eco*RI fragment excised from pI1 (above), was ligated between the *Bam*HI and *Eco*RI sites of the pVI3 partial digestion product, resulting in fusion of nucleotides 1-33 of the IE3 orf to nucleotides 293-3930 of the gene 62 orf (by linker sequences), creating pVHS1. The VHS1 chimeric polypeptide, which consists predominantly of 140k sequences, is thus expressed from SV40 regulatory sequences. Ligation of *Bam*HI 5' overhanging ends to *Kpn*I 3' overhanging ends was facilitated by inclusion of an 8-mer *Bam*HI-*Kpn*I adaptor oligonucleotide, ^{5}'GATCGTAC^{3}', in the ligations.

2.5. Plasmids pVHS1 and pHVZ1 Activate Gene Expression with an Efficiency Similar to That of p175

Plasmid pHVZ1 was found to be indistinguishable from its parent plasmid (p175) in transfection assays (Figure 15 and Table 4), which indicates that the activity of p175 does not reflect a reduced efficiency of SV40 promoter sequences relative to gene 62 promoter sequences. Somewhat unexpectedly, however, pVHS1 (which is composed predominantly of p140 sequences), rather than having a phenotype like p140, was found to be equivalent to p175 both in the absence, and in the presence of p111 (Figure 15 and Table 4). Alterations in 140k coding sequences in pVHS1 complicate the interpretation of this result. Either the promoter-leader sequences in p140 and p175 do indeed influence their phenotypes in transfections, or

Figure 15. CAT assays showing the relative *trans*-activation activities of p140, p175, and the chimeric plasmids, pVHS1 and pHVZ1. The ability of the test plasmids to activate expression from pgDCAT, which contains the HSV-1 glycoprotein D (gD) promoter linked to the coding sequences of the CAT reporter gene, was compared in transfection assays. The CAT extracts were diluted 1:5 in the assays shown (all subsequent Figures show the results of assaying neat extract). **(a).** Activation alone. W.S. HeLa cells were transfected with 4ug pgDCAT plus 4ug of either pUC9 (negative control), p140, p175, pVHS1, or pHVZ1, as indicated. The construction and structure of pVHS1 and pHVZ1 is described in Section 3C.2.4(a). **(b).** Activation of pgDCAT in the presence of Vmw110. The CAT assays in panel (b) represent transfection of cells with pgDCAT, test plasmid, and in addition, 4ug of plasmid p111, which expresses Vmw110. The total amount of plasmid DNA in transfections was equalized by addition of pUC9 where appropriate. These assays graphically demonstrate the powerful *trans*-activation of pgDCAT by p140 alone (panel (a)), and demonstrate synergistic activation by the combination of Vmw175 and Vmw110 (the track in panel (b) marked pUC9 shows the low level of activation of pgDCAT by p111 alone-compare to panel (a)). The chimeric plasmids have phenotypes like that of p175.

(a)
(b)
pUC9
p140
p175
pVHS1
pHVZ1

Plasmid	*Trans*-Activation Activity	
	Alone	+ p111
p140	478 (88)	313 (114)
p175	20 (13)	431 (129)
pVHS1	53 (10)	667 (33)
pHVZ1	91 (32)	715 (304)

Table 4. *Trans*-activation by the chimeric plasmids pVHS1 and pHVZ1. The ability of plasmids pVHS1 and pHVZ1 to activate expression from pgDCAT was compared to that of the parental plasmids in transfection assays (Section 3C.2.5). The CAT assays of a typical experiment are shown in Figure 15. The percentage conversion per microgram protein was calculated for each sample as described in Section 2B.7 using appropriate extract dilutions. The results shown are the mean of three independant experiments, with the standard deviation given in brackets. These results demonstrate the powerful *trans*-activation by p140 alone and also synergistic activation by p111 in the presence of p175. The chimeric plasmids have phenotypes similar to that of p175.

alternatively, the phenotype of pVHS1 reflects these coding sequence alterations. To distinguish between these possibilities, it was necessary to construct two related plasmids (p140SV and p175VZ) by exchanging fragments encoding promoter-leader sequences in isolation. This is described in the next section.

It is especially interesting that pVHS1 activates the gD promoter in synergy with p111. Since only 11 amino acids of the VHS1 polypeptide are derived from Vmw175, the most likely interpretation of this result is that the ability of VHS1 to function in synergy with Vmw110 reflects an intrinsic property of 140k. In agreement with this view, synergistic activation of both the VZV and HSV-1 tk promoters by 140k and Vmw110 has been reported (Cabirac *et al.*, 1990). The failure to detect this synergy when using pgDCAT presumably reflects a property of this reporter plasmid, rather than an inherent functional difference between 140k and Vmw175. Supposing the gD promoter were maximally activated by 140k alone, then synergy with Vmw110 would not be detected as CAT activity would not be increased. If, however, the intrinsic activity of 140k were reduced (perhaps by small alterations such as those in pVHS1) and the gD promoter was no longer maximally activated, then the ability of 140k to function in synergy with Vmw110 might be revealed.

2.6. Construction of Plasmids p140SV and p175VZ

To enable construction of chimeric plasmids in which only promoter-leader sequences were exchanged between p140 and p175, it was first necessary to generate derivatives of these plasmids (p140ET and p175ET) with unique *Eco*RI sites located just upstream of the 5' ends of their respective transcripts. The construction of p140ET by site-directed mutagenesis is described in Section 3B.1.3.

Plasmid p175 was modified by converting the *Bst*EII site at position +239 of the p175 leader sequences (65 nucleotides upstream of the ATG codon) to an EcoRI site, to yield p175ET. Plasmid p175 was partially digested with

*Bst*EII (as it contains a second *Bst*EII site) and singly cut linear plasmid molecules isolated. A partially double-stranded oligonucleotide linker was designed in which the central region contained an *Eco*RI site, while the 5' overhangs were complimentary to those of the *Bst*EII site. Thus the oligonucleotide ^{5}'GT(G/C)ACGAATTC^{3}' was synthesised and ligated directly with the *Bst*EII linearized p175 DNA (following phosphorylation of 5' ends), and the desired clone isolated.

Plasmids p140SV and p175VZ resulted from the exchange of *Bam*HI-*Eco*RI and *Kpn*I-*Eco*RI fragments (containing promoter-leader sequences) between p140ET and p175ET. Their construction followed the scheme outlined for construction of pVHS1 and pHVZ1 (Figure 14, Section 2.4), p140ET replacing pVI3, and p175ET replacing pI1.

2.7. Promoter-Leader Sequences do not Influence the Phenotypes of p140 and p175 in Transfection Assays

The effect of promoter-leader sequences on the phenotypes of p140 and p175 were assessed by determining the phenotypes of p140SV and p175VZ in transfection assays.

The CAT assays from a single experiment are shown in Figure 16. The activity of p140 in this experiment was reduced 5- to 10-fold relative to that of earlier experiments (Figure 16 and Table 4). Furthermore, inclusion of p111 in the transfection reduced the activity of p140 by 2- to 3-fold. A similar reduction was observed in later assays where, in addition, synergistic activation by p111 in the presence of p175 was only about 5- to 10-fold, values of 30- to 100-fold being more typical (Section 3C.2.3 and Table 4). This is reflected in the results given in Table 5. This effect is probably attributable to an alteration in the state of the cells (reflecting, for example, variability in batches of serum). Similar variation in transfection data as a result of minor alterations in experimental conditions has been reported (Everett, 1988). Nevertheless, the assay still clearly differentiated between p175 and p140, and moreover, between

Figure 16. Promoter-leader sequences do not influence the phenotypes of p140 and p175 in transfection assays. VZV gene 62 and SV40 promoter-leader sequences in p140 and p175 respectively, were exchanged to create p140SV and p175VZ (see Section 3C.2.4(c)). Thus VZV 140k and Vmw175 coding sequences in these chimeric plasmids are expressed from from heterologous promoter-leader sequences. The ability of p140SV and p175VZ to activate expression from pgDCAT was compared with that of the parental plasmids in transfection assays. **(a).** Activation alone. W.S. HeLa cells were transfected with 4ug pgDCAT plus 4ug of either pUC9 (negative control), p140, p175, p140SV, or p175VZ, as indicated. **(b).** Activation of pgDCAT in the presence of Vmw110. The CAT assays in panel (b) represent transfection of cells with pgDCAT, test plasmid, and in addition, 4ug of plasmid p111, which expresses Vmw110. The total amount of plasmid DNA in transfections was equalized by addition of pUC9 where appropriate. These assays demonstrate that p140SV and p175VZ have phenotypes like that of their parental plasmids.

(a)
(b)
pUC9
p140
p175
p140SV
p175VZ

Table 5. Promoter-leader sequences do not influence the activities of p140 and p175 in transfection assays. The ability of plasmids p140SV and p175VZ to activate expression from pgDCAT was compared with that of the parental plasmids in transfection assays (Section 3C.2.4). The CAT assays of a single experiment are shown in Figure 16. The percentage conversion per microgram protein was calculated for each sample as described in Section 2B.7 using appropriate extract dilutions. The results shown give the mean and standard deviation (brackets) of at least three independent experiments. Note that the activity of p140 is reduced compared to earlier experiments (Table 4), with a further reduction of 2- to 3-fold being observed in the presence of p111. Furthermore, activation by p111 in the presence of p175 was significantly reduced compared to earlier experiments (Table 4). Nevertheless, the assay still clearly differentiates between p140 and p175, and more importantly between pVHS1 (and pHVZ1) and p140, and demonstrates that p140SV and p175VZ have phenotypes like those of their parent plasmids.

Plasmid	*Trans*-Activation Activity	
	Alone	+ p111
p140	92 (26)	25 (5)
p175	9 (3)	30 (11)
pVHS1	6 (1)	42 (10)
pHVZ1	12 (4)	24 (8)
p140SV	84 (18)	26 (8)
p175VZ	5 (2)	28 (19)

pVHS1 (and pHVZ1) and p140 (Table 5). Plasmids p140SV and p175VZ activated the gD promoter with comparable efficiency to their parent plasmids (p140 and p175 respectively), thus demonstrating that the promoter-leader sequences in p140 and p175 do not influence their phenotypes in transfection assays.

3C.3. CONSTRUCTION AND CHARACTERIZATION OF PLASMIDS WITH DEFINED DELETIONS IN THE N-TERMINAL CODING SEQUENCES OF 140k

The phenotype of plasmid pVHS1 appears, therefore, to be a consequence of alterations in coding sequences (rather than regulatory sequences). During construction of pVHS1, the N-terminal 98 amino acids of 140k were removed, and are replaced with the N-terminal 11 amino acids of Vmw175. Thus, either the N-terminal 98 amino acids of 140k have a critical role in activation, or alternatively, the N-terminal residues derived from Vmw175 somehow reduce the activity of residual 140k sequences. The latter seems unlikely as five of these 11 Vmw175 residues are conserved in the N-terminus of 140k (Figure 17). Therefore, defined deletions were created within residues 1-98 of 140k in order to assess their possible role in *trans*-activation. This is described in the following sections.

3.1. Construction of Plasmid p140del1

Plasmids pVI1 and pVI3 contain single *Eco*RI linkers inserted into the gene 62 orf at nucleotides 142 and 292 respectively (Section 3C.1.1). Plasmid p140del1, in which an in-frame deletion removes nucleotides 142-292 of the orf (codons 48-98), was created from pVI1 and pVI3 as follows. The *Bam*HI-*Eco*RI fragment of pVI3, representing gene 62 sequences from -1146 to nucleotide 292 of the orf, was excised (as described in Section 3C.2.4), and replaced by the corresponding fragment from pVI1, which extends to only nucleotide 142 of the orf. (The single nucleotide deletion in pVI1 noted in Section 3C.1.1 is located 3' to the *Eco*RI linker and will not be contained in the fragment

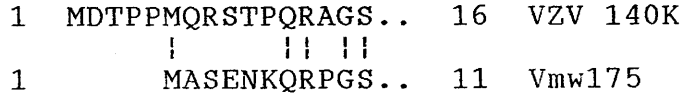


Figure 17. Limited N-terminal homology between VZV 140k and Vmw175. The N-terminal 16 amino acids of VZV 140k are shown aligned with the N-terminal 11 amino acids of Vmw175. Five out of eleven of the Vmw175 residues are conserved in VZV 140k.

used in this procedure.)

3.2. p140del1 Activates Gene Expression as Efficiently as p140

Coding sequences deleted from gene 62 during construction of p140del1 include a stretch of 19 amino acids, nine of which are acidic, a property associated with the activation domains of a class of transactivators which includes GAL4 of *Saccharomyces cerevisiae* (Ma and Ptashne, 1987), and Vmw65 of HSV-1 (Triezenberg *et al.*, 1988; Sadowski *et al.*, 1988)(see Section 1B.3.1.[d]). However, activation of gene expression in transfection assays by plasmids p140del1 and p140 is indistinguishable in the absence of Vmw110 (Figure 18A and Table 6), thus demonstrating that amino acid residues 48-98 of 140k are not essential for fully efficient *trans*-activation by the polypeptide.

3.3. Construction of p140delM1 and p140delM2

Two further defined deletions were created within the N-terminus of 140k by M13 mutagenesis, one removing nucleotides 7-57 (codons 3-19), and a second removing nucleotides 58-117 (codons 20-39). The single-stranded template DNA used for mutagenesis was prepared from M13E13 (described in Section 3B.1.3), which contains gene 62 sequences from -1146 to nucleotide 1412 of the orf (on a *Bam*HI-*Eco*RI fragment) ligated between the *Bam*HI and *Eco*RI sites of M13 mp18 (nucleotide +57 of gene 62 having been mutated from A to G to create an *Eco*RI site at +57). The procedure for M13 deletion mutagenesis is essentially as described for point mutagenesis (Section 2B.23), except that the mutagenic oligonucleotides contain deletion rather than point mutations. Following mutagenesis, mutant phage were plaque purified and their sequence confirmed by dideoxy sequencing.

The mutations thus created were transferred to p140 by replacing the wt *Bam*HI-*Eco*RV fragment, which encodes gene 62 sequences from -1146 to nucleotide 1245 of the orf, with the corresponding *Bam*HI-*Eco*RV fragments from

the mutant M13 clones, which contain the deletions. Plasmid p140 was partially digested with BamHI, followed by complete digestion with *Eco*RV. The appropriate partial digestion product was isolated (from which the above wt *Bam*HI-*Eco*RV fragment had been excised), and ligated directly with the mutant *Bam*HI-*Eco*RV fragments, yielding p140delM1 and p140delM2.

3.4. The Deletions Contained in p140delM1 and p140delM2 do not Impair *Trans*-Activation

Plasmids p140delM1 and p140delM2 were found to activate the gD promoter as efficiently as p140 in transfection assays (Figure 19), demonstrating that amino acid residues 3-19, and 20-39, are also dispensable for fully efficient activation. The degree of synergy between p175 and p111 was low (2 fold), and activation by p140 was again reduced by cotransfection with p111, as noted in Section 3C.2.7). Despite these variations, all previously characterised plasmids behaved in a predictable manner.

3C.4. What is the Basis of the Reduced Activity of the VHS1 Polypeptide?

The importance of residues 1-98 of 140k in *trans*-activation was suggested following analysis of pVHS1 (Section 3C.2.5) and p140SV (Section 3C.2.7). However, the results obtained with p140del1 (Section 3C.3.2), and with p140delM1 and p140delM2 (Section 3C.3.4), argue against the importance of these residues. It is possible that the deletions in the above constructs, which only remove portions of residues 1-98, are insufficient to alter the activity of 140k. With this in mind, plasmid p140del98 was constructed in which gene 62 sequences from nucleotide +57 to nucleotide 292 (codon 98) of the orf are deleted. The polypeptide expressed by p140del98 has not been characterized, but must be N-terminally truncated to at least amino acid 99. (Codon 99, an ATG codon, may serve as a site for initiation of translation, permitting expression of a polypeptide lacking amino acids 1-98, although

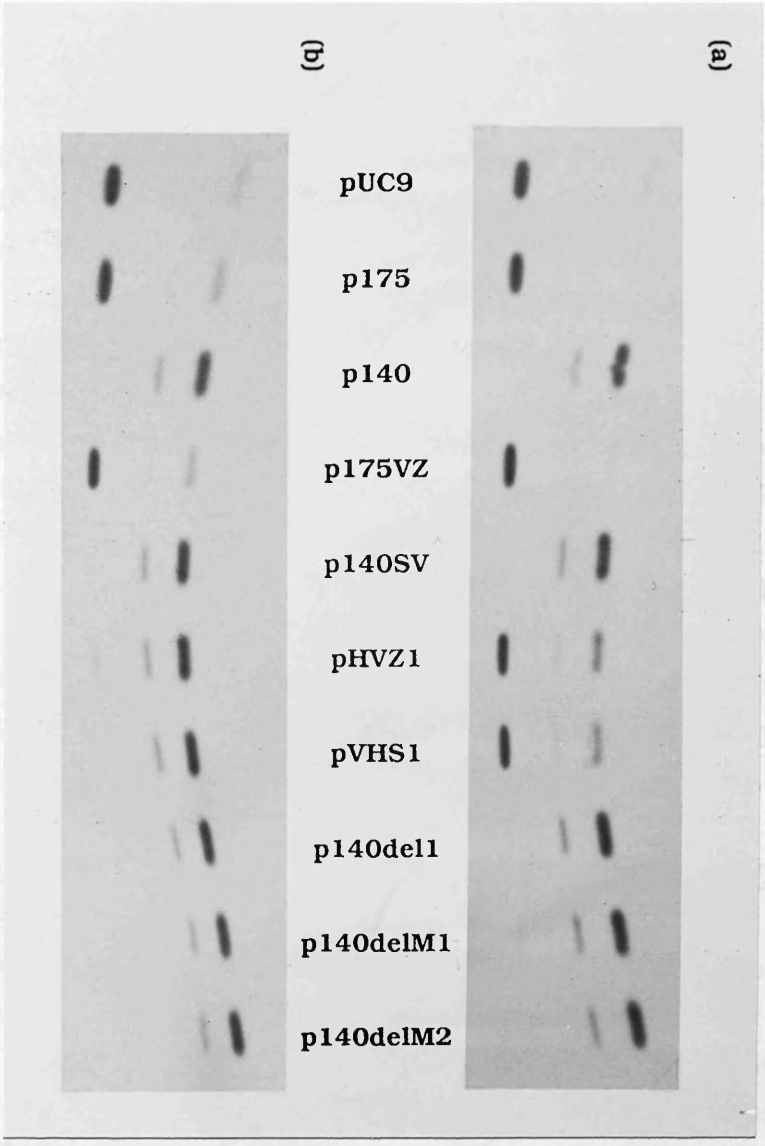
Figure 18. CAT assays showing that p140del1 activates gene expression as efficiently as p140. Activation of pgDCAT by p140del1 and p140 was compared in transfection assays. Plasmid p140del1 contains an in-frame deletion removing codons 48-98 of the VZV 140k orf. **(a).** Activation alone. W.S. HeLa cells were transfected with 4ug pgDCAT plus 4ug of either pUC9 (negative control), p140, p175, pHVZ1, pVHS1, or p140del1, as indicated. **(b).** Activation of pgDCAT in the presence of Vmw110. The CAT assays in panel (b) represent transfection of cells with pgDCAT, test plasmid, and in addition, 4ug of plasmid p111, which expresses Vmw110. The total amount of plasmid DNA in transfections was equalized by addition of pUC9 where appropriate. Note that synergistic activation by p175 in the presence of p111 is reduced compared to earlier assays in Figures 15 and 16 (compare the track marked p175 in panel (b) with that in (a)). This is discussed in Section 3C.2.4(d). However, the assay still clearly differentiates between p140 and p175, and shows that p140del1 activates pgDCAT as efficiently as does p140.

(a)
pUC9
p175
p140
pHVZ1
pVHS1
p140del1
(b)

Plasmid	*Trans*-Activation Activity	
	Alone	+ p111
p140	132	20
p175	12	27
pVHS1	7	60
pHVZ1	15	20
p140del1	156	71

Table 6. Plasmid p140del1 activates gene expression as efficiently as does p140. The ability of plasmid p140del1 to activate expression from pgDCAT was compared to that of p140 in transfection assays (Section 3C.3.2). The CAT assays of a single experiment are shown in Figure 18. The percentage conversion per microgram protein was calculated for each sample as described in Section 2B.7 using appropriate extract dilutions. The results shown are from a single experiment. A number of independent experiments were performed, and although the transfection efficiency varied between experiments, the pattern of results obtained was essentially consistent with those shown.

Figure 19. CAT assays showing that p140delM1 and p140delM2 activate gene expression as efficiently as p140. Activation of pgDCAT by p140delM1, p140delM2, p140, and other previously characterized plasmids was compared in transfection assays. Plasmids p140delM1 and p140delM2 contain in-frame deletions removing codons 3-19 and 20-39 respectively of the VZV 140k orf. **(a).** Activation alone. W.S. HeLa cells were transfected with 4ug pgDCAT plus 4ug of either pUC9 (negative control), p175, p140, p175VZ, p140SV, pHVZ1, pVHS1, p140del1, p140delM1, or p140delM2, as indicated. **(b).** Activation of pgDCAT in the presence of Vmw110. The CAT assays in panel (b) represent transfection of cells with pgDCAT, test plasmid, and in addition, 4ug of plasmid p111, which expresses Vmw110. The total amount of plasmid DNA in transfections was equalized by addition of pUC9 where appropriate. A single experiment was performed and the results were not quantitated.


(b)
(a)
pUC9
p175
p140
p175VZ
p140SV
pHVZ1
pVHS1
p140del1
p140delM1
p140delM2

initiation could occur further downstream resulting in even greater truncation.) The finding that the activity of p140del98 is 50-100% of that obtained with p140 (results not shown) is further evidence that the N-terminal 98 residues of 140k are non-essential in *trans*-activation transfection assays.

It appears then that the Vmw175 residues in pVHS1 are decreasing the activity of residual 140k sequences which, in isolation, are fully functional. By analogy, it might be predicted that removal of these same sequences from the N-terminus of Vmw175 (as occurs in pHVZ1) would result in the polypeptides activity being increased. However, pHVZ1 exhibits an activity that is indistinguishable from wt p175. Thus whatever the mechanism by which these sequences mediate their effect in pVHS1, it presumably does not reflect a similar role in Vmw175. That is, the low activity of Vmw175 compared to 140k inferred from transfection assays is not attributable to these sequences. Their effect in pVHS1 is probably highly dependent on the particular environment in which they are located, and thus confined to pVHS1. As such, it is unlikely that pVHS1 is revealing anything of significance regarding the function of 140k or Vmw175.

3C.5. LIMITATIONS OF TRANSFECTION ASSAYS IN THE STUDY OF PROTEIN FUNCTION

When studying the function of a polypeptide by determining the effects of small insertion or deletion mutations (within the coding sequences) in transfection assays (or by analysis of chimeric polypeptides), it is assumed that wt and mutant plasmids are expressing equivalent amounts of polypeptides, and thus any change in activity is attributed to an alteration in the intrinsic activity of the polypeptide. In many cases this assumption is doubtless correct. Nevertheless, there is clearly the potential for such alterations to affect the levels of a protein which are expressed. For example, the mRNA might become less stable, or might have an altered secondary

structure which reduces the rate of translation initiation or elongation. Alternatively, the protein product may have reduced solubility or stability. These complications should be borne in mind when interpreting such data. Indeed the phenotype of pVHS1 may be explained by just such effects since the alterations in 140k/Vmw175 coding sequences are in the vicinity of the Vmw175 ATG start codon (at which translation of the VHS1 polypeptide presumably initiates), and may thus reduce the stability of the mRNA or the rate of initiation or elongation of translation as outlined.

3D. REPRESSION OF EXPRESSION FROM THE VZV GENE 62 PROMOTER BY VZV 140K AND HSV-1 Vmw175 AND THEIR MUTANT DERIVATIVES

During HSV-1 infection of tissue culture cells, expression of IE gene 3 is subject to autoregulation or repression by its product, Vmw175 (see Sections 1B.3.2 and 1C.2.4). Repression of the IE-3 promoter by Vmw175 has been reproduced in transfection assays (O'Hare and Hayward, 1985a), and it has been demonstrated that region 2 polypeptide sequences have a critical role in the autoregulation phenotype (Paterson and Everett, 1988a; DeLuca and Schaffer, 1988; Shepard *et al.*, 1989). Since the sequences comprising region 2 of Vmw175 and VZV 140k are among the most highly conserved between the two proteins (McGeoch *et al.*, 1986), it was of interest to determine whether VZV 140k exhibited similar autoregulation of its own promoter. To this end plasmid p140CAT was constructed in which the promoter-leader sequences of VZV gene 62 drive the expression of the CAT gene (Figure 20). The following sections describe the results of co-transfection experiments utilizing p140CAT in which the ability of VZV 140k to repress gene 62 promoter activity was assessed. It has been reported that gene 62 promoter activity is increased about 15-fold by co-transfection of p140CAT with a plasmid expressing Vmw65 (McKee *et al.*, 1990), and transfection assays were carried out both in the absence and in the presence of stimulation by Vmw65. The results demonstrate that 140k strongly represses both the basal and Vmw65-stimulated levels of expression from the gene 62 promoter, as does Vmw175. Moreover, genetic evidence was obtained which indicates that the two polypeptides repress gene expression by a related mechanism.

3D.1. Plasmids

Plasmid p140CAT contains VZV gene 62 promoter and upstream sequences from the *ClaI* site (-1146) to nucleotide +57 cloned upstream of the chloramphenicol acetyl transferase (CAT) gene in the vector pCAT, which was derived from pBLW2 (Gaffney et al, 1985). A *SmaI-BglII*

fragment representing the above sequences was isolated from p140BT (see Section 3B.1.3), and inserted between the *Sma*I and *Bam*HI sites of pCAT to generate p140CAT (Figure 20).

p140del1CAT and p140del2CAT are largely identical to p140CAT, but contain deletions within the gene 62 capsite-leader region. Fragments containing these deletions were isolated from plasmids pTM14CAT and pTM15CAT (Tom A. McKee, Ph.D thesis, University of Glasgow, 1990). Plasmid pTM14CAT (Figure 21) contains an *Xba*I-*Hin*dIII fragment representing sequences upstream of gene 62 from *Dde*I (-781) to *Sca*I (-22) inserted upstream of the CAT gene in a derivative of pFJ3 (Rixon and McLauchlan, 1990). The *Xba*I and *Hin*dIII sites are derived from polylinker sequences flanking the VZV insert in the parent plasmids. Plasmid pTM14CAT was modified by inserting a double stranded oligonucleotide encoding VZV sequences from the *Afl*III site (-33) to +20 to create pTM15CAT (Figure 21). The oligonucleotide was designed to have 5'-overhanging ends complimentary to those produced by *Afl*III and *Bam*HI. pTM14CAT was partially digested with *Afl*III and then cleaved to completion with *Bam*HI (which cleaves a BamHI site in the polylinker sequences located between the VZV insert and the CAT gene), and the oligonucleotide ligated between the *Afl*III and *Bam*HI sites to produce pTM15CAT. Thus in pTM15CAT VZV sequences from -22 to +20 replace the polylinker sequences located between the VZV insert and the CAT gene in pTM14CAT.

Plasmids pTM14CAT and pTM15CAT lack VZV sequences from *Cla*I (-1146) to *Dde*I (-781) present in p140CAT, and thus cannot be compared directly to p140CAT in transfection assays to determine the effect of the capsite-leader deletions. Thus *Xho*I-*Eco*RI fragments containing gene 62 upstream sequences (and including the region harbouring the deletions and the 5'-end of the CAT gene) were excised from pTM15CAT and pTM14CAT, and replace the full length *Xho*I-*Eco*RI fragment of p140CAT to create p140del1CAT and p140del2CAT respectively (Figure 21). p140del1CAT is identical to p140CAT except that it lacks residues +20 to

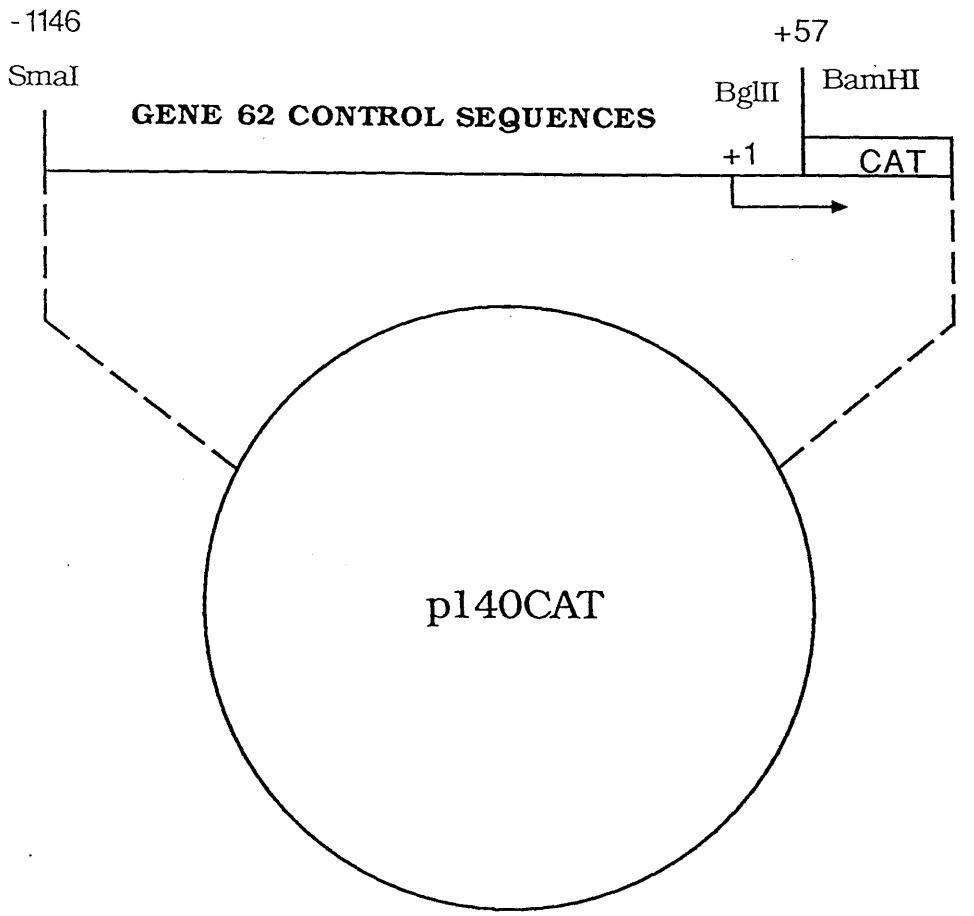
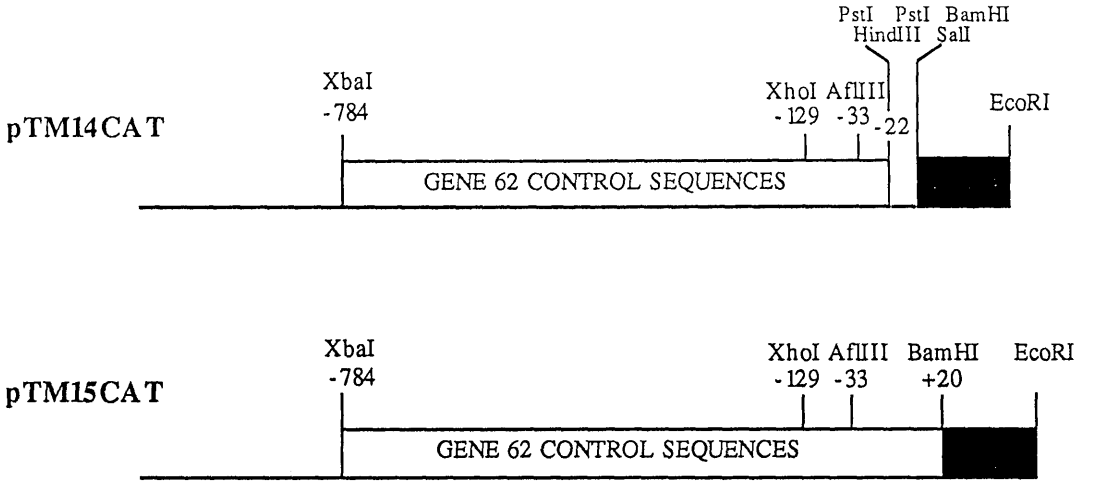


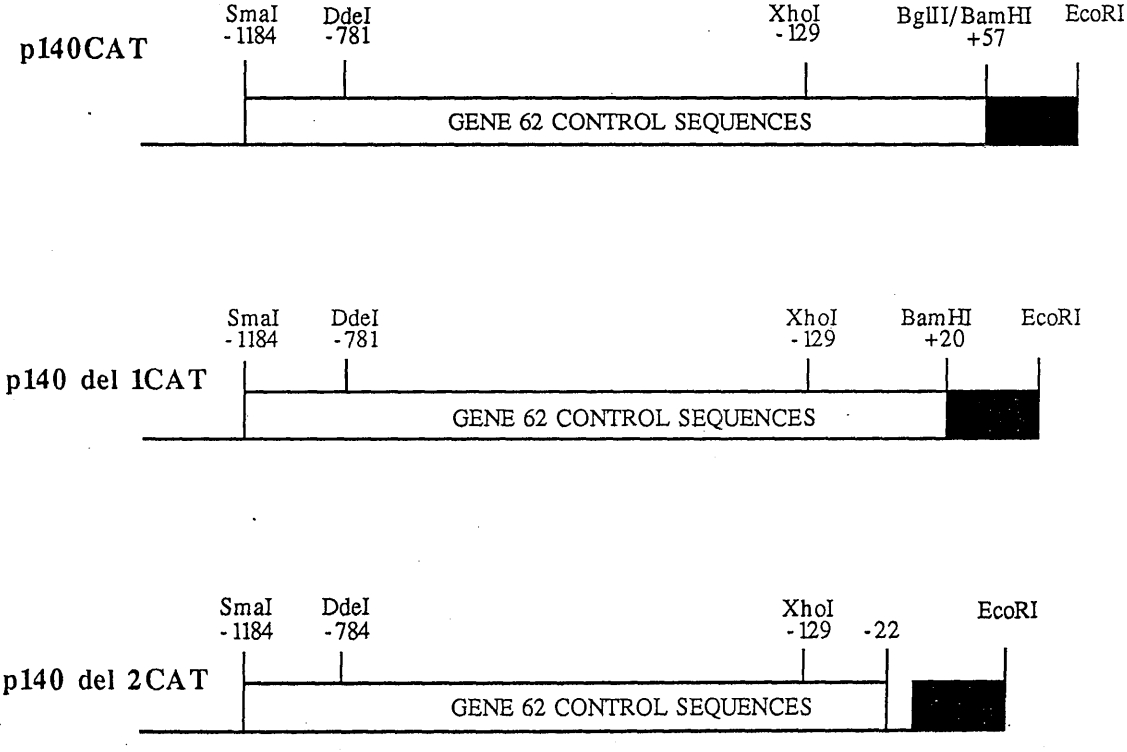
Figure 20. Structure of plasmid p140CAT. A *SmaI-BglII* fragment representing VZV gene 62 sequences from -1146 (*ClaI*) to +57 (and containing the complete promoter of the gene) was isolated from plasmid p140BT (McKee *et al.*, 1990) and inserted between the *Sma*I and *Bam*HI sites of the vector pCAT, upstream of the chloramphenicol acetyl transferase (CAT) coding sequences, to generate p140CAT. The transcription start site (+1) is indicated. The *Sma*I site is derived from pUC9 polylinker sequences located 5' to the VZV insert in p140BT. The *Bgl*II site is the result of a two-step mutagenesis protocol in which nucleotide +57 (of gene 62) was first mutated, changing the sequence from AAATTC to GAATTC, the resulting *Eco*RI site was subsequently modified by linker insertion to create the unique *Bgl*II site in p140BT.

Figure 21. Plasmids containing VZV gene 62 control sequences linked to the CAT gene. VZV gene 62 sequences are represented by the open boxes, and the solid boxes represent N-terminal coding sequences of the CAT reporter gene. Some restriction enzyme recognition sites and their coordinates (relative to the transcription start site, +1) are marked. a) Plasmid pTM14CAT contains VZV gene 62 sequences from *Xba*I (-784) to *Sca*I (-22) linked to the CAT gene. Polylinker sequences containing the restriction enzyme sites *Pst*I-*Hind*III-*Pst*I-*Sal*I-*Bam*HI are located between the VZV insert and the CAT gene in pTM14CAT. Plasmid pTM15CAT was created by inserting a double-stranded oligonucleotide representing VZV gene 62 sequences from *Afl*III (-33) to +20 between the *Afl*III and *Bam*HI sites of pTM14CAT, thus replacing the polylinker sequences in pTM14CAT with VZV sequences from -22 to +20. b) Plasmid p140CAT contains VZV gene 62 sequences from *Sma*I (-1184) to +57 (*Bgl*II) linked to the CAT gene (see Section 3D.1). Plasmids p140del1CAT and p140del2CAT were created by replacing the *Xho*I (-129) to *Eco*RI (CAT) fragment (representing VZV sequences from -129 to +57 linked to the CAT gene) with the corresponding *Xho*I-*Eco*RI fragments from pTM15CAT and pTM14CAT respectively (which contain deletions in the capsite-leader region).

a)


pTM14CAT
XbaI
-784
PstI
HindIII
PstI
SalI
BamHI
XhoI
-129
AflIII
-33
-22
EcoRI
GENE 62 CONTROL SEQUENCES
pTM15CAT
XbaI
-784
XhoI
-129
AflIII
-33
BamHI
+20
EcoRI
GENE 62 CONTROL SEQUENCES

b)


p140CAT
SmaI
-1184
DdeI
-781
XhoI
-129
BglII/BamHI
+57
EcoRI
GENE 62 CONTROL SEQUENCES
p140 del 1CAT
SmaI
-1184
DdeI
-781
XhoI
-129
BamHI
+20
EcoRI
GENE 62 CONTROL SEQUENCES
p140 del 2CAT
SmaI
-1184
DdeI
-784
XhoI
-129
-22
EcoRI
GENE 62 CONTROL SEQUENCES

+57 of gene 62; p140del2CAT lacks residues -22 to +57 and thus the bona fide VZV transcription start site.

3D.2. The Assay

BHK cells, at a density of 10^6 cells per 50mm dish, were transfected (as described in Section 2B.6.1) with 4ug p140CAT in the presence or absence of pMC1 (which expresses Vmw65) and increasing amounts of p140SV. The total amount of plasmid DNA in each transfection was equalised to 14ug by addition of pUC9. Plasmid p140SV contains the VZV 140k transcription unit (without its own promoter) linked to the SV40 early promoter and enhancer region (see Section 3C.2.6), and was used to express VZV 140k. The use of p140SV (rather than a plasmid which expresses VZV 140k from the gene 62 promoter) reduces difficulties in the interpretation of the results which would arise from promoter competition effects and through repression of the gene 62 promoter in the VZV 140k expression plasmid. CAT extracts were prepared 48 hours after transfection and CAT activities determined as described in Section 2B.7. The protein concentrations of the extracts were determined (Section 2B.8) and the percentage conversion from substrate to product per microgram protein calculated. The CAT activities of extracts prepared from cells transfected with test plasmid were expressed as a percentage of the control value (no test plasmid), and the results depicted graphically.

3D.3. REPRESSION EXPERIMENTS

3.1. Repression by VZV 140k

The results of a typical repression experiment are shown in Figure 22 and are depicted graphically in Figure 23. Transfection of p140CAT alone into cells resulted in 4% conversion per microgram protein (which represents a total conversion of 36% in the assay), and inclusion of pMC1 in the transfection increased CAT activity by 30-fold (determined using suitable extract dilutions). The results clearly show that both the basal and Vmw65-stimulated

levels of CAT activity from p140CAT were repressed by up to 50-fold by the addition of increasing amounts of p140SV. The repression is not due to promoter competition effects since transfection with equivalent molar amounts of pSVEB (which contains the SV40 promoter region present in p140SV, but lacks coding sequences) resulted in levels of repression which were insignificant compared to those induced by p140SV. Similar results were obtained using Vero cells (data not shown).

It should be noted that repression of the gene 62 promoter by p140SV was most clearly observed when the levels of CAT activity in control transfections were high (about 50-200 fold above background). If the cells transfected poorly then the degree of repression was correspondingly reduced (as might be expected). Since repression was always most clearly observed with high level expression from the gene 62 promoter, most experiments which will be described included pMC1.

3.2. Vmw175 Represses the Gene 62 Promoter, But VZV 140k Does Not Repress the HSV-1 IE-3 Promoter

The results presented above clearly demonstrate that co-transfection of p140SV represses the gene 62 promoter. Given the functional similarities of VZV 140k and Vmw175 in transactivation transfection assays (Everett, 1984; Inchauspe *et al.*, 1989; Cabirac *et al.*, 1990), it was of interest to determine whether the two proteins were functionally interchangeable in repression transfection assays. Therefore p140CAT was co-transfected with increasing amounts of p175 (expression of Vmw175 in p175, like that of 140k in p140SV, is driven by the SV40 early promoter region). The results show that Vmw175 also represses the gene 62 promoter with an efficiency similar to that of VZV 140k (Figure 24). The converse experiment to determine whether 140k is able to repress the HSV-1 IE-3 promoter was performed in Vero cells, the cell-type in which repression of the IE-3 promoter by Vmw175 was first demonstrated (O'Hare and Hayward, 1985a). (As noted above,

140k efficiently represses the gene 62 promoter in Vero cells as well as BHK cells.) Plasmid pIE3CAT (in which the HSV-1 IE-3 promoter drives expression of the CAT gene) replaced p140CAT as reporter plasmid in these experiments. Figure 25 shows the results of a single experiment in which pIE3CAT was cotransfected into cells with pMC1 and increasing amounts of test plasmids. The pMC1 stimulated level of expression from pIE3CAT was of the same order as that from pMC1 stimulated p140CAT. IE-3 promoter activity showed a small reduction in the presence of pSVEB, a maximum of 2-fold being observed at the highest level of plasmid. Titration of p175 led to a greater reduction in activity, being 4-fold in the presence of 8ug p175. Although this was less efficient than in previous assays (Paterson and Everett, 1988a), it does confirm repression of the IE3 promoter by Vmw175. In marked contrast, titration of p140SV resulted in an increase in IE-3 promoter activity of 2- to 4-fold. In agreement with this, it was found that in BHK cells p140SV strongly transactivated the basal level of expression from the IE-3 promoter (in the absence of pMC1) approximately 40-fold (data not shown). Therefore, although both VZV 140k and Vmw175 repress their respective promoters, the mechanisms by which this occurs are not entirely interchangeable.

3.3. Repression of the Gene 62 Promoter by VZV 140k and Vmw175 Requires the Integrity of Homology Region 2

The integrity of homology region 2 of Vmw175 (amino acid residues 315-484) has been shown to be of crucial importance for repression of the IE-3 promoter (Paterson and Everett, 1988a; DeLuca and Schaffer, 1988; Shepard *et al.*, 1989). To determine whether region 2 of Vmw175 was also involved in the repression of the gene 62 promoter, plasmid pI13 (Paterson and Everett, 1988a) was co-transfected in increasing amounts with p140CAT. Plasmid pI13 contains an in-frame insertion of four amino acids into the proline codon at position 324 of Vmw175 (within region 2; Paterson and Everett, 1988a). The effect of the

mutation is to essentially eliminate repression of the IE-3 promoter by Vmw175 (Paterson and Everett, 1988a). The same effect was observed in co-transfections of p140CAT and pI13 (Figure 24). Therefore the same region of Vmw175 is involved in the repression of both the gene 62 and IE-3 promoters.

Plasmid pVI8 encodes a mutant form of VZV 140k which is analogous to the protein expressed by pI13. It was derived from p140 (as described in section 3C.1.1) and contains a mutation resulting in the insertion of four amino acids into the serine codon at position 472 of 140k, which lies within region 2 (amino acid residues 467-641). Like pI13, pVI8 was unable to repress p140CAT (Figure 24). The interpretation of this result is potentially complicated since pVI8 uses the gene 62 promoter instead of the SV40 early promoter in p140SV. This raises the possibility that the lack of repression by pVI8 is not due to the mutation in region 2 of VZV 140K but that autoregulation of VI8 polypeptide expression from pVI8 results in insufficient protein to repress the gene 62 promoter in p140CAT as well. However, it is difficult to envisage how the VI8 polypeptide might repress its own expression from pVI8 without also reducing gene 62 promoter activity in p140CAT. In addition, in *trans*-activation transfection assays the VI8 mutation, like that of pI13 (Paterson and Everett, 1988a), eliminates the ability of plasmid pVI8 to *trans*-activate the HSV-1 glycoprotein D (gD) promoter (which is activated 50-100-fold by 140k; see section 3C.1.2). Therefore it is most likely that, as with the mutations in region 2 of Vmw175 (Paterson and Everett, 1988a; Deluca and Schaffer, 1988; Shepard *et al.*, 1989), the VI8 mutation results in an inactive polypeptide. The experiment therefore demonstrates that the same homologous regions of Vmw175 and VZV 140k are involved in the repression of their respective promoters.

Figure 22. CAT assays showing repression of the VZV gene 62 promoter by VZV 140k. (a). Repression of the *cis*-activated level of expression from p140CAT. BHK cells were transfected with 4ug p140CAT alone (tracks 1 and 6) or with in addition increasing amounts of p140SV (tracks 2-5; 1, 2, 4 and 8ug respectively) or pSVEB (tracks 7-10; 0.5, 1, 2 and 4ug respectively). The negative control plasmid, pSVEB, lacks coding sequences present in p140SV and is consequently approximately half the size. Hence the actual amounts used in transfections were 0.5, 1, 2 and 4ug to maintain a molar equivalence of common sequences. **(b).** Repression of the Vmw65 *trans*-activated level of expression from p140CAT. This experiment was performed essentially as described in (a), except all transfections contained in addition 2ug of plasmid pMC1, which expresses Vmw65. Tracks 1 and 6 show the strong stimulation of p140CAT by pMC1. Cotransfection of increasing amounts of p140SV causes repression of both the *cis*- and *trans*-activated levels of expression from p140CAT not caused by cotransfection of pSVEB, which contains only promoter sequences. These results are depicted graphically in Figure 23 taking account of the protein concentrations in the extracts.

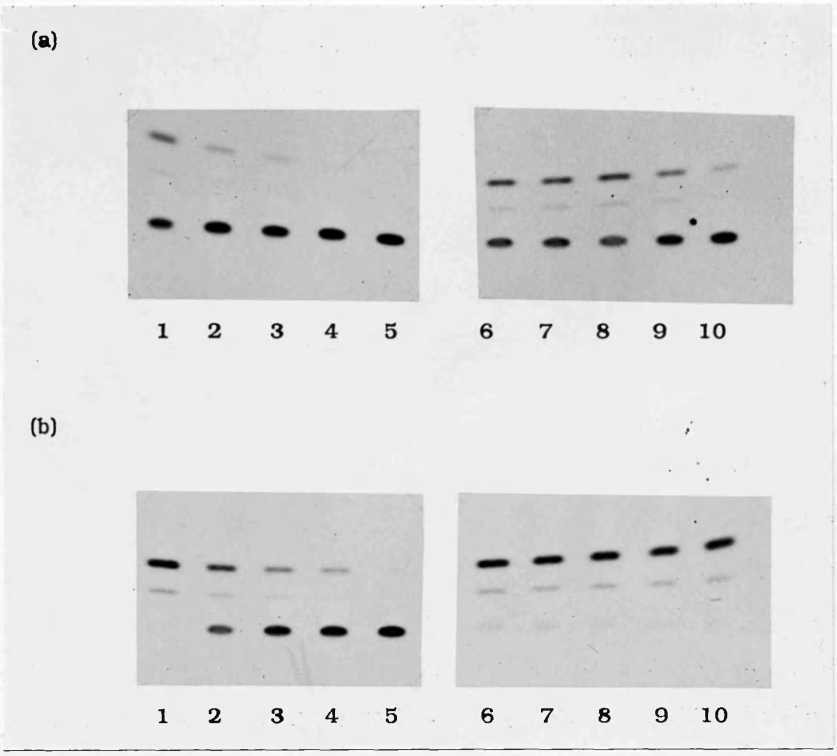

(a)
1 2 3 4 5 6 7 8 9 10
(b)
1 2 3 4 5 6 7 8 9 10

Figure 23. Graph showing repression of the *cis*- and *trans*-activated levels of expression from VZV gene 62 promoter-leader sequences (in p140CAT) by VZV140k. BHK cells were transfected with 4µg of p140CAT and increasing amounts of p140SV (which expresses VZV 140k; 1, 2, 4 and 8µg) or pSVEB (0.5, 1, 2 and 4µg), in the presence or absence of pMC1 (which expresses Vmw65). The negative control plasmid, pSVEB, lacks coding sequences present in p140SV and is consequently approximately half the size. Hence the actual amounts used in transfections were 0.5, 1, 2, and 4ug to maintain a molar equivalence of common sequences. The relative CAT activities (determined as described in the text) from a typical titration experiment, the CAT assays of which are shown in Figure 22, are shown plotted against the amount of test plasmid DNA added. The degree of repression observed in titration experiments was variable, and almost certainly reflects the variation in levels of expression from the gene 62 promoter noted in the text. However, the experiment in the presence of pMC1 has been repeated many times, and in all cases the pattern of results was consistent with those above. The titration in the absence of pMC1 was performed twice, and in both cases the results clearly showed repression.

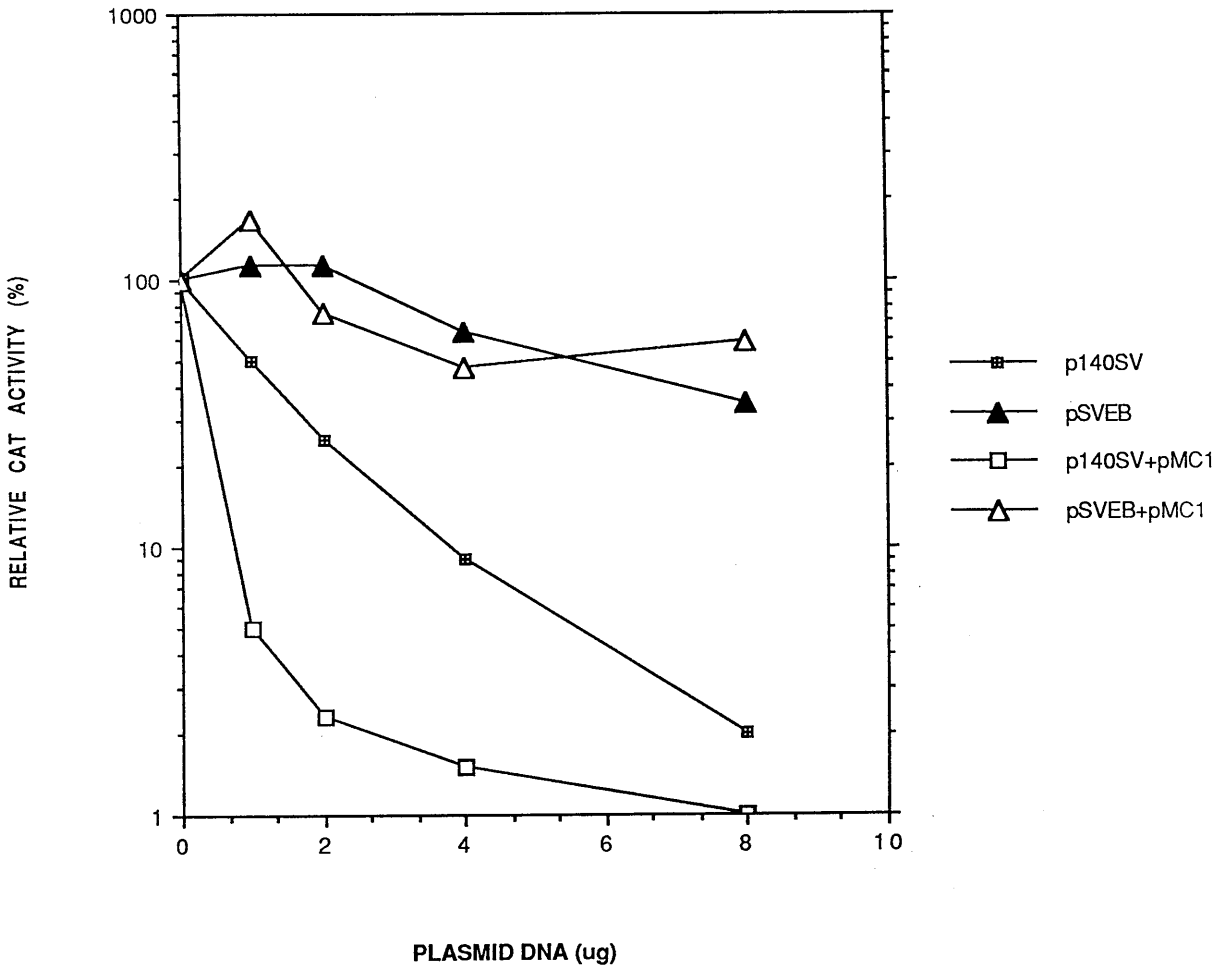
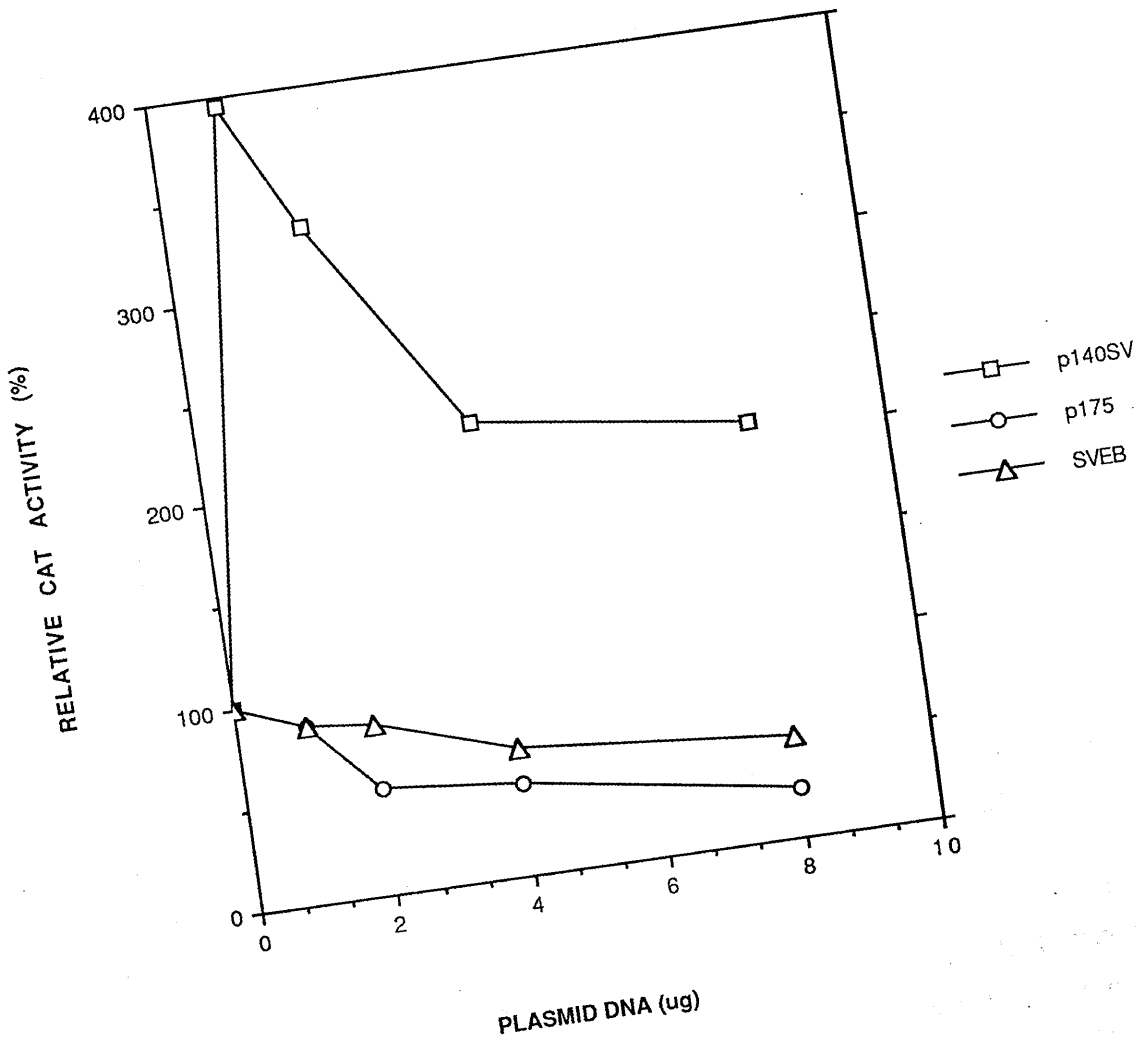

1000
100
10
1
0
2
4
6
8
10
RELATIVE CAT ACTIVITY (%)
PLASMID DNA (ug)
p140SV
pSVEB
p140SV+pMC1
pSVEB+pMC1

Figure 24. Graph showing repression of expression from VZV gene 62 promoter-leader sequences (in p140CAT) by VZV140k, Vmw175, and their mutant derivatives. BHK cells were transfected with 4µg of p140CAT and increasing amounts of test plasmids (1, 2, 4 and 8µg) or pSVEB (0.5, 1, 2 and 4µg; see the legend to Figure 23), in the presence of pMC1 (which expresses Vmw65). The test plasmids used express the following polypeptides: VZV 140k (p140SV); Vmw175 (p175); VIδ and I13, insertion mutant derivatives of 140k and Vmw175 respectively (pVIδ and pI13). The relative CAT activities (determined as described in the text) are shown plotted against the amount of test plasmid DNA added. The data shown is derived from a typical titration experiment. This experiment has been repeated three times, and in all cases the insertion mutants failed to effect significant repression of p140CAT.

Figure 25. Graph showing activation (rather than repression) of expression from HSV-1 IE gene 3 promoter-leader sequences (in pIE3CAT) by VZV 140k. Vero cells were transfected with 4µg of pIE3CAT and increasing amounts of test plasmids (1, 2, 4 and 8µg) or pSVEB (0.5, 1, 2 and 4µg; see the legend to Figure 23), in the presence of pMC1 (which expresses Vmw65). The test plasmids used express VZV 140k (p140SV) and Vmw175 (p175). The relative CAT activities (determined as described in the text) are shown plotted against the amount of test plasmid DNA added. The data shown is derived from a single titration experiment.


400
300
200
100
0
0
2
4
6
8
10
RELATIVE CAT ACTIVITY (%)
PLASMID DNA (ug)
p140SV
p175
SVEB

3.4. Does Repression of the Gene 62 Promoter by VZV 140k and Vmw175 Involve Recognition of a Specific DNA Sequence Motif ?

The mechanism of repression of the IE-3 promoter by Vmw175 requires a functional Vmw175 DNA binding domain (Paterson and Everett, 1988b; Shepard *et al.*, 1989) and the presence of a cognate DNA binding site at the cap-site (Roberts *et al.*, 1988). This binding site includes a consensus sequence (ATCGTC) common to many, but not all, sequences with which Vmw175 can associate (Muller, 1987; Faber and Wilcox, 1986b, 1988; Michael *et al.*, 1988). In an attempt to determine whether analogous sequences in the region of the gene 62 cap site were required for repression by VZV 140k and Vmw175, plasmids p140del1CAT and p140del2CAT were utilized in repression transfection assays. These plasmids are deletion variants of p140CAT in which promoter sequences upstream of the TATA box are intact, but leader or capsite-leader sequences are deleted. Thus p140del1CAT and p140del2CAT are truncated at positions +20 and -22 respectively, whereas VZV sequences in p140CAT extend to nucleotide +57.

It was found that the intrinsic activities of the deleted promoters in p140del1CAT and p140del2CAT were consistently reduced by at least 10-fold relative to p140CAT (results not shown), which implies that sequences in the gene 62 leader region contribute to the efficiency of its expression. The activity of both promoters was increased by 15- to 30-fold when pMC1 was present in transfections (as with the intact promoter), but promoter activity was still at least 10-fold below that of the pMC1 activated gene 62 promoter. As noted above, low basal levels of promoter activity result in relatively poor levels of repression, and it was not possible to draw unequivocal conclusions from the results. The reduced activity of p140del1CAT might be explained by the absence of the *bona fide* capsite, and that of p140del2CAT by the presence of the polylinker sequences located between the VZV insert and the CAT coding sequences. Therefore, a

better approach would probably be to create small insertions or deletions in p140CAT.

3E. VZV 140K DNA BINDING STUDIES

The HSV-1 homologue of VZV 140k, Vmw175, is a DNA binding protein which binds to sequences which include the consensus, ATCGTC (Beard *et al.*, 1986; Faber and Wilcox, 1986b; Kristie and Roizman, 1986b; Tedder *et al.*, 1989). Region 2 of Vmw175 contains sequences that are essential for binding to this consensus motif (DeLuca and Schaffer, 1988; Paterson and Everett, 1988b; Shepard *et al.*, 1989). Vmw175 also binds (probably with lower affinity) to other sites, although these are somewhat diverse and deviate significantly from the above consensus (Michael *et al.*, 1988; Michael and Roizman, 1990). Given the extensive similarities between VZV 140k and Vmw175, it was of interest to determine whether 140k binds to specific DNA sequences, particularly as the sequences comprising region 2 of Vmw175 are so highly conserved in VZV 140k.

Repression of the VZV gene 62 promoter by VZV 140k has been demonstrated in transfection assays (Section 3D.3.1). Significantly, genetic evidence obtained in these studies suggests that, like repression of the HSV-1 IE3 promoter by Vmw175, this involves binding by VZV 140k to specific sequences in the gene 62 promoter, presumably in the vicinity of the capsite (see Section 3D.3.3). Therefore, in an attempt to demonstrate sequence-specific binding by 140k, gel retardation studies were undertaken using extracts containing VZV 140k and a panel of probes representing the entire gene 62 promoter-leader region. In addition, the possibility that 140k might be capable of binding to ATCGTC consensus motifs was investigated.

3E.1. Probes Used in Gel Retardation Assays

The promoter sequences which control expression of VZV gene 62 have been defined by deletion analysis (McKee *et al.*, 1990). Promoter activity is contained within residues -410 to +57; constitutive (basal) promoter activity is associated with residues -131 to +57, while residues -410 to -131 (which contain an octamer/TAATGARAT element) confer responsiveness to Vmw65 (see Section

1B.4.2). Therefore, a series of probes representing sequences between -410 and +57 were prepared.

Plasmid p140UP6 contains VZV gene 62 sequences from *Cla*I (-1146) to nucleotide 142 of the orf on a *Bam*HI-*Eco*RI fragment (derived from pVI1; Section 3C.1.1) inserted between the *Bam*HI and *Eco*RI sites of pUC9. DNA fragments representing VZV sequences from *Sal*I (-410) to *Ava*I (+43) were derived from p140UP6 by restriction enzyme digestion, and end-labelled for use in gel retardation assays (Section 2B.18.2). A fragment representing gene 62 sequences from *Taq*I (-51) to +57 (*Bgl*II) was derived from p140BT (Section 3B.1.3) and similarly end-labelled. The locations of these fragments are summarized in Figure 26 and Table 7; a loose copy of these can be found at the back of the thesis.

For clarity, the VZV gene 62 probes are divided into three groups as follows:

Capsite probes

Three partially overlapping probes containing the gene 62 capsite were utilized. The largest probe, CAP1, contains sequences from -131 to +43, and thus the majority of the basal promoter sequences. CAP2 contains sequences from -51 to +61, including the proposed TATA box at -28. The smallest probe, CAP3, contains sequences between -23 and +43, and thus does not contain any recognisable promoter elements.

Proximal promoter region probe

The UP1 probe (-131 to -23) contains all recognisable promoter elements from within the basal promoter region, but does not contain any sequences from the capsite-leader region.

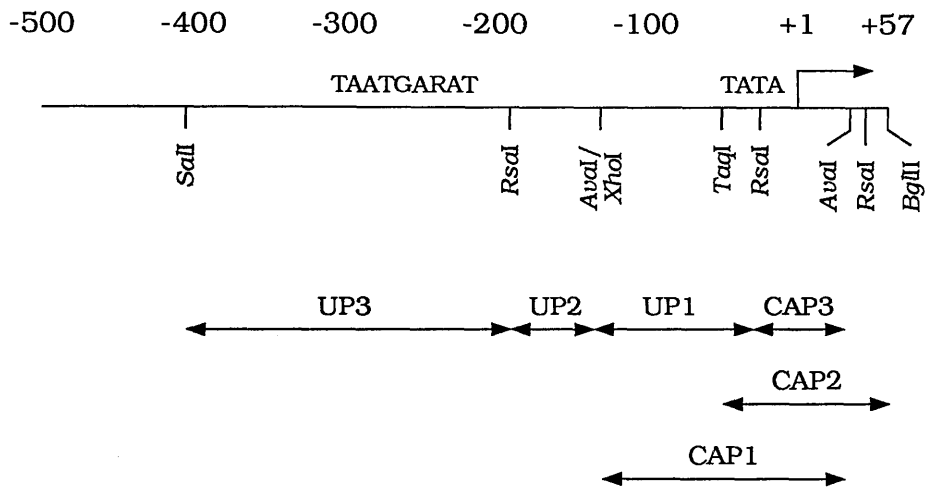
Far-upstream promoter region probes

The UP2 (-188 to -131) and UP3 (-410 to -188) probes represent sequences from the upstream regulatory region; UP3 contains the octamer/TAATGARAT motif.

The HSV-1 IE Gene 3 Capsite Probe (IE3 probe) contains a consensus ATCGTC motif, and was prepared from the 45bp *Bam*HI-*Ava*I fragment which spans the IE gene 3

Figure 26. Locations of the VZV gene 62 fragments from which were derived the probes used in gel retardation assays. VZV gene 62 sequences from -500 to +57 are represented by the solid line. The transcription start site, +1, is indicated by the arrow. McKee *et al.* (1990) demonstrated that sequences between *Xho*I (-131) and +57 constituted a proximal promoter region responsible for basal promoter activity, whereas far-upstream sequences between *Sal*I (-410) and *Xho*I (-131) conferred responsiveness to Vmw65 of HSV-1. The approximate location of an octamer/TAATGARAT motif (centered on nucleotide -255) implicated in the response to Vmw65 is shown. The cleavage sites of a number of restriction enzymes used to excise fragments (from p140UP6 and p140BT; see Section 3E.1) for the preparation of probes are marked. (The *Bgl*II site in p140BT was created by site-directed mutagenesis of p140 as described in Section 3B.1.3.) The fragments from which each of the probes were prepared are indicated by the arrows. This information is summarized in the Table below.

Table 7. Summary of the fragments from which VZV gene 62 promoter-regulatory region probes were derived. The various probes were prepared from VZV gene 62 fragments as shown. The fragments were end-labelled by "filling in" the 5' overhanging ends of the restriction sites shown in bold (with Klenow or T4 polymerase and [α-^{32}P]dNTPs).



Probe	Gene 62 Fragment	
	From	To
CAP1	**XhoI (-131)**	AvaI (+43)
CAP2	TaqI (-51)	**BglII (+57)**
CAP3	RsaI (-23)	**AvaI (+43)**
UP1	**AvaI (-131)**	RsaI (-23)
UP2	RsaI (-188)	**AvaI (-131)**
UP3	**SalI (-410)**	RsaI (-188)

capsite (Muller, 1987).

3E.2. GEL RETARDATION ASSAYS WITH VZV INFECTED CELL EXTRACTS

2.1. Extract Preparation

Initial experiments utilized extracts prepared from either F.HeLa or HFL cells. F.HeLa cells have been used in previous studies on Vmw175 DNA binding, whilst HFL cells were selected as they are permissive for VZV infections. Later experiments used CV1 cells, which grow better in tissue culture and are also permissive for VZV growth. Where an experiment was performed in HFL and CV1 cells, the results were similar.

HFL or CV1 cells were seeded into 90mm dishes and infected (at around 80% confluence) by addition of VZV infected cells (HFL or CV1 accordingly) representing 1/16 of a 175cm^2 flask. Where appropriate, infected HFL cells were used to infect F.HeLa cells in an analogous fashion. Nuclear extracts (NE) or whole cell extracts (WCE) were generally prepared from cells 48h following infection, as described (Section 2B.9). The protein concentration of extracts was determined by the method of Bradford (1976; Section 2B.8), such that equal amounts of protein from different extracts were used in each assay.

2.2. Gel Retardation Assays with Extracts Prepared from F.HeLa and HFL cells

The following section describes the results obtained from gel retardation experiments which utilized mock and VZV infected extracts prepared from HFL and F.HeLa cells. To avoid unnecessary repetition, these results are summarized and discussed together at the end of the Section.

Binding to the Capsite Region

Figure 27 shows the complexes formed when increasing amounts of mock or VZV infected F.HeLa WCE were incubated with the CAP1 probe (-131 to +43). Incubation

with 0.8µg of mock extract (lane 2) generated three complexes, F1, F2, and F3. Incubation with progressively more extract resulted in the appearance of a slower migrating complex, X (lane 6), with a concomitant decrease in the intensity of F1-F3, which probably reflects binding of multiple factors to each probe molecule. An identical profile was obtained when increasing amounts of VZV WCE were incubated with CAP1 (lanes 7-11); no complex specific to VZV infection of F.HeLa cells was identified.

The above experiment was repeated using extracts prepared from HFL cells (Figure 28). The mock infected HFL extract formed three complexes with the CAP1 probe designated H1, H2, and H3 (lane 2; H2 and H3 are only clearly visible in the original autoradiograph). Extract prepared from VZV infected cells at 24h p.i. formed these same complexes (lane 4). In addition, some minor complexes are visible above HI (lane 4); identical complexes are also formed in the mock (lane 2; visible in the original autoradiograph). However, incubation of CAP1 with extract prepared at 48h p.i generated a novel complex designated V48 (lane 6).

Binding to Proximal Promoter Sequences

Binding to the UP1 fragment was assayed using mock and VZV 48h p.i. HFL extracts (Figure 29). The mock extract formed complexes corresponding to H1, H2 and H3 (H3 is visible in the original autoradiograph). Addition of 8 and 16µg of mock extract also formed complexes of lower mobility than H1 (lanes 5,6). Incubation of UP1 with 48h p.i. extract formed an additional weak complex which probably corresponds to the V48 complex formed with the CAP1 probe (the CAP1 and UP1 probes are in fact partially overlapping; see Figure 26).

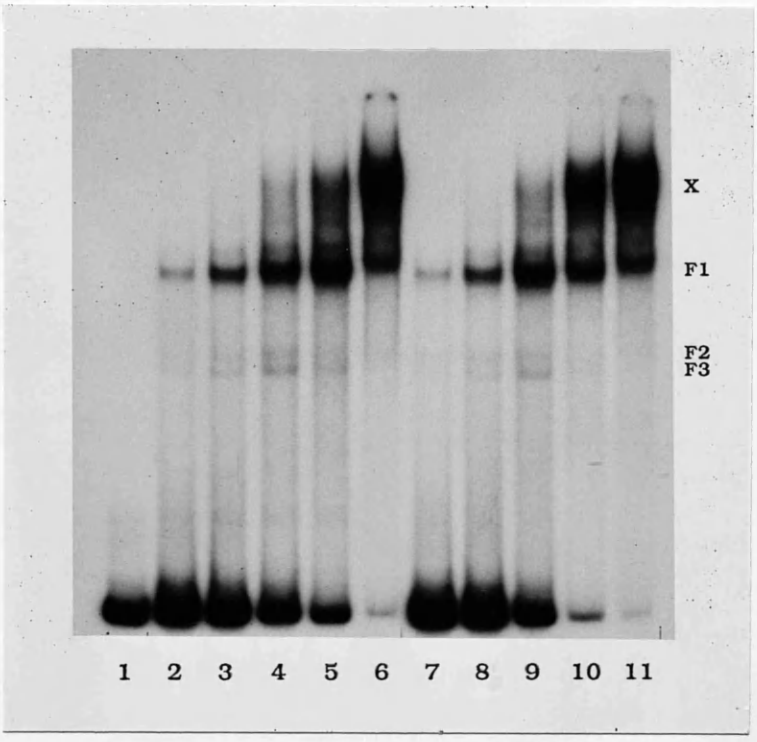
Binding to Far-Upstream Sequences

Complexes formed with the UP3 probe were analysed (Figure 30). Mock extract formed H1 and H2 and additional complexes of lower mobility (lanes 2-6). Incubation of probe with 48h p.i. extract formed reduced amounts of H1 and H2 (relative to the mock), and several very weak novel

Figure 27. DNA binding assay with whole cell extracts (WCE) prepared from mock and VZV infected F.HeLa cells. The CAP1 probe was incubated with increasing amounts of mock WCE (lanes 2-6) or VZV infected WCE (lanes 7-11). The total amount of protein in each incubation was as follows:

Lane	Mock Extract (µg)	VZV Extract (µg)
1	.	.
2	0.8	.
3	1.6	.
4	4.0	.
5	8.0	.
6	16.0	.
7	.	0.8
8	.	1.6
9	.	4.0
10	.	8.0
11	.	16.0

Lane 1 represents probe incubated in the absence of extract. The positions of complexes X, F1, F2 and F3 are marked.


X
F1
F2
F3
1 2 3 4 5 6 7 8 9 10 11

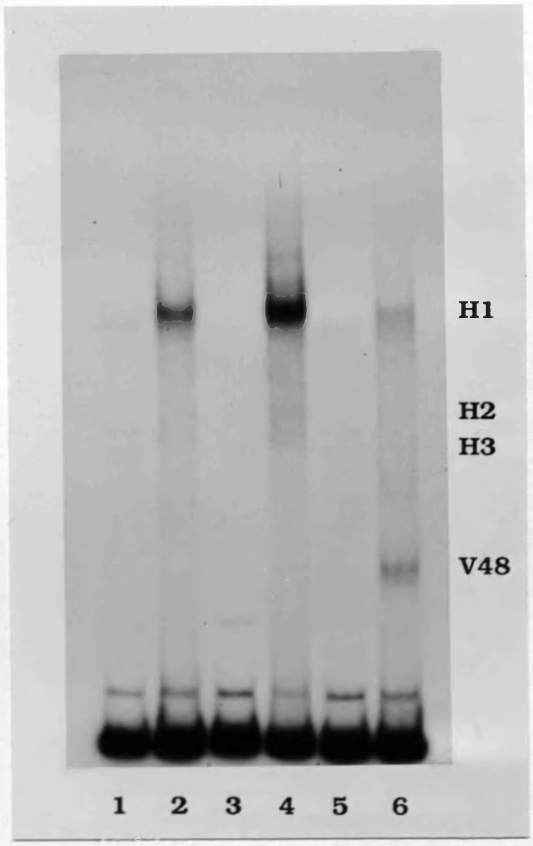


Figure 28. DNA binding assay with whole cell extracts (WCE) preprared from mock and VZV infected HFL cells. The CAP1 probe was incubated with mock WCE (lanes 1,2) and with VZV infected WCE prepared at 24h post-infection (lanes 3,4) or 48h post-infection (lanes 5,6). Incubations contained either 3µg (lanes 1,3,5) or 16µg (lanes 2,4,6) of total cell protein. The positions of complexes H1, H2, H3 and V48 are marked.

Figure 29. DNA binding assay with whole cell extracts (WCE) prepared from mock and VZV infected HFL cells. The UP1 probe was incubated with increasing amounts of mock WCE (lanes 2-6) or VZV infected WCE (lanes 7-11). The total amount of protein in each incubation was as follows:

Lane	Mock Extract (µg)	VZV Extract (µg)
1	.	.
2	0.8	.
3	1.6	.
4	4.0	.
5	8.0	.
6	16.0	.
7	.	0.8
8	.	1.6
9	.	4.0
10	.	8.0
11	.	16.0

Lane 1 represents probe incubated in the absence of extract. The positions of complexes H1, H2, H3 and V48 are marked.

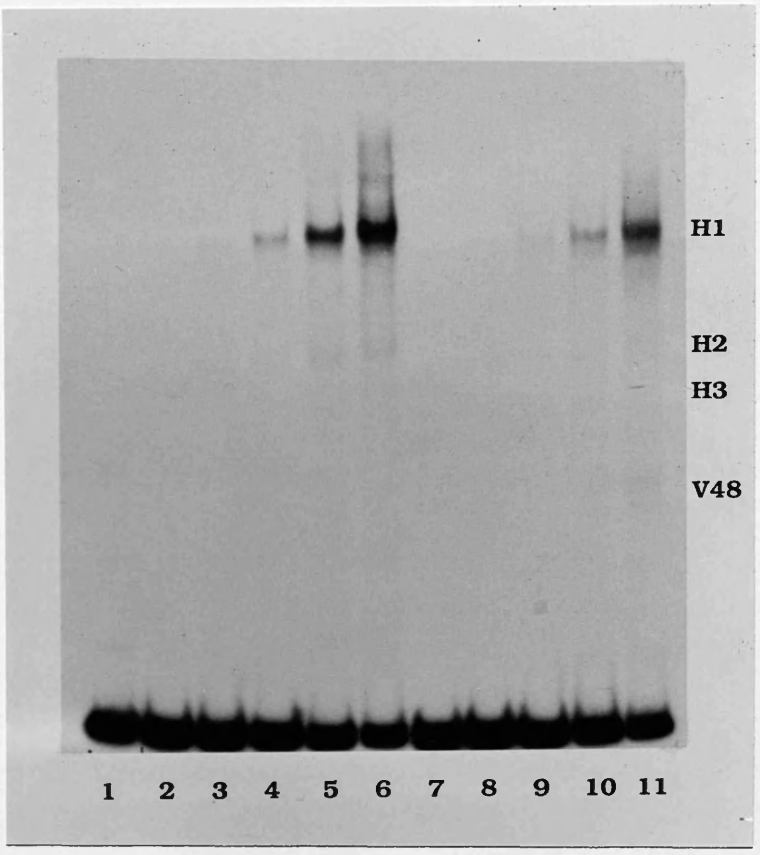
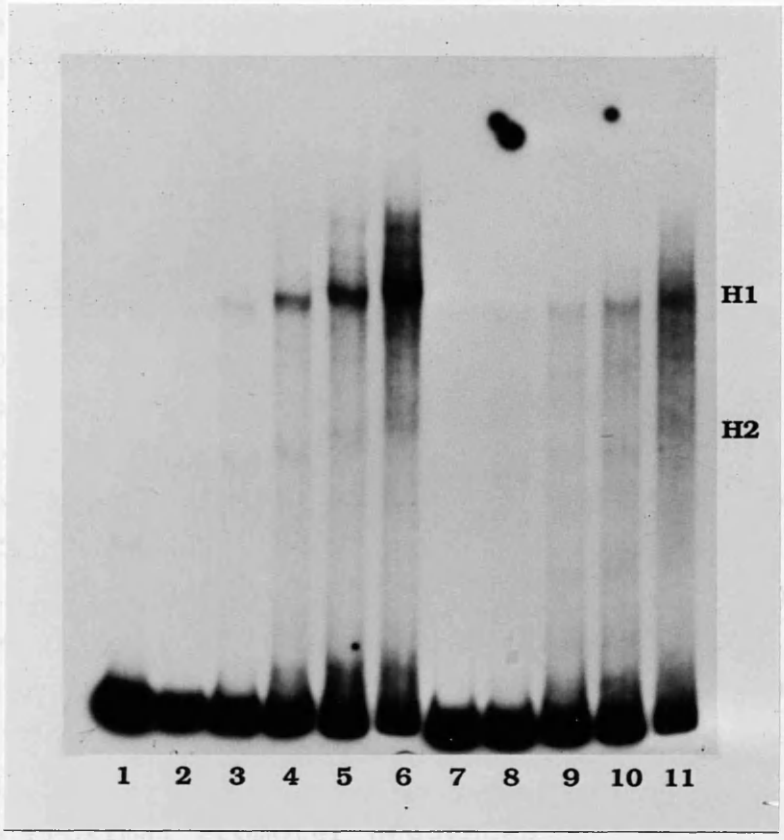

H1
H2
H3
V48
1 2 3 4 5 6 7 8 9 10 11

Figure 30. DNA binding assay with whole cell extracts (WCE) prepared from mock and VZV infected HFL cells. The UP3 probe was incubated with increasing amounts of mock WCE (lanes 2-6) or VZV infected WCE (lanes 7-11). The total amount of protein in each incubation was as follows:

Lane	Mock Extract (µg)	VZV Extract (µg)
1	.	.
2	0.8	.
3	1.6	.
4	4.0	.
5	8.0	.
6	16.0	.
7	.	0.8
8	.	1.6
9	.	4.0
10	.	8.0
11	.	16.0

Lane 1 represents probe incubated in the absence of extract. The positions of complexes H1 and H2 are marked.


H1
H2
1 2 3 4 5 6 7 8 9 10 11

complexes. Experiments in which binding to the UP2 probe was assayed yielded similar results (not shown).

Summary of Protein Binding Studies with HFL Extracts

Incubation of probes representing VZV gene 62 promoter-leader sequences with extract prepared from VZV infected HFL cells generated several novel complexes. Of these, the V48 complex was the most prominent and reproducibly observed. The mobility of the V48 complex is indicative of binding by a relatively low molecular weight polypeptide, and it is therefore unlikely that V48 contains VZV 140k.

2.3. Gel Retardation Assays With Extracts Prepared From CV1 Cells

Binding to the CAP3 (-23 to +43) and UP1 (-131 to -23) probes was assayed using mock and VZV infected extracts prepared from CV1 cells (see Figure 31).

Binding to the Capsite Region

Mock CV1 extract formed three complexes, C2-C4, with the CAP3 probe (lanes 4,5). In addition to these complexes, the CV1 48h p.i. extract formed a complex of comparable mobility to the V48 complex formed with HFL extracts (compare lane 3 with lanes 6,7), as well as a number of other minor novel complexes. Note that in this experiment the V48 complex appears to be a doublet.

Binding to Proximal Promoter Sequences

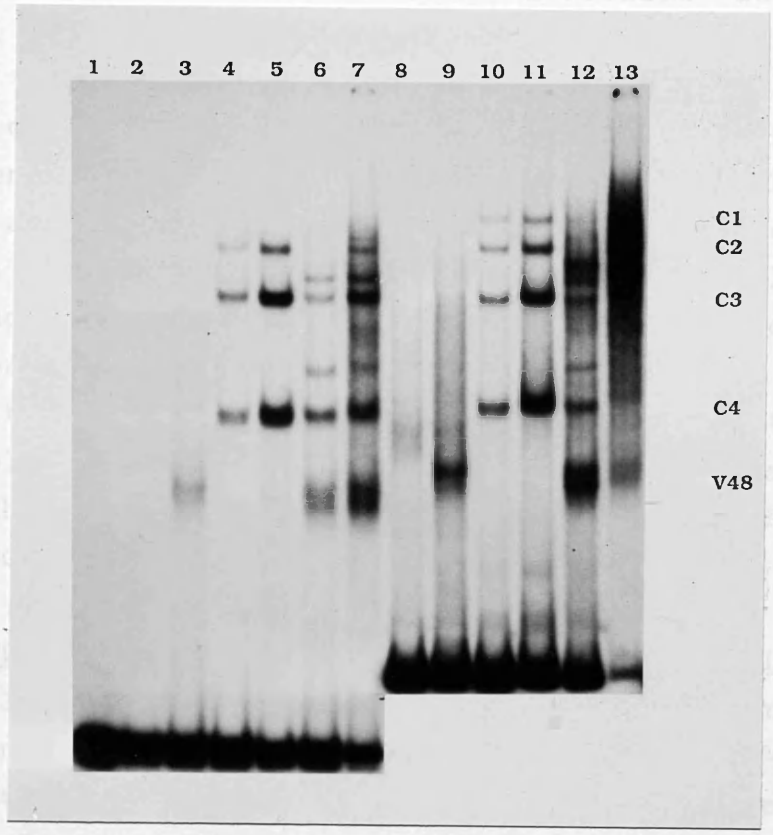
Incubation of mock CV1 extract with the UP1 probe formed complexes C2-C4, and, in addition, a slower migrating complex, C1 (lanes 10,11). A number of novel complexes were formed when CV1 48h p.i. extract was incubated with UP1 (lane 12). Most of these novel complexes were comparable to those formed with CAP3 (compare lanes 6 and 7 with lane 12), and included a complex corresponding to V48 (compare lane 9 with lane 12).

That the novel complexes are formed with two different probes indicates that they represent either non-specific binding or binding to a common site in both probes. As stated in Section 3E.2.2, the mobility of the

Figure 31. DNA binding assay with nuclear extracts (NE) prepared from mock and VZV infected HFL and CV1 cells. The CAP3 probe was incubated without extract (lane 1), or with mock HFL NE (lane 2), VZV infected HFL NE (lane 3), mock CV1 NE (lanes 4,5), or VZV infected CV1 NE (lanes 6,7). This was repeated using the UP1 probe. Thus UP1 was incubated with mock HFL NE (lane 8), VZV infected HFL NE (lane 9), mock CV1 NE (lanes 10,11), or VZV infected CV1 NE (lanes 12,13). The total amount of protein in each incubation is summarized below:

Lane	Extract	Amount (µg)	Probe
1			CAP3
2	Mock HFL	3.0	"
3	VZV HFL	3.0	"
4	Mock CV1	1.0	"
5	mock CV1	4.0	"
6	VZV CV1	1.0	"
7	VZV CV1	4.0	"
8	Mock HFL	3.0	UP1
9	VZV HFL	3.0	"
10	Mock CV1	1.0	"
11	Mock CV1	4.0	"
12	VZV CV1	1.0	"
13	VZV CV1	4.0	"

The positions of complexes C1, C2, C3, C4 and V48 are marked.


1 2 3 4 5 6 7 8 9 10 11 12 13
C1
C2
C3
C4
V48

V48 complex is not consistent with it containing VZV 140k. However, the possibility that V48 or another novel complex contains 140k, or a proteolytic fragment of 140k, cannot be excluded. In order to investigate this possibility further, monoclonal antibodies or antisera reactive against a range of VZV 140k epitopes would be required. Unfortunately, these were not available during these studies. In the above experiment (Figure 31), it is notable that incubation of either probe with equivalent amounts of CV1 mock and 48h p.i. extracts did not form equivalent amounts of the host derived complexes, C1-C4; reduced amounts (particularly of C2) were formed with the 48h p.i. extract (compare lanes 4 and 10 with lanes 6 and 12). Since reduced formation of C1-C4 was accompanied by the appearance of the various novel complexes (including V48), it is tempting to speculate that the latter contain a proteolytic fragment of one or more of the host factors. If so, this suggests that VZV infection of HFL and CV1 cells induces a protease activity.

3E.3. GEL RETARDATION ASSAYS WITH TRANSFECTED CELL EXTRACTS

Interpretation of the results of gel retardation experiments conducted using VZV infected cell extracts is complicated by the presence of other viral proteins in the extract. In particular, it is difficult to prove whether or not a complex contains VZV 140k. This problem can be avoided by using extracts prepared from cells transfected with a plasmid expressing only VZV 140k. Therefore, F.HeLa cells were transfected with plasmid p140 and extracts prepared for use in gel retardation experiments. (N.B. An authentic complex containing Vmw175 is formed when the HSV-1 IE3 probe is incubated with extracts prepared from cells transfected with plasmid p175, which expresses Vmw175; Paterson and Everett, 1988b).

Binding to VZV Gene 62 Promoter-Leader Sequences

The CAP1 (Figure 32), UP1 (Figure 33), UP2 (Figure 34) and UP3 probes (Figure 35) were incubated with increasing amounts of mock (prepared from cells which were

Figure 32. DNA binding assay with nuclear extracts prepared from transfected F.HeLa cells. The CAP1 probe was incubated with increasing amounts of mock nuclear extract (lanes 2-6) or p140-transfected nuclear extract (lanes 7-11). The total amount of protein in each incubation was as follows:

Lane	Mock Extract (µg)	p140 Extract (µg)
1	.	.
2	0.3	.
3	0.6	.
4	1.4	.
5	2.8	.
6	5.6	.
7	.	0.3
8	.	0.6
9	.	1.4
10	.	2.8
11	.	5.6

Lane 1 contains probe incubated in the absence of extract.

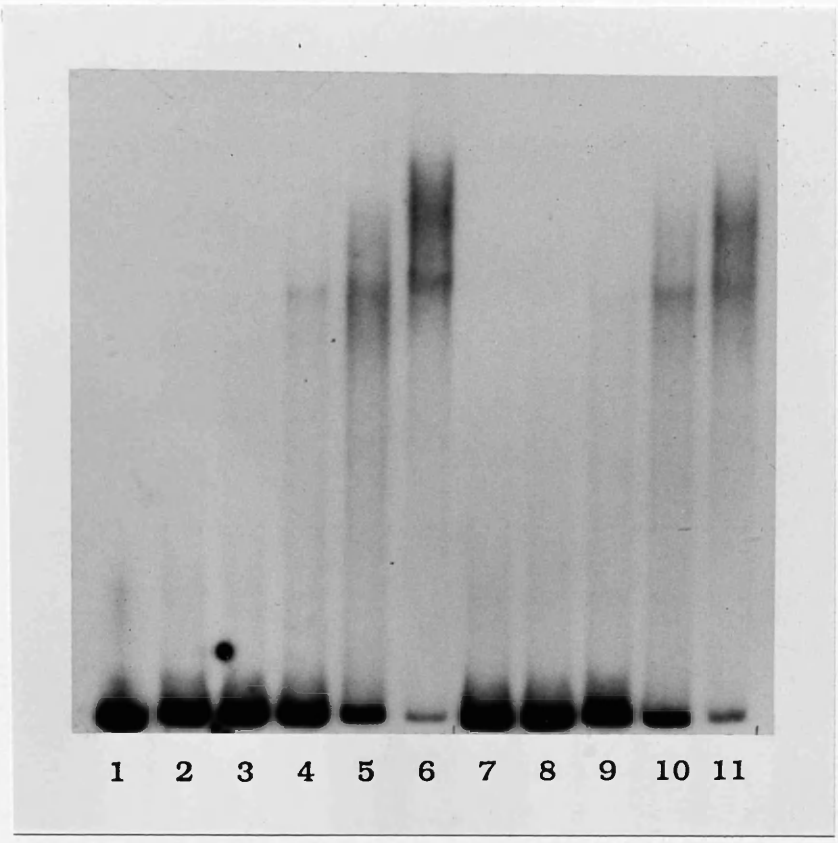

1 2 3 4 5 6 7 8 9 10 11

Figure 33. DNA binding assay with nuclear extracts prepared from transfected F.HeLa cells. The UP1 probe was incubated with increasing amounts of mock nuclear extract (lanes 2-6) or p140-transfected nuclear extract (lanes 7-11). The total amount of protein in each incubation was as follows:

Lane	Mock Extract (µg)	p140 Extract (µg)
1	.	.
2	0.3	.
3	0.6	.
4	1.4	.
5	2.8	.
6	5.6	.
7	.	0.3
8	.	0.6
9	.	1.4
10	.	2.8
11	.	5.6

Lane 1 contains probe incubated in the absence of extract.

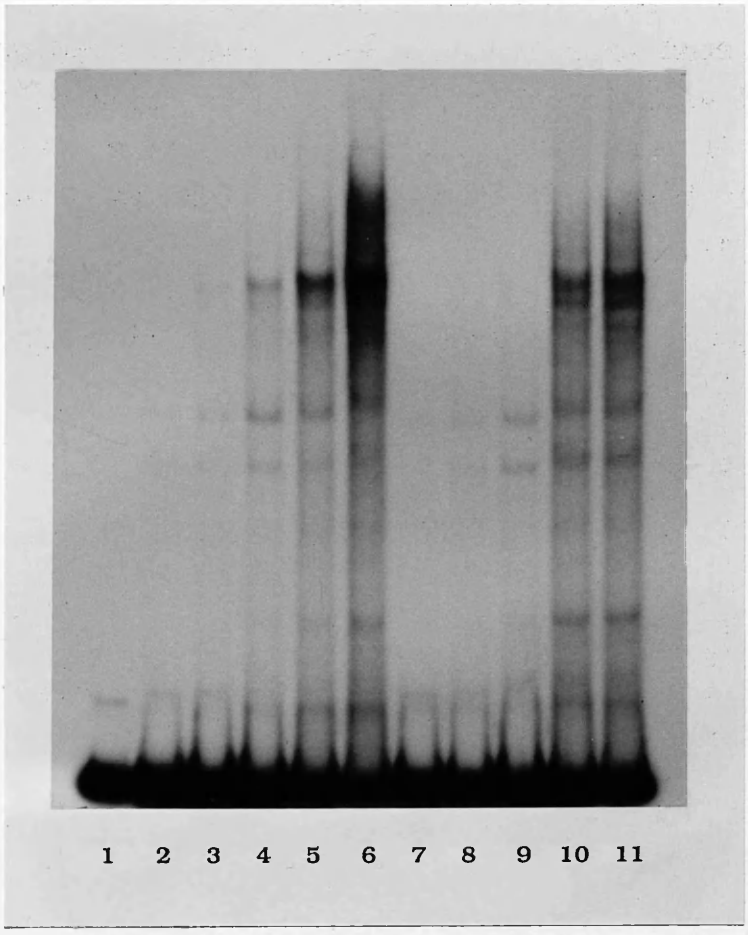

1 2 3 4 5 6 7 8 9 10 11

Figure 34. DNA binding assay with nuclear extracts prepared from transfected F.HeLa cells. The UP2 probe was incubated with increasing amounts of mock nuclear extract (lanes 2-6) or p140-transfected nuclear extract (lanes 7-11). The total amount of protein in each incubation was as follows:

Lane	Mock Extract (µg)	p140 Extract (µg)
1	.	.
2	0.3	.
3	0.6	.
4	1.4	.
5	2.8	.
6	5.6	.
7	.	0.3
8	.	0.6
9	.	1.4
10	.	2.8
11	.	5.6

Lane 1 contains probe incubated in the absence of extract.

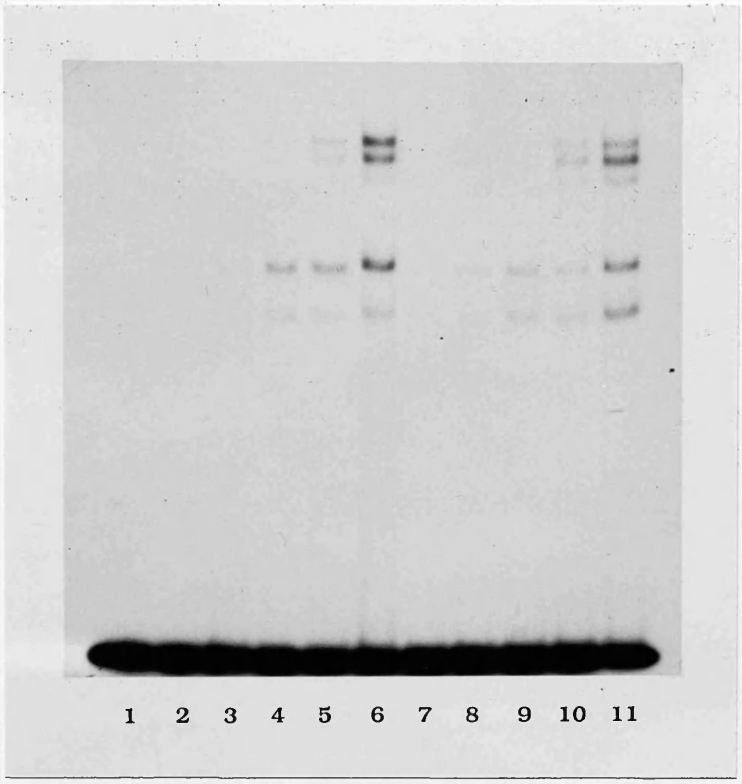
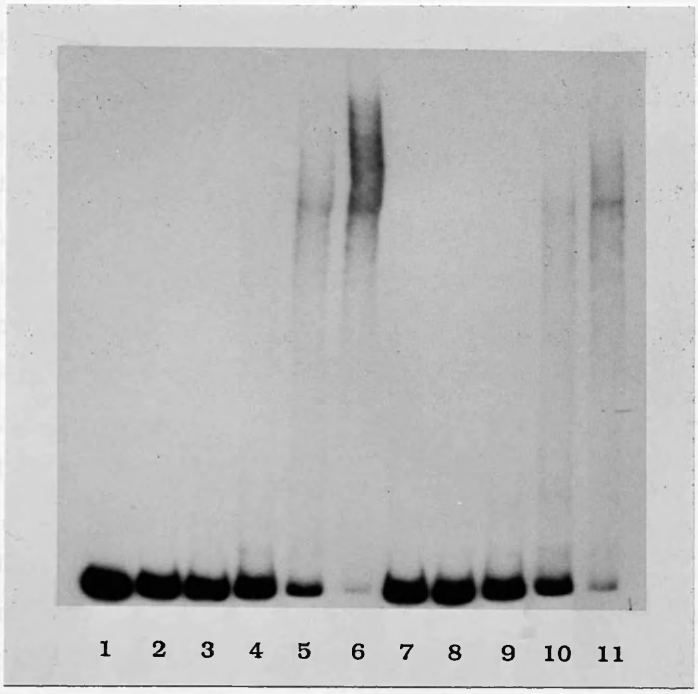

1 2 3 4 5 6 7 8 9 10 11

Figure 35. DNA binding assay with nuclear extracts prepared from transfected F.HeLa cells. The UP3 probe was incubated with increasing amounts of mock nuclear extract (lanes 2-6) or p140-transfected nuclear extract (lanes 7-11). The total amount of protein in each incubation was as follows:

Lane	Mock Extract (µg)	p140 Extract (µg)
1	.	.
2	0.3	.
3	0.6	.
4	1.4	.
5	2.8	.
6	5.6	.
7	.	0.3
8	.	0.6
9	.	1.4
10	.	2.8
11	.	5.6

Lane 1 contains probe incubated in the absence of extract.


1 2 3 4 5 6 7 8 9 10 11

not transfected) or p140 transfected F.HeLa extract. In all experiments, the complexes formed with mock and p140-transfected F.HeLa extracts were essentially identical, though the relative intensities of certain of the complexes formed did vary slightly between the two extracts (compare, for example, lanes 6 and 11, Figure 34). Since similar variations were observed with several probes they were not considered to be significant.

3E.4. GEL RETARDATION ASSAYS WITH EXTRACTS PREPARED FROM CELLS INFECTED WITH HSV-1 OR HSV-140

The experiments described above using VZV-infected and p140 transfected cell extracts did not demonstrate DNA binding by 140k. Amounts of 140k in these extracts (relative to host factors) were almost certainly very low and, consequently, these results must be considered indeterminate since binding would be difficult to detect against such a background unless it were of high affinity. In order to investigate DNA binding further, extracts containing higher amounts of VZV 140k were required. A recombinant virus, HSV-140, in which the VZV 140k coding sequences replace those of Vmw175 in an HSV-1 virus genome, has been constructed (Section 3B). During HSV-140 infection of tissue culture cells, 140k is expressed as an early protein, with high levels of expression persisting late into infection. Notably, nuclear extracts prepared from HSV-140 infected cells contain copious amounts of 140k (Section 3F.2).

Hence, BHK cells were infected with HSV-1 (10pfu/cell) or HSV-140 (100-200 particles/cell), and nuclear extracts prepared at 5h p.a. and 12-14h p.a. respectively. Extracts were also prepared from uninfected cells.

Binding to HSV-1 IE gene 3 Capsite Sequences

The ability of Vmw175 to bind DNA sequences containing the consensus, ATCGTC, is critically dependant upon the integrity of region 2 polypeptide sequences. As noted in Section 1C.3, there is extensive homology between

the sequences comprising region 2 of VZV 140k and Vmw175. Therefore, in order to determine whether 140k, like Vmw175, is capable of binding to an ATCGTC consensus motif, HSV-140 extracts were incubated with the IE3 probe. When the IE3 probe was incubated with mock extract prepared from either F.HeLa or BHK cells, two major complexes, M1 and M2, and several minor complexes were formed (Figure 36, lanes 1 and 10). As expected, incubation of the IE3 probe with extract prepared from HSV-1 infected F.HeLa cells (kindly provided by Dr. T. Paterson) formed the typically strong complex which contains Vmw175 (lane 11). An identical complex was formed with the extract prepared from HSV-1 infected BHK cells (lane 2). In contrast, the HSV-140 extract did not form any novel complexes with the IE3 probe (lane 3), inferring that VZV 140k does not bind to the sequence ATCGTC.

Binding to Gene 62 Capsite Sequences

Incubation of the CAP3 probe with mock BHK extract formed complexes M1 and M2, and a number of other minor complexes (Figure 36, lane 7). The HSV-1 extract formed these same complexes, and an additional minor complex of lower mobility than M1 (lane 8). However, this complex was not formed when different competitor DNA was used (see Figure 37, lanes 5 and 11), and thus probably does not reflect sequence specific binding by Vmw175. The HSV-140 extract did not form any novel complexes with CAP3 (Figure 36, lane 9).

A number of experiments were performed in which the incubation conditions were varied: NP40 was removed from the incubation buffer; a number of different competitor DNAs were used; incubation was carried out on ice (rather than 25°C). Additionally, gels were run at 4°C. Figure 37 shows the results of such an experiment. Parallel incubations were carried out in TEN-P40 and TE (minus NP40) buffers, using as competitor DNA polydI:polydC or salmon testes DNA (ssDNA). The complexes formed were largely unaffected by these modifications, although the intensity of some of the minor complexes was reduced, indicating that

Figure 36. DNA binding assay with nuclear extracts (NE) prepared from mock, HSV-1, and HSV-140 infected BHK cells. Each set of three lanes represents incubation of the specified probe with extract in the following order: mock NE, HSV-1 infected NE, HSV-140 infected NE. The extracts assayed in lanes 10 and 11 (kindl y provided by Dr. Trevor Paterson), which serve as controls in this experiment, were prepared from mock and HSV-1 infected F.HeLa cells. The extracts were incubated with the UP1 (lanes 4,5,6) and CAP3 (lanes 7,8,9) probes derived from VZV gene 62 sequences, and a probe which spans the HSV-1 IE-3 capsite (lanes 1,2,3,10,11). This is summarized below:

Lane	Probe	Extract
1	IE-3	Mock
2	"	HSV-1
3	"	HSV-140
4	UP1	Mock
5	"	HSV-1
6	"	HSV-140
7	CAP3	Mock
8	"	HSV-1
9	"	HSV-140
10	IE-3	Mock (TP)
11	"	HSV-1 (TP)

The positions of comlexes M1 and M2 are marked.

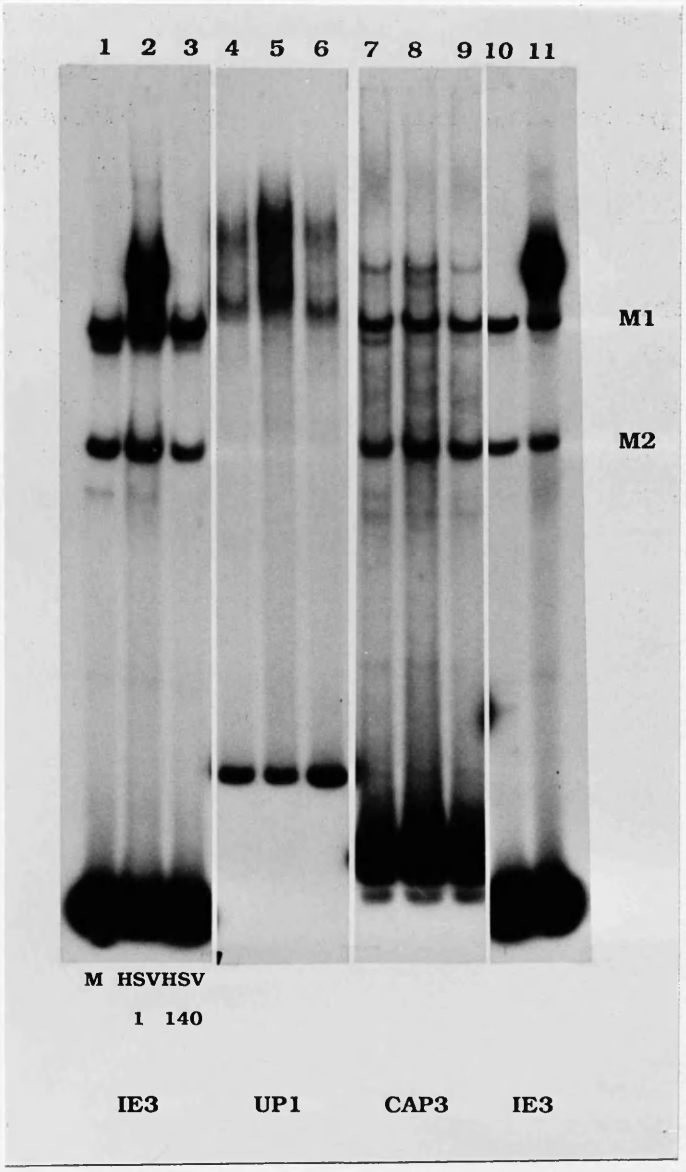
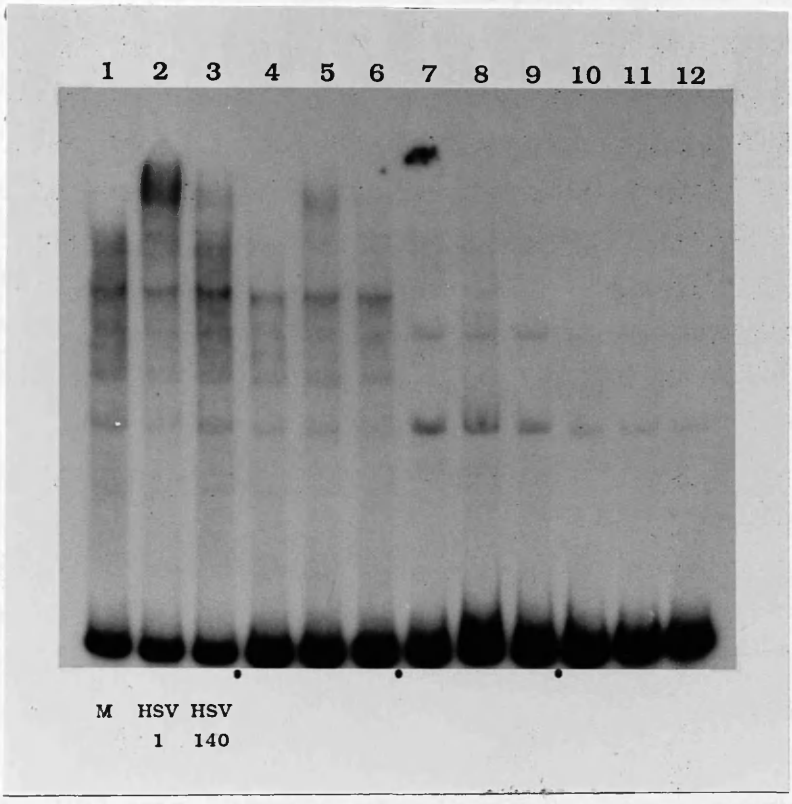

1 2 3 4 5 6 7 8 9 10 11
M1
M2
M HSVHSV
1 140
IE3
UP1
CAP3
IE3

Figure 38. The effect of different competitor DNAs on complex formation with the UP3 probe. Each set of three lanes represents incubation of the UP3 probe (under the specified conditions) with extracts in the following order: Mock (M), HSV-1 infected extract, HSV-140 infected extract. The experiment used as competitor DNA 1ug or 4ug of polydI:dC (lanes 1,2,3 and 4,5,6 respectively) and 1ug or 4ug of salmon testes DNA (ssDNA; lanes 7,8,9 and 10,11,12 respectively). This is summarized below:

Lane	Extract	Competitor (ug)
1	M	dI:dC (1)
2	HSV-1	"
3	HSV-140	"
4	M	dI:dC (4)
5	HSV-1	"
6	HSV-140	"
7	M	ssDNA (1)
8	HSV-1	"
9	HSV-140	"
10	M	ssDNA (4)
11	HSV-1	"
12	HSV-140	"


1 2 3 4 5 6 7 8 9 10 11 12
M
HSV 1
HSV 140

they reflect non-specific binding of host derived proteins. A minor complex migrating slightly ahead of the host complex M1 was formed with HSV-140 extract; nevertheless, an identical complex was also formed with mock extract. (The intensity of this minor complex varied between extracts and was very weak in the mock in this experiment. However, a stronger comparable complex can be seen in the mock in Figure 36, lane 1). Identical results were obtained from a similar experiment in which adenovirus major late promoter sequences were used as competitor (not shown).

Binding to Proximal Promoter Sequences

Mock, HSV-1 infected and HSV-140 infected extracts were incubated with the UP1 probe (Figure 36, lanes 4,5,6). The infected cell extracts did not form novel complexes. (The increased complex formation with the HSV-1 extract probably reflects the addition of slightly more protein.)

Binding to Far-Upstream Sequences

Binding to the UP3 probe was assayed using polydI:polydC or ssDNA as competitor (Figure 38). Complex formation was altered depending on the competitor used, with polydI:polydC competing non-specific binding less efficiently than ssDNA. Thus many of the complexes formed in lanes 1-6 are absent from lanes 7-12. Furthermore, binding to UP3 was largely abolished in the presence of 4µg ssDNA, and no specific complexes were observed. Binding to the UP2 probe was not assayed with these extracts, but was assayed using partially purified 140k (see Section 3E.6).

4.1. Proteinase K Treatment of HSV-140 Infected-Cell Nuclear Extract

The results described in preceding sections indicate that if the VZV 140k polypeptide binds to DNA, then it does so with low affinity. In this situation, it is likely that binding of host derived proteins to the probe would preclude detection of complexes containing 140k. With this in mind, a further experiment was performed following a recent report by Everett *et al.* (1990). They showed that proteinase K (PK) treatment of extract containing Vmw175

(prior to, or following incubation with probe) liberated a protease-resistant domain which not only formed a specific complex with the IE3 probe, but apparently did so with greater affinity than the intact protein. Furthermore, host derived binding proteins appeared to be inactivated by prior treatment of the extract with PK since, in this case, mock complexes were not formed. Thus an analogous experiment was performed in which HSV-140 infected extract were pre-treated with PK.

Mock or HSV-140 extract was added to incubation buffer and treated with PK (by addition of 1-6ng) for 10min at room temperature. Following this PMSF was added to a final concentration of 0.5 mM, then probe (CAP 2) added, and the sample incubated on ice for 30min. PK treatment of incubation buffer containing HSV-1 extract (by addition of 2ng PK) was performed in parallel, prior to addition of CAP 2 or IE3 probe. These results are shown in Figure 39. Pre-treatment of mock extract with PK prevented formation of complexes M1 and M2 (although several rapidly migrating minor complexes were formed; lanes 1,2,3). In agreement with the results of Everett *et al.*, PK treated HSV-1 extract formed a strong complex with the IE3 probe (lane 8). In contrast, the PK treated HSV-140 extract did not form any novel complexes with the CAP2 probe (some of the minor complexes in lanes 5 and 6 are not visible in lanes 2 and 3 in the photograph, but are visible in the original autoradiograph). Since it is not known whether or not VZV 140k contains a protease resistant domain comprising similar polypeptide sequences to those contained in Vmw175, no firm conclusions can be drawn from this experiment.

3E.5. VZV 140K BINDS NON-SPECIFICALLY TO DNA

Hay and Hay (1980) employed DNA-cellulose chromatography to demonstrate that most of the HSV-1 IE proteins bind DNA to some extent *in vitro*. In an analogous experiment, the ability of 140k to bind to a calf-thymus DNA column was investigated. BHK cells were

infected with HSV-140 at a multiplicity of 100ppc, labelled with [^{35}S]-methionine from 10-12h p.a., and nuclear extracts prepared. Nuclear extract was also prepared from BHK cells infected with HSV-1 (10pfu/cell) under immediate-early conditions (the infection and labelling conditions were exactly as described in Section 3B.2.4 for preparation of IE extracts for SDS-PAGE). A suitable column was packed with calf-thymus DNA-cellulose (to a bed volume-0.3ml) which had been pre-soaked in DC buffer for 3h (on ice), and washed extensively with DC buffer. 5-10µl of nuclear extract (equivalent to 20-40µg protein) was diluted to a final volume of 200µl in DC buffer and loaded onto the column. The flow through was collected and the column washed twice with DC, again collecting the eluates. Elution of bound protein was achieved by washing the column with DC containing increasing concentrations of salt (0.1, 0.2, 0.3, 0.6, and 2M), collecting eluates at all stages. Finally, approximately 2-3µg total uninfected cell protein were added to eluates (as carrier) and proteins precipitated by addition of 4 volumes of acetone (-20°C/2h). Precipitated proteins were reconstituted in 20µl boiling mix and analysed by SDS-PAGE (Figure 40). Vmw175 bound to the column (although some protein eluted in the washes), and was eluted predominantly between 0.3 and 2M salt. Vmw110 bound more tightly and eluted in the 0.6 and 2M salt washes. These results are in good agreement with those of earlier experiments (Dr. Roger Everett, pers. comm.). A protein of identical mobility to that identified as VZV 140k in Section 3B.2.2. was retained by the column and eluted at higher salt concentrations. The elution profile of VZV 140k was essentially like that of Vmw175, a small amount eluting in the wash steps, while the majority eluted between 0.3 and 2M salt. Thus 140k, like the IE proteins of HSV-1, appears to bind non-specifically to DNA *in vitro*. However, it should be borne in mind that retention of VZV 140k on a DNA-cellulose column using crude extracts does not prove a direct intraction between 140k and DNA.

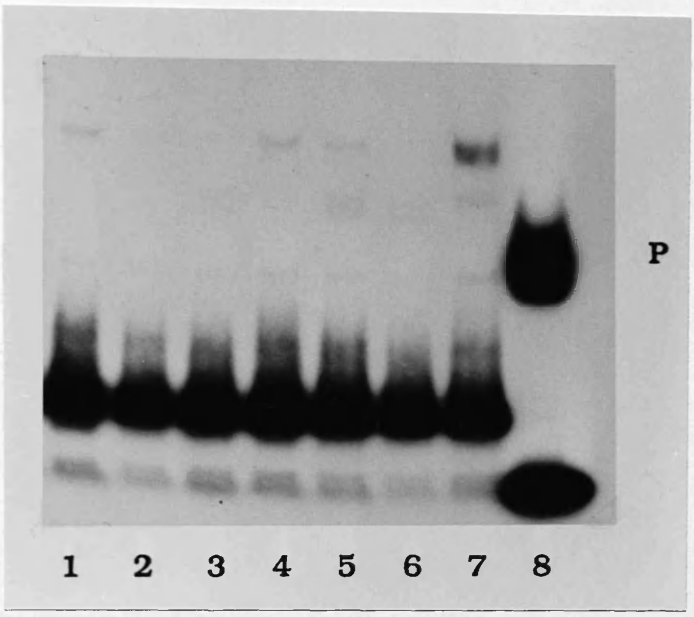


Figure 39. Proteinase K treatment of BHK cell nuclear extracts. Mock, HSV-1, or HSV-140 extract was added to incubation buffer and treated with proteinase K (PK; 1-6ng) for 10 mins at room temperature as detailed below:

Lane	Extract	PK (ng)
1	M	1
2	M	2
3	M	6
4	HSV-140	1
5	HSV-140	2
6	HSV-140	6
7	HSV-1	2
8	HSV-1	2

The PK-treated extracts were incubated with the CAP2 probe (lanes 1-7) or a probe spanning the HSV-1 IE-3 capsite (lane 8). The position of complex P is marked.

Figure 40. VZV 140k binds to a calf-thymus DNA cellulose column. [^{35}S]methionine labelled nuclear extracts (NE) were prepared from HSV-140 infected BHK cells, and from BHK cells infected with HSV-1 under immediate-early (IE) conditions, as described in Section 3E.5. An aliquot of each NE extract (containing approximately 20-40ug of protein) was diluted to 200ul in DC buffer and loaded onto a calf-thymus DNA cellulose column. The flow through, which contained unbound protein, was collected, and the column washed twice with DC buffer, collecting the eluates (Wash 1 and Wash 2). Bound protein was eluted by washing the column with DC buffer containing increasing concentrations of salt (0.1M, 0.2M, 0.3M, 0.6M, and 2M), collecting eluates following each wash. Proteins contained in each of the eluates were precipitated as described in the text, reconstituted in boiling mix, and analysed by SDS-PAGE. The upper and lower panels show the results of parallel experiments with HSV-1 IE NE and HSV-140 NE respectively. The outer lanes contain samples of crude nuclear extract for comparison; the postions of Vmw175, Vmw110, and VZV 140k are marked.

HSV-1
Vmw175
Vmw110
Crude
Unbound
Wash 1
Wash 2
0.1M
0.2M
0.3M
0.6M
2.0M
Crude
HSV-140
VZV140k

3E.6. PARTIALLY PURIFIED 140K DOES NOT BIND TO GENE 62 PROMOTER SEQUENCES

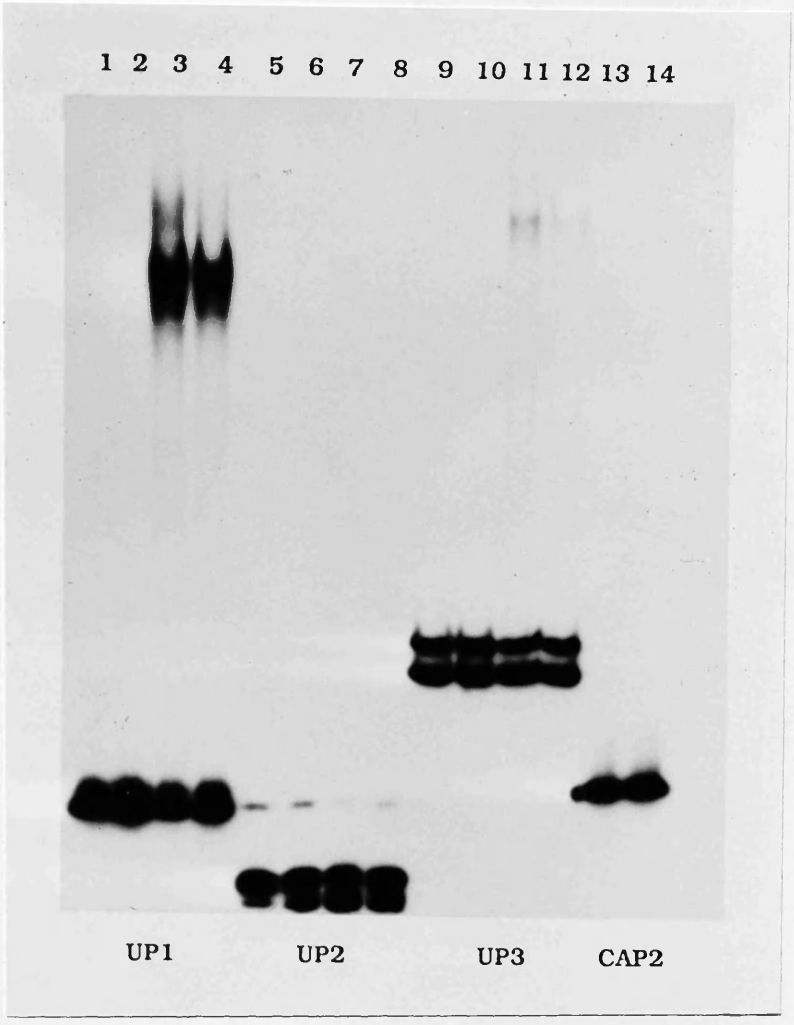
As mentioned previously (Section 3E.4.1), it is unlikely that low affinity binding of 140k would be detected using crude nuclear extracts. It was thus decided to attempt the purification of 140k from HSV-140 infected cells to facilitate further studies. During this work, it was found that 140k in crude nuclear extract became insoluble when the salt concentration was reduced to below 0.2M (Section 3F.3). Since previous experiments were performed at a salt concentration of 0.1M or less, it is conceivable that non-specific aggregation of 140k with other proteins in the extract might have precluded formation of a protein/DNA complex.

Partially purified 140k was obtained by FPLC gel filtration chromatography of crude nuclear extract as described (Section 3F.3.1). The column was run in Super buffer, which is essentially TEN-40 containing 0.3M salt, and thus binding activity could be assayed by direct addition of polydI:polydC and probe to aliquots of appropriate fractions. The experiment described utilized the UP1, 2 and 3, and CAP2 probes, which represent the entire gene 62 promoter-leader region. The column fractions assayed were fraction 9, which contained 140k, and fraction 15, which did not contain any detectable 140k (as judged by SDS-PAGE). Since even partially purified 140k (in fraction 8) became insoluble when the salt concentration was reduced below 0.2M (Section 3F.3.1), binding assays were performed at a salt concentration of 0.3M. Fraction 9 did not form a complex with any of the probes (Figure 41). However, fraction 15 formed a strong complex with the UP1 probe (and also a weak complex with UP3; Figure 41). It has previously been suggested that either CCAAT or ATF/CRE binding factors form a complex with sequences contained within the UP1 probe, (T. A. McKee, PhD Thesis, 1990), and thus fraction 15 may contain one or other of these activities.

An obvious pitfall of this approach is that low affinity binding may be impaired or abolished at a salt

Figure 41. DNA binding assay with FPLC fractions containing partially purified VZV 140k and a set of probes representing the entire VZV gene 62 promoter region. Nuclear extract was prepared from HSV-140 infected BHK cells and polypeptides fractionated by FPLC gel filtration chromatography as described in the text. DNA binding assays utilized fraction 9, which contained VZV 140k (lanes 1,2,5,6,9,10,13,14), and fraction 15, which did not contain any detectable VZV 140k (lanes 3,4,7,8,11,12). These fractions were incubated with the following probes: UP1 (lanes 1-4); UP2 (lanes 5-8); UP3 (lanes 9-12); and CAP2 (lanes 13,14). Incubations contained 1, 2, 3, or 5µg of polydI:polydC as competitor as detailed below:

Lane	Fraction	dI:dC (µg)	Probe
1	9	1	UP1
2	9	3	"
3	15	1	"
4	15	3	"
5	9	1	UP2
6	9	3	"
7	15	1	"
8	15	3	"
9	9	2	UP3
10	9	5	"
11	15	2	"
12	15	5	"
13	9	1	CAP2
14	9	3	"


1 2 3 4 5 6 7 8 9 10 11 12 13 14
UP1
UP2
UP3
CAP2

concentration of 0.3M. (The assay was also performed at 0.2M salt and still binding was not detected; not shown.) Purified 140k (which would hopefully remain soluble at reduced salt concentrations) will be required for further investigation of binding by the intact protein. Such material was not available while these studies were in progress.

3F. PARTIAL PURIFICATION OF VZV 140K FROM HSV-140 INFECTED CELLS

The purification of 140k was undertaken to facilitate further studies on its DNA binding properties. It was envisaged that purified 140k would also permit additional biochemical studies to be undertaken, such as the development of an *in vitro* transcription system. A partial purification of 140k was achieved, but problems were encountered with the solubility of the polypeptide which prevented further purification being attempted.

3F.1. Cells and Infection Conditions

BHK cells were used for extract preparation throughout. Cells were seeded at a density of $2x10^7$ per 140mm dish, infected with HSV-140 (50-200ppc), and labelled with [^{35}S]-methionine (2mCi per dish) from 12-14h p.a. Nuclear extracts were prepared from labelled cells as described below. Since the efficiency of gene expression varied somewhat between stocks of virus, the exact moi used was determined individually for each stock to optimize expression of 140k (whilst avoiding extensive cpe and/or nuclear disruption).

3F.2. Modification of the Nuclear Extraction Protocol

The standard nuclear extraction protocol (Section 2B.9.1) involves first isolating nuclei (at very low salt concentration; 10mM), following which DNA binding proteins are eluted from the nucleus by resuspending nuclei in high salt buffer (0.4M). This procedure was modified as follows (exact details are given in Section 2B.9.2). Nuclei were isolated and resuspended in low salt buffer (final concentration, 0.09M) and a low salt nuclear extract made. This was repeated twice more. Following this residual nuclear proteins were eluted by incubating nuclei in high salt buffer (final salt concentration, 0.24M). The effect of these alterations was determined by SDS-PAGE analysis of equal volumes of each extract (Figure 42). Significant amounts of many polypeptides (particularly of lower

molecular weight) were removed by the low salt extractions, whereas 140k was present in the high salt extract, in which it was enriched compared to a standard NE. This modified procedure was used to generate extract for subsequent purification.

3F.3. PURIFICATION BY FPLC

Initially, FPLC ion-exchange (MONO-Q) chromatography was investigated. The recovery of material from the MONO-Q column was found to be only 10-20% (determined by liquid scintillation counting of aliquots of the fractions). Material appeared to be retained by the pre-column filter (rather than being tightly bound to the column), suggesting that the low recovery was a consequence of aggregation of protein(s) in the extract to form high molecular weight complexes unable to pass through the filter. The solubility of proteins in the crude extract at low salt was thus investigated.

100µl aliquots of nuclear extract were dialysed overnight at 4°C against modified buffer c containing 0.1 or 0.2M salt. The dialysed sample was then centrifuged at 10,000rpm for 15 min in the Sorval SS34 rotor. Pelleted material was reconstituted in 100µl boiling mix, and 20µl of boiling mix added to 5µl of supernatant. These samples (20µl of each) were analysed by SDS-PAGE, Figure 43. It is assumed that proteins found in the pellet were insoluble under the conditions of dialysis. 140k was found to be soluble at 0.2M, but insoluble at 0.1M.

3.1. Partial Purification of 140k by Gel-Filtration Chromatography

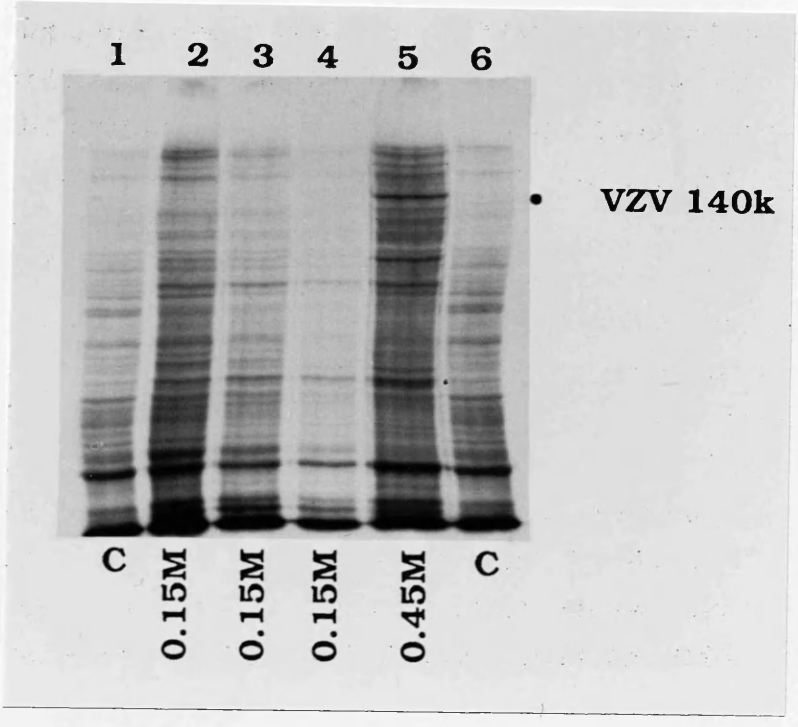
Metzler and Wilcox (1985) noted similar solubility problems during their purification of Vmw175. They found, however, that following gel-filtration chromatography, the partially purified protein was soluble at salt concentrations below 0.1M. A similar approach was adopted in the purification of 140k in the hope that it too would be soluble when partially purified.

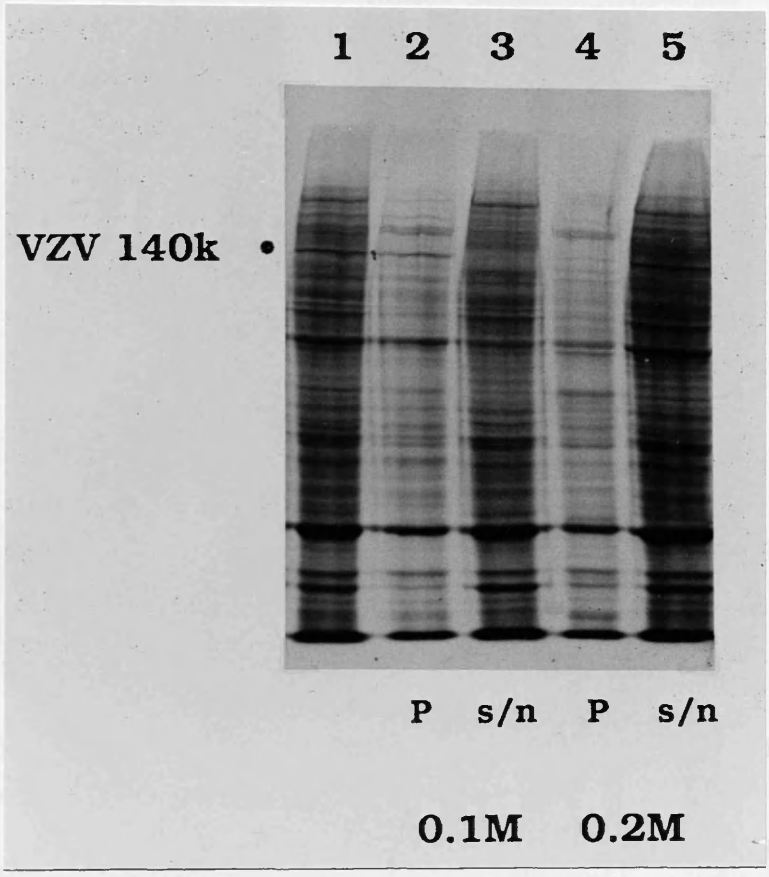
An FPLC Superose 12 column was selected. Since it was intended to assay column fractions for DNA binding activity, columns were run in Super buffer which is essentially TEN-P40 (as used in DNA binding assays) to which had been added β-mercaptoethanol and salt (to 0.3M). Each run generally used 200µl of extract (equivalent to $6x10^7$ cells). The column was run at a flow rate of 0.2 ml/min, and 0.5ml fractions collected from 25-75 mins (elution volume of 6-15ml; the void volume of the column was 6ml) These fractions were analysed by SDS-PAGE (7µl of 3x boiling mix added to 13µl of fraction)(Figure 44). 140k eluted in fractions 8-12 (9-11ml) and was well separated from the majority of lower molecular weight proteins.

Before further purification was attempted, the solubility of partially purified 140k contained in fractions 8-12 was determined. Fraction 8 was divided into two aliquots which were then dialysed against buffer containing 50 or 100mM salt, and analysed as described above (Section 3F.3). It was found that 140k was insoluble at both salt concentrations (other proteins in the fraction displayed partial solubility also.) The addition of 1% NP40 failed to overcome this lack of solubility (results not shown).

That 140k in the FPLC (Superose) fractions is insoluble at low salt concentrations presents a considerable problem with regard to further purification, since many of the steps which might subsequently be employed (ion-exchange chromatography, chromatofocussing etc.) demand that sample be applied at low salt concentrations (i.e. below 0.1M). Thus until 140k can be obtained in a form which remains soluble at low salt concentrations further purification from HSV-140 infected cells will not be possible.

Figure 42. Modified nuclear extraction protocol. BHK cells were infected with 50-200 ppc of HSV-140, and labelled with [^{35}S]methionine from 12-14h p.a. Cells were isolated and lysed in low salt buffer as described, and nuclei pelleted by centrifugation. The supernatant from this step, which contained cytoplasmic proteins, was retained. Nuclei were resuspended in 0.15M salt buffer, incubated on ice for 15 min, nuclei pelleted, and the 0.15M extract reserved. This process was repeated twice more, following which nuclei were resuspended in 0.45M salt buffer and a high salt extract prepared. Equal volumes of the cytoplasmic fraction (C; lanes 1 and 6), each of the sequential 0.15M nuclear extracts (lanes 2, 3 and 4), and the final 0.45M nuclear extract (lane 5) were analysed by SDS-PAGE. The position of VZV 140k in the 0.45M extract is marked. The 0.15M salt extractions remove significant amounts of nuclear proteins and VZV 140k is thus enriched in the 0.45M extract.


1
2
3
4
5
6
VZV 140k
C
0.15M
0.15M
0.15M
0.45M
C


1 2 3 4 5
VZV 140k
P s/n P s/n
0.1M 0.2M

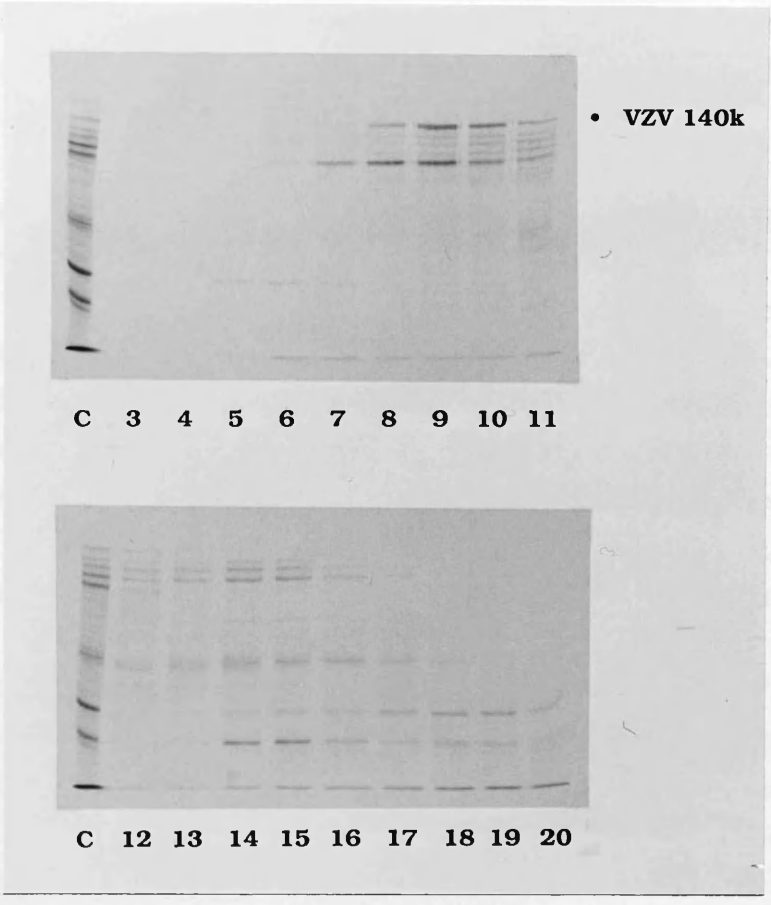


Figure 44. Partial purification of VZV 140k by FPLC gel filtration chromatography. [^{35}S]methionine labelled nuclear extract was prepared by the modified protocol described in the text. This material was fractionated by chromatography on an FPLC superose column. Twenty 0.5ml fractions representing an elution volume of 5-15ml were collected, and equal aliquots of fractions 3-20 (elution volume 6-15ml) analysed by SDS-PAGE. The sample in the extreme left lane of each gel (marked C) is an aliquot of crude nuclear extract for comparison. The position of VZV 140k, which eluted predominantly in fractions 9 and 10, is marked.

4. DISCUSSION

The studies described in this thesis sought to determine the functions and properties of the VZV 140k polypeptide. The results obtained are discussed in relation to the properties of Vmw175, and their implications for VZV gene regulation considered. Finally, some areas for further study are outlined.

4.1. Functional Homology Between VZV 140k and Vmw175

Several groups have reported that VZV 140k, like Vmw175, is a powerful *trans*-activator in transfection assays (Everett, 1984; Everett and Dunlop, 1984; Inchauspe *et al.*, 1989; Cabirac *et al.*, 1990). Further functional relatedness between the two polypeptides has been demonstrated by Felser *et al.* (1987, 1988), who showed that VZV 140k was able to complement the growth of viruses with *ts* and deletion mutations in Vmw175. The studies described with the HSV-1 recombinant virus, HSV-140, sought to analyse this complementation in greater depth. In summary, it was found that VZV 140k was able to complement, to a large extent, for loss of Vmw175 function. The phenotype of HSV-140, particularly those aspects pertaining to the functions of VZV 140k, are discussed in detail below.

HSV-140, unlike wt virus, failed to form clearly defined plaques. At first sight, this finding appears to be at variance with the results of Felser *et al.* (1988), who described plaque formation by HSV-1 Vmw175 mutants on stably transformed cell lines expressing VZV 140k. However, different sequences control the expression of VZV 140k in their studies and those reported here, which may explain this apparent contradiction. Thus VZV 140k is expressed from the HSV-1 IE-3 promoter in HSV-140 and, since this promoter is not subject to autoregulation (see below), VZV 140k accumulates to high levels as infection proceeds (relative to Vmw175 in a normal wt virus infection). In contrast, VZV 140k is expressed from the authentic gene 62 promoter in the cell lines of Felser *et al.*, and hence presumably subject to autoregulation. Therefore, levels of

VZV 140k during their experiments were probably much lower. It is conceivable that the presence of significant amounts of VZV 140k throughout the later stages of HSV-140 infections might alter the balance of gene regulation. Subtle alterations in gene expression might lead to a reduced rate of virus assembly and maturation, which could in turn delay the appearance of cpe, and possibly account for the lack of plaque formation.

Comparison of viral polypeptide expression during HSV-140 and wt virus infections (at high multiplicity) demonstrated that a majority of predominant viral polypeptides were expressed in similar amounts and with similar kinetics. However, it should be stressed that this analysis would not detect subtle changes in the expression of polypeptides which are of low abundance during a normal wt virus infection. Indeed, the defect in DNA synthesis may well reflect the aberrant expression of an early function involved in DNA replication. It is likely that the reduced expression of certain late polypeptides is a consequence of less efficient DNA replication.

Intriguingly, gene expression during low multiplicity HSV-140 infections is markedly reduced. This aspect of the phenotype of HSV-140 is strikingly reminiscent of the phenotypes of HSV-1 viruses with mutations in Vmw65 and Vmw110 (Stow and Stow, 1986; Sacks and Schaffer, 1987; Ace *et al.*, 1989; Everett, 1989). The deficient growth of viruses lacking Vmw110 function is apparently a consequence of reduced viral gene expression (Everett, 1989). Furthermore, the Vmw65 mutant *in*1814 expresses reduced amounts of Vmw110 and exhibits partial complementation when grown on cells transfected with a plasmid expressing Vmw110 (Ace *et al.*, 1989). It would thus appear that at the outset of low multiplicity infections, if Vmw110 is absent or underexpressed, then Vmw175 is not of itself able to efficiently induce viral gene expression. It has been proposed that synergistic activation by Vmw175 and Vmw110 in transfection assays might reflect a mechanism whereby Vmw110 augments viral gene expression during

infection. Therefore, is the multiplicity dependence of HSV-140 gene expression a consequence of inefficient activation of viral promoters by VZV 140k, either alone or in combination with Vmw110? This would seem unlikely given that VZV 140k has been shown to *trans*-activate the HSV-1 glycoprotein D (gD) and thymidine kinase (tk) gene promoters much more efficiently than Vmw175 in transfection assays (Section 3C, this thesis; Cabirac *et al.*, 1990), and synergistic activation of the tk promoter by VZV 140k and Vmw110 has been reported (Cabirac *et al.*, 1990). However, these observations in transfection assays may not accurately reflect the situation at the outset of infection, when the relative amounts of the *trans*-activator polypeptides and target promoters is likely to be different.

Surprisingly, VZV 140K is poorly expressed in a cycloheximide reversal experiment, despite being expressed from the HSV-1 IE-3 promoter. IE-3 promoter sequences upstream of position +27 (the site used for the linkage to VZV gene 62 sequences in HSV-140), have been shown to be sufficient to confer IE gene specificity upon heterologous sequences (Cordingly et al, 1983), and therefore it was expected that VZV 140k would be expressed as a normal HSV-1 IE gene in HSV-140. (The failure of VZV 140k to autoregulate the IE-3 promoter is discussed below.) Since much of the IE-3 and VZV gene 62 untranslated leader sequences are absent from the hybrid RNA encoded by HSV-140, a number of explanations for the lack of expression of VZV 140k following cycloheximide reversal can be envisaged. For example, the gene may not be transcribed. Alternatively, the mRNA might be intrinsically unstable and fail to accumulate, or may be inefficiently translated under these conditions. Whatever the reason, these results suggest that the nature of the transcribed sequences may play a role in the expression of IE genes after cycloheximide reversal.

The absence of VZV 140K in a cycloheximide reversal experiment, which is unphysiological, is probably not

significant with regard to the growth properties of HSV-140 in normal infections. The polypeptide profiles at early times in HSV-140 and wt virus infections (at high multiplicity) were essentially indistinguishable, which argues that sufficient amounts of VZV 140K are produced at the outset of infection to ensure efficient induction of early gene expression.

4.1.1. *Trans*-Activation and Autoregulation by VZV 140k

The virus studies discussed above demonstrate that VZV 140k is able to *trans*-activate many HSV-1 promoters. The *trans*-activation properties of VZV 140k were analysed further in transfection assays. It was found that a small in-frame insertion into homology region 2 of the polypeptide abolished *trans*-activation activity. Significantly, the integrity of region 2 sequences of Vmw175 has been shown to be essential for *trans*-activation (Paterson and Everett, 1988a; Shepard *et al.*, 1989), which suggests that the mechanism of *trans*-activation by VZV 140k is likely to be similar to that of Vmw175, a model of which is discussed in Section 1D.7.1.

The results described in Section 3D demonstrate repression or autoregulation of the VZV gene 62 promoter by VZV140k, thus defining a new function of the polypeptide. Interestingly, Vmw175 is able to repress the VZV gene 62 promoter with a similar efficiency to VZV 140k, but VZV 140k is unable to repress the IE-3 promoter. The failure of VZV 140k to repress the IE-3 promoter in transfection experiments is consistent with the properties of the recombinant virus, HSV-140; infection by HSV-140 leads to high levels of VZV 140k expression which continues late into infection.

The finding that the integrity of homology region 2 of VZV 140k and Vmw175 is required for repression of the VZV gene 62 promoter is clearly of interest since it indicates that both polypeptides repress VZV gene 62 by a mechanism similar to that described for repression of the HSV-1 IE-3 promoter by Vmw175 (see Section 1C.2.4). This

requires a specific DNA recognition sequence at the IE-3 cap site (Roberts et al., 1988), and a functional Vmw175 DNA binding domain (Paterson and Everett, 1988b; Shepard et al., 1989). Therefore, in an attempt to identify a VZV 140k DNA recognition element, sequences in the gene 62 capsite-leader region were deleted. These experiments were, however, inconclusive. A more productive approach might be to direct nucleotide alterations or smaller deletion mutations into the otherwise intact capsite-leader in p140CAT.

4.1.2. DNA Binding

The immediate-early polypeptides of HSV-1 bind non-specifically to DNA *in vitro* (Powell and Purifoy, 1976; Hay and Hay, 1980). A similar approach to that used by Hay and Hay was employed to investigate whether or not VZV 140k binds DNA. It was found that VZV 140k was retained by a DNA-cellulose column and had a similar elution profile to Vmw175. This is indirect evidence that VZV 140k binds to DNA, although possible artefacts such as partial insolubility of VZV 140k under the conditions used to load the column (thus leading to retention) cannot be excluded.

The repression studies discussed above provide genetic evidence that both VZV 140k and Vmw175 bind to specific sequences in the VZV gene 62 promoter-leader region. Thus gel retardation assays were undertaken with a range of probes representing the entire gene 62 promoter-leader region (-410 to +57) as defined by Mckee *et al.* (1990). These studies did not demonstrate sequence-specific binding of either polypeptide to sequences in this region. It should be stressed that detection of protein-DNA interactions in gel retardation assays requires that the interaction be relatively stable under the incubation/gel conditions used. It is possible that a different approach (e.g. footprinting) may yet detect binding of VZV 140k to DNA.

The sequence-specific DNA binding function of Vmw175 resides in a discrete domain of the polypeptide

comprising predominantly region 2 sequences. This domain, either expressed as a fusion protein in bacteria, or liberated from the intact polypeptide by proteolytic digestion, is able to bind DNA with a similar affinity and specificity to the intact polypeptide (Everett *et al.*, 1990; Wu and Wilcox, 1990). More recent data from Wu and Wilcox (XVth International Herpesvirus Workshop; Pers. Comm.) has demonstrated binding of region 2 of VZV 140k (expressed as a *trpE* fusion protein in *E.coli*) to specific sequences in the VZV gene 62 promoter-regulatory region; their results are discussed in more detail below. The failure of the experiments described in this thesis to detect such binding probably reflects both the low affinity of the binding (as noted by Wu and Wilcox, see below) and the solubility problems encountered with the intact polypeptide in both crude extracts and in partially purified form.

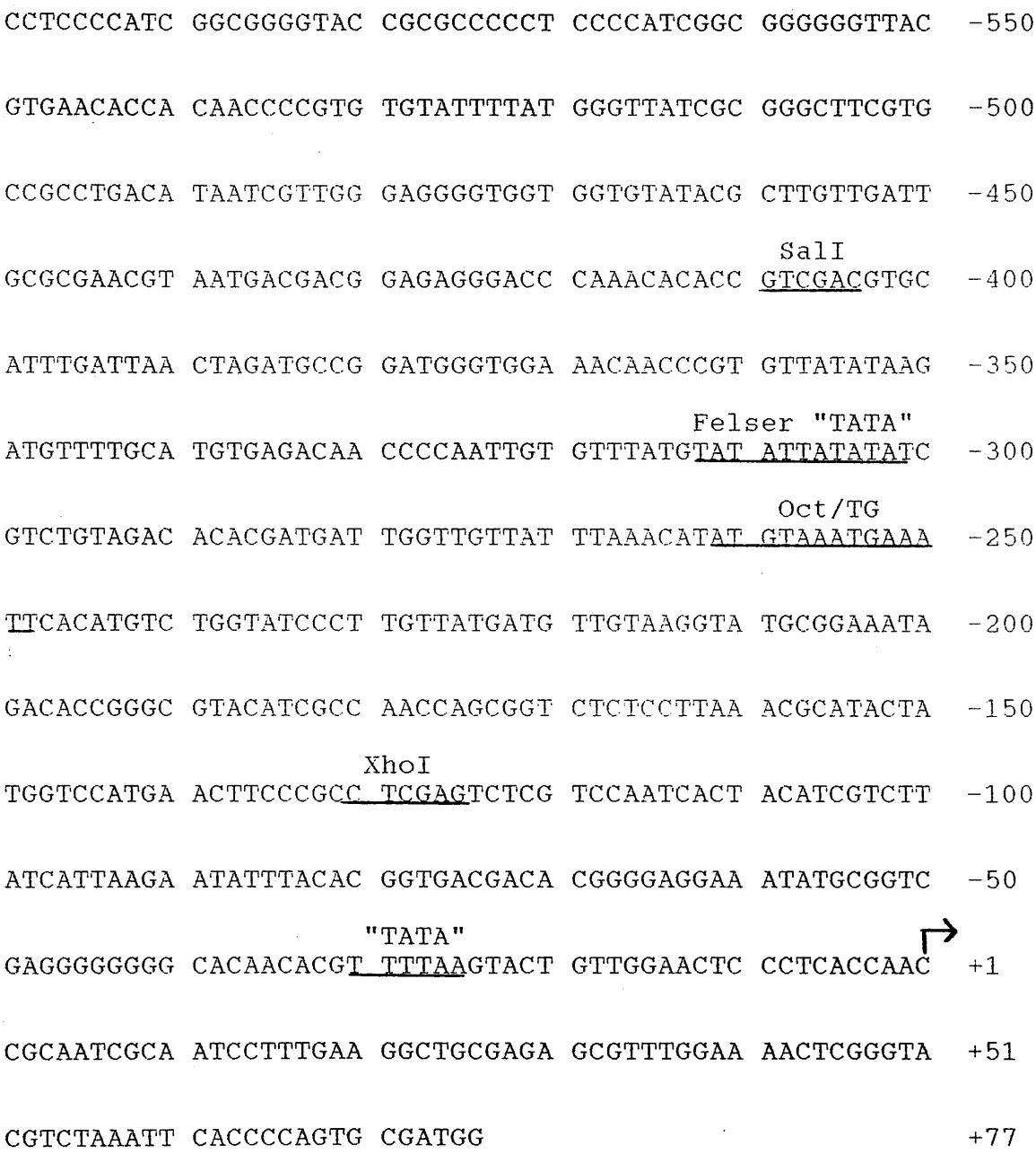
Wu and Wilcox expressed region 2 of VZV 140k, and the corresponding sequences from Vmw175 and the PRV-IE polypeptide, as *TrpE* fusion proteins in *E.Coli*. These fusion proteins were partially purified and utilized in DNAse footprinting and gel retardation assays with probes representing VZV gene 62 sequences from -410 to +95, essentially as investigated by the experiments described in this thesis. In addition, binding to probes spanning the HSV-1 IE-3 and PRV IE gene capsites was investigated. In comparison to the HSV-1 and PRV fusion proteins, the VZV 140k fusion protein (designated FP617) bound with much lower affinity to all sequences analysed. FP617 was found to bind to a number of sites in the VZV promoter-leader region; the highest affinity binding was to a site located between -311 and -282, with binding to a number of weaker sites located between -200 and +95 also being detected. Interestingly, the sequence specificity of FP617 and the Vmw175 fusion protein, FP449 (and also the PRV fusion protein), was found to be similar, the higher affinity sites all containing the sequence $^{5'}$ATCGT$^{3'}$. Strikingly, these residues are identical to those shown to be most

important for binding of Vmw175 to the consensus ATCGTC motif at the IE-3 capsite (see Section 1C.1.3). However, FP449 did not appear to bind to the high affinity site (-311 to -282) in the VZV promoter (which contains an ATCGT motif). Thus the fusion proteins appear to have similar sequence recognition characteristics, though these are not indistinguishable.

What are the implications of these results for the proposed mechanism of autoregulation of the VZV gene 62 promoter by VZV 140k and Vmw175? The genetic evidence discussed above suggests that repression involves binding to specific sequences, presumably at or near the gene 62 capsite. However, the FP617 fusion protein binds most strongly to a site located between -311 and -282, far upstream of the experimentally determined capsite, and binding to the capsite region itself is very weak. The far-upstream site is, however, located immediately downstream of what Felser *et al.* (1988) originally proposed (on the basis of sequence analysis alone) to be the gene 62 TATA box (see Figure 45). Wu and Wilcox propose that gene 62 may contain two capsites, one as determined by McKee *et al.* (1990), which is designated +1, and a second located around 300 bp upstream of this at around -300, downstream of the proposed "Felser" TATA box at -312. If this hypothetical "upstream" capsite were functional and constituted a predominant start site (at least early in infection), then by analogy with repression of the HSV-1 IE-3 promoter by Vmw175, binding of VZV 140k to this site might bring about repression of the gene 62 promoter.

There is as yet no experimental evidence for the existence of this second capsite, and it is unlikely to be important in the expression of gene 62 for the following reasons. The sequence elements predicted by Felser *et al.* (1988) are located between -664 and -280, whereas McKee *et al.* (1990) demonstrated that basal promoter activity was contained in a region between -131 and +57, and that sequences upstream of -410 had no effect on either basal or Vmw65 induced levels of promoter activity. Furthermore, if

Figure 45. VZV gene 62 control Region. Sequences upstream of VZV gene 62 between -599 and +77 are shown. The orf begins at the ATG codon at +74. McKee *et al.* (1990) analysed the sequences controlling expression of gene 62. Basal promoter activity was found to reside between *Xho*I (-131) and +57. The location of the experimentally determined capsite is indicated by the arrow, and the location of the presumed TATA box (-30) is shown. Sequences between *Sal*I (-410) and *Xho*I (-131) were found to confer responsiveness to Vmw65, and the location of an octamer/TAATGARAT motif (centered on -255) implicated in this response is shown. The TATA box proposed by Felser *et al.* (1988) is indicated (centered on -306).


CCTCCCCATC GGCGGGGTAC CGCGCCCCCT CCCCATCGGC GGGGGGTTAC -550
GTGAACACCA CAACCCCGTG TGTATTTTAT GGGTTATCGC GGGCTTCGTG -500
CCGCCTGACA TAATCGTTGG GAGGGGTGGT GGTGTATACG CTTGTTGATT -450
SalI
GCGCGAACGT AATGACGACG GAGAGGGACC CAAACACACC GTCGACGTGC -400
ATTTGATTAA CTAGATGCCG GATGGGTGGA AACAACCCGT GTTATATAAG -350
Felser "TATA"
ATGTTTTGCA TGTGAGACAA CCCCAATTGT GTTTATGTAT ATTATATATC -300
Oct/TG
GTCTGTAGAC ACACGATGAT TGGTTGTTAT TTAAACATAT GTAAATGAAA -250
TTCACATGTC TGGTATCCCT TGTTATGATG TTGTAAGGTA TGCGGAAATA -200
GACACCGGGC GTACATCGCC AACCAGCGGT CTCTCCTTAA ACGCATACTA -150
XhoI
TGGTCCATGA ACTTCCCGCC TCGAGTCTCG TCCAATCACT ACATCGTCTT -100
ATCATTAAGA ATATTTACAC GGTGACGACA CGGGGAGGAA ATATGCGGTC -50
"TATA"
GAGGGGGGGG CACAACACGT TTTAAGTACT GTTGGAACTC CCTCACCAAC +1
CGCAATCGCA ATCCTTTGAA GGCTGCGAGA GCGTTTGGAA AACTCGGGTA +51
CGTCTAAATT CACCCCAGTG CGATGG +77

a mRNA were transcribed from this upstream capsite, its untranslated-leader region would contain ten AUG codons upstream of the predicted AUG initiation codon, each of which is followed by an in-frame termination codon. Even if potential splice donor and acceptor sites (located at -212 and +69 respectively) were utilized, the leader would still contain five AUG codons followed by in-frame termination codons upstream of the orf. It is likely that translation of VZV 140k coding sequences encoded by an mRNA with these characteristics would be extremely inefficient. In contrast, the untranslated-leader sequence of mRNA transcribed from the capsite at +1 contains no AUG codons upstream of the orf. Thus, this second "hypothetical" capsite, if indeed it exists, is unlikely to be of significant importance in the expression of VZV 140k during virus infection.

It is informative to also consider repression of the VZV gene 62 promoter by Vmw175. Thus although Vmw175 represses the gene 62 promoter as efficiently as VZV 140k, the FP449 fusion protein, which binds relatively strongly to the IE-3 capsite, binds only weakly to sites in the gene 62 promoter, none of which overlap, or are located close to either the experimental or hypothetical capsites. This is intriguing since repression of the IE-3 promoter by Vmw175 appears to require that the binding site is located at or close to the capsite. Finally, although FP617 apparently binds as efficiently to the consensus motif located at the HSV-1 IE-3 capsite as it does to sites in the gene 62 promoter, VZV 140k strongly represses the gene 62 promoter yet activates the IE-3 promoter.

Genetic evidence suggests that DNA binding is central to the mechanism whereby VZV 140k and Vmw175 repress expression from the VZV gene 62 promoter. However, it is not clear from the DNA binding studies discussed above whether this involves binding to the capsite alone, or whether binding to a number of sites might be involved. Clearly, further experimentation will be required to delineate the mechanism whereby these polypeptides repress

the VZV gene 62 promoter.

4.2. Gene Regulation and the Biology of VZV

The regulation of expression of one of the predicted VZV IE genes, gene 62, has been studied in transfection assays. McKee *et al.* (1990) defined a promoter for VZV gene 62 which was shown to respond to the HSV-1 virion *trans*-activator polypeptide, Vmw65. The VZV homologue of Vmw65, encoded by VZV gene 10, was found to be unable to *trans*-activate gene 62, and it was concluded that a cellular polypeptide might replace Vmw65 function or activate VZV IE gene expression directly.

The transfection studies described in this thesis demonstrate autoregulation of the VZV gene 62 promoter by VZV 140k. Repression of the HSV-1 IE-3 promoter by Vmw175 in transfection assays presumably reflects a homeostatic mechanism whereby levels of Vmw175 are stabilized and high level expression is prevented as infection proceeds. It is proposed that expression of VZV 140k during VZV infection will likewise be subject to autoregulation. Autoregulation of expression of VZV 140k and Vmw175 might be important with respect to the biology of VZV and HSV-1, since overexpression of these *trans*-activator polypeptides at late times in infection might disrupt gene expression. Indeed, this might account for some of the properties of HSV-140.

The results presented in this thesis and elsewhere provide compelling evidence that VZV 140k has a pivotal role in the growth of VZV *in vivo*. As such, modulation of the efficiency of expression of VZV 140k may be important in determining the biological properties of VZV. For example, differential expression of VZV 140k in different cell types could have a profound effect on the outcome of VZV infection. VZV encodes three other polypeptides with homology to HSV-1 IE genes. The product of VZV gene 4 is able to partially complement HSV-1 mutants with lesions in Vmw63, and is thus also likely to be important in the growth of VZV. The potential role of the predicted VZV

equivalent of Vmw68 remains to be determined.

Finally, these studies do not indicate whether or not VZV genes are regulated with "early" or "late" kinetics. It will be necessary to analyse gene expression during VZV infection to address this question.

4.3. Future Prospects

The work presented in this thesis has increased our knowledge of the properties and functions of VZV 140k. A number of areas for potential future study will be outlined.

VZV 140k is a powerful *trans*-activator of viral promoters, and is also able to repress expression from its own promoter, that of gene 62. Homology region 2 of VZV 140k, like that of Vmw175, is important for both of these functions. By extension, it is likely that homology region 4 of VZV 140k will be functionally important, at least in *trans*-activation. It would, therefore, be of interest to study the properties of regions 2 and 4 in more detail. One approach which could be taken would be to construct chimeric genes in which region 2 sequences (and also the C-terminal end of region 1, which appears to be important in the DNA binding properties of Vmw175), region 4 sequences, or both were exchanged between VZV 140k and Vmw175. These chimeric genes could then be studied in a number of ways:

The *trans*-activation properties of the polypeptides expressed by the chimeric genes could be investigated in transfection assays. This would determine whether the powerful *trans*-activation of the HSV-1 gD and VZV tk promoters by VZV 140k compared to Vmw175 (in the absence of Vmw110) reflects sequence alterations within or outwith the conserved regions of the polypeptides.

Region 2 of VZV 140k and Vmw175 represent distinct DNA binding domains. The DNA binding properties of the chimeric polypeptides (particularly affinity) should, therefore, reflect those of the polypeptide from which region 2 sequences are derived. Analysis of repression of

the HSV-1 IE-3 and VZV gene 62 promoters by these chimeric polypeptides should help to clarify the relationship between the DNA binding and repression properties of VZV 140k and Vmw175, which is at present somewhat opaque.

Finally, these chimeric genes could be recombined into D30EBA, and the resulting recombinant viruses characterized. Thus it would be possible to determine whether the defects in HSV-140 reflect the properties of regions 2 and/or 4 of VZV 140k, or whether they reflect those of the non-conserved regions. It would hopefully be possible to relate the findings of transfection studies to the growth properties of the viruses. The effect of overexpressing VZV 140k on the growth of HSV-140 should also be assessed by constructing a virus in which VZV 140k is expressed from the gene 62 promoter, rather than the HSV-1 IE-3 promoter. If overexpression of VZV 140k were found to account for some of the properties of HSV-140, then expression of chimeric polypeptides in recombinant viruses would utilize a promoter shown to be repressed (in transfection assays) by the polypeptide in question.

These studies could be extended to include the corresponding homologous region 2 sequences of the PRV and EHV-1 IE polypeptides.

REFERENCES

ABMAYR, S.M., FELDMAN, L.T., & ROEDER, R.G. (1985). In vitro stimulation of specific RNA polymerase II-mediated transcription by the pseudorabies virus immediate early protein. Cell 43, 821-829.

ABMAYR, S.M., WORKMAN, J.L., & ROEDER, R.G. (1988). The pseudorabies immediate early protein stimulates in vitro transcription by facilitating TFIID:promoter interactions. Genes and Development 2, 542-553.

ACE, C.I., DALRYMPLE, M.A., RAMSAY, F.A., PRESTON, V.G., & PRESTON, C.M. (1988). Mutational analysis of the herpes simplex virus type 1 *trans*-inducing factor Vmw65. Journal of General Virology 69, 2595-2605.

ACE, C.I., McKEE, T.A., RYAN, M.J., CAMERON, J.M., & PRESTON, C.M. (1989). Construction and characterisation of a herpes simplex virus type 1 mutant unable to transinduce immediate early gene expression. Journal of Virology 63, 2260-2269.

ACHONG, B.G., & MEURISE, E.V. (1968). Observation on the fine structure of varicella virus in cultivated human amnion cells. Journal of General Virology 3, 305-308.

ALFORD, C.A., & BRITT, W.J. (1984). Cytomegalovirus. In: "Virology", pp 629-660. Ed. B.N. Fields. Raven press, New York.

ALLISON, L.A., & INGLES, C.J. (1989). Mutations in RNA polymerase II enhance or suppress mutations in *GAL4*. Proceedings of the National Academy of Sciences (USA) 86, 2794-2798.

ALLISON, L.A., MOYLE, M., SHALES, M., & INGLES, C.J. (1985). Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell 42, 599-610.

ALLISON, L.A., WONG, J.K-C., FITZPATRICK, V.D., MOYLE, M., & INGLES, C.J. (1988). The C-terminal domain of the largest subunit of RNA polymerase II of *saccharomyces cerevisiae, drosophila melanogaster,* and mammals: a conserved structure with an essential function. Molecular and Cellular Biology 8, 321-329.

ApRHYS, C.M., CIUFO, D.M., O'NEILL, E.A., KELLY, T.J., & HAYWARD, G.S. (1989). Overlapping octamer and TAATGARAT motifs in herpes simplex virus immediate-early promoters represent independant binding sites for cellular nuclear factor III. Journal of Virology 63, 2798-2812.

ARSENAKIS, M., CAMPADELLI-FIUME, G., & ROIZMAN, B. (1988). Regulation of glycoprotein D synthesis: Does α4, the major regulatory protein of herpes simplex virus 1, regulate late genes both positively and negatively. Journal of Virology **62**, 148-158.

ARVIN, A.M., KOROPCHAK, C.M., WILLIAMS, B.R.G., GRUMET, F.C., & FOUNG, S.K.H. (1986). Early immune response in healthy and immunocompromised subjects with primary varicella-zoster virus infection. Journal of Infectious Diseases **154**, 422-429.

ASANO, Y., ITAKURA, N., HIROISHI, Y., HIROSE, S., OZAKI, T., YAZAKI, T., YAMANISHI, K., & TAKAHASHI, M. (1985). Viral replication and immunological responses in children naturally infected with varicella-zoster virus and in varicella vaccine recipients. Journal of Infectious diseases **152**, 863-868.

ASANO, Y., & TAKAHASHI, M. (1979). Studies on the polypeptides of varicella-zoster (V-Z) virus. I. Detection of varicella-zoster virus polypeptides in infected cells. Biken Journal 22, 81-89.

ASANO, Y., & TAKAHASHI, M. (1980). Studies on the polypeptides of varicella-zoster virus (VZV). II. Synthesis of viral polypeptides in infected cells. Biken Journal 23, 95-106.

ASHER, Y., KELLER, M., & BECKER, Y. (1969). Incorporation of lipids into herpes simplex virus particles. Journal of General Virology **4**, 65-76.

BAGCHI, S., RAYCHAUDHURI, P., & NEVINS, R. (1990). Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: A novel mechanism for E1A *trans*-activation. Cell **62**, 659-669.

BARINGER, J.R., & SWOVELAND, P. (1973). Recovery of herpes simplex virus from human trigeminal ganglia. New England Journal of Medicine **288**, 648-650.

BARTOLOMEI, M.S., HALDEN, N.F., CULLEN, C.R., & CORDEN, J.L. (1988). Genetic analysis of the repetitive carboxy-terminal domain of the largest subunit of mouse RNA polymerase II. Molecular and Cellular Biology 8, 330-339.

BATTERSON, W., & ROIZMAN, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. Journal of Virology **46**, 371-377.

BEARD, P., FABER, S., WILCOX, K.W., & PIZER, L.I. (1986). Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. Proceedings of the National Academy of Sciences (USA) **83**, 4016-4020.

BENOIST, C., & CHAMBON, P. (1981). *In vivo* sequence requirements of the SV40 early promoter region. Nature **290**, 304-310.

BEN-PORAT, T., HOFFMAN, P., BROWN, L., FELDMAN, L., & BLANKENSHIP, M.L. (1982). Partial characterization of temperature-sensitive mutants of pseudorabies virus. Virology **122**, 252-267.

BEN-PORAT, T., VEACH, R.A., & IHARA, S. (1983). Localization of regions of homology between the genomes of herpes simplex virus, type 1, and pseudorabies virus. Virology **127**, 194-204.

BERG, J.M. (1986). Potential metal-binding domains in nucleic acid binding proteins. Science **232**, 485-487.

BERGER, S.L., CRESS, W.D., CRESS, A., TRIEZENBERG, S.J., & GUARENTE, L. (1990). Selective inhibition of activated but not basal transcription by the acidic activation of VP16: evidence for transcriptional adaptors. Cell **61**, 1199-1208.

BERLIN, B.S., & CAMPBELL, T. (1970). Hospital acquired herpes zoster following exposure to chickenpox. Journal of the American Medical Association **211**, 1831-1833.

BOYER, H.W., & ROULLAND-DOUSSOIX, D. (1969). A complementation analysis of the restriction modification of DNA in *Escherichia coli*. Journal of Molecular Biology **41**, 459-472.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry **72**, 248-254.

BRANDL, C.J., AND STRUHL, K. (1989). Yeast GCN4 transcriptional activator protein interacts with RNA polymerase II *in vitro*. Proceedings of the National Academy of Sciences (USA) **86**, 2652-2656.

BROWN, S.M., & HARLAND, J. (1987). Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11 and US12 and two in which R_S has been extended by 6kb to 0.91 map units with loss of U_S sequences between 0.94 and the U_S/TR_S junction. Journal of General Virology 68, 1-18.

BROWN, S.M., RITCHIE, D.A., & SUBAK-SHARPE, J.H. (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. Journal of General Virology **18**, 329-346.

BURATOWSKI, S., HAHN, S., SHARP, P.A., & GUARENTE, L. (1988). Function of a yeast TATA element-binding protein in a mammalian transcription system. Nature **334**, 37-42.

BURATOWSKI, S., HAHN, S., GUARENTE, L., & SHARPE, P.A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56**, 549-561.

BURKE, B.L., STEELE, R.W., WOOD, J.S., CAIN, T.D., & MARMER, D. (1982). Immune responses to varicella in the aged. Archives of Internal Medicine **142**, 291-293.

BURTON, Z.F., ORTOLAN, L.G., & GREENBLATT, J. (1986). Proteins that bind to RNA polymerase II are required for accurate initiation of transcription at the adenovirus 2 major late promoter. EMBO Journal 5, 2923-2930.

BURTON, Z.F., KILLEEN, M., SOPTA, M., ORTOLAN, L.G., & GREENBLATT, J. (1988). RAP 30/74: a general initiation factor that binds to RNA polymerase II. Molecular and Cellular Biology 8, 1602-1613.

BZIK, D.J., & PRESTON, C.M. (1986). Analyses of DNA sequences which regulate the transcription of herpes simplex virus immediate-early gene 3: DNA sequences required for enhancer-like activity and response to *trans*-activation by a virion polypeptide. Nucleic Acids Research 14, 929-943.

CABIRAC, G.F., MAHALINGHAM, R., WELLISH, M., & GILDEN, D.H. (1990). Trans-activation of viral tk promoters by proteins encoded by varicella zoster virus open reading frames 61 and 62. Virus Research **15**, 57-68.

CABRAL, G.A., COURTNEY, R.J., SCHAFFER, P.A., & MARCIANO-CABRAL, F. (1980). Ultrastructural characterization of an early, nonstructural polypeptide of herpes simplex virus type 1. Journal of Virology **33**, 1192-1198.

CADENA, D.L., & DAHMUS, M.E. (1987). Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. Journal of Biological Chemistry **262**, 12468-12474.

CAMPBELL, M.E.M., PALFREYMAN, J.W., & PRESTON, C.M. (1984). Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for stimulation of immediate-early transcription. Journal of Molecular Biology **180**, 1-19.

CAMPBELL, M.E.M., & PRESTON, C.M. (1987). DNA sequences which regulate the expression of the pseudorabies virus major immediate early gene. Virology **157**, 307-316.

CASEY, T.A., RUYECHAN, W.T., FLORA, M.N., REINHOLD, W., STRAUS, S.E., & HAY, J. (1985). Fine mapping and sequencing of a variable segment in the inverted repeat region of varicella-zoster virus DNA. Journal of Virology **54**, 639-642.

CAUGHMAN, G.B., STACZEK, J., & O'CALLAGHAN, D.J. (1985). Equine herpesvirus type 1 infected cell polypeptides: Evidence for immediate early/early/late regulation of viral gene expression. Virology **145**, 49-61.

CAUNT, A.E. (1963). The growth of varicella-zoster virus in human thyroid tissue cultures. Lancet **ii**, 982-983.

CAUNT, A.E., & TAYLOR-ROBINSON, D. (1964). Cell-free varicella-zoster virus in tissue culture. Journal of Hygiene **62**, 413-424.

CAVALLINI, B., FAUS, I., MATTHES, H., CHIPOULET, J.M., WINSOR, B., EGLY, J.M., & CHAMBON, P. (1989). Cloning of the gene encoding the yeast protein BTF1Y, which can substitute for the human TATA box-binding factor. Proceedings of the National Academy of Sciences (USA) **86**, 9803-9807.

CHALLBERG, M. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. Proceedings of the National Academy of Sciences (USA) **83**, 9094-9098.

CHASMAN, D.I., LEATHERWOOD, J., CAREY, M., PTASHNE, M., & KORNBERG, R.D. (1989). Activation of yeast polymerase II transcription by herpesvirus VP16 and GAL4 derivatives *in vitro*. Molecular and Cellular Biology **9**, 4746-4749.

CHENG, Y-C., TSOU, T.Y., HACKSTADT, T., & MALLAVIA, L.P. (1979). Induction of thymidine kinase and DNAse in varicella-zoster virus-infected cells and properties of the virus-induced enzymes. Journal of Virology **31**, 172-177.

CHEUNG, A.K. (1989). DNA nucleotide sequence analysis of the immediate-early gene of pseudorabies virus. Nucleic Acids Research **17**, 4637-4646.

CLEMENTS, J.B., WATSON, R.J., & WILKIE, N.W. (1977). Temporal regulation of herpes simplex virus transcription: Location of transcripts on the viral genome. Cell **12**, 275-285.

COEN, D.M., WEINHEIMER, S.P., & McKNIGHT, S.L. (1986). A genetic approach to promoter recognition during trans induction of viral gene expression. Science **234**, 53-59.

CONLEY, A.J., KNIPE, D.M., JONES, P.C., & ROIZMAN, B. (1981). Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by *in vitro* mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. Journal of Virology 37, 191-206.

COOK, M.L., & STEVENS, J.G. (1968). Labile coat: reason for noninfectious cell-free varicella-zoster virus in culture. Journal of Virology 2, 1458-1464.

COOK, M.L., & STEVENS, J.G. (1970). Replication of varicella-zoster virus in cell culture: an ultrastructural study. Journal of Ultrastructure Research 32, 334-350.

CORDINGLY, M.G., CAMPBELL, M.E.M., & PRESTON, C.M. (1983). Functional analysis of a herpes simplex virus type 1 promoter: identification of far upstream regulatory sequences. Nucleic Acids Research 11, 2347-2365.

CORSALO, C.M. & PEARSON, M.L. (1981). Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. Somatic Cell Genetics 7, 603-616.

COSTANZO, F., CAMPADELLI-FIUME, G., FOA-TOMASI, L., & CASSAI, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. Journal of Virology 21, 996-1001.

COURTNEY, R.J., SCHAFFER, P.A., & POWELL, K.L. (1976). Synthesis of virus-specific polypeptides by temperature-sensitive mutants of herpes simplex virus type 1. Virology 75, 306-318.

COUSENS, D.J., GREAVES, R., GODING, C.R., & O'HARE, P. (1989). The C-terminal 79 amino acids of the herpes simplex virus regulatory protein, Vmw65, efficiently activate transcription in yeast and mammalian cells in chimeric DNA-binding proteins. EMBO Journal 8, 2337-2342.

CROEN, K.D., OSTROVE, J.M., DRAGOVIC, L.J., SMIALEK, J.E., & STRAUS, S.E. (1987). Latent herpes simplex virus in human trigeminal ganglia. Detection of an immediate early gene "anti sense" transcript by in situ hybridization. New England Journal of Medicine 317, 1427-1432.

CROEN, K.D., OSTROVE, J.M., DRAGOVIC, L.J., & STRAUS, S.E. (1988). Patterns of gene expression and sites of latency in human nerve ganglia are different for varicella-zoster and herpes simplex viruses. Proceedings of the National Academy of Sciences (USA) 85, 9773-9777.

CROMLISH, W.A., ABMAYR, S.M., WORKMAN, J.L., HORIKOSHI, M., & ROEDER, R.G. (1989). Transcriptionally active immediate-early protein of pseudorabies virus binds to specific sites on class II gene promoters. Journal of Virology 63, 1869-1876.

DALRYMPLE, M.A., McGEOCH, D.J., DAVISON, A.J., & PRESTON, C.M. (1985). DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate-early promoters. Nucleic Acids Research 13, 7865-7879.

DAVIDSON, I., & STOW, N.D. (1985). Expression of an immediate-early polypeptide and activation of a viral origin of DNA replication in cells containing a fragment of herpes simplex virus DNA. Virology 141, 77-88.

DAVISON, A.J. (1984). Structure of the genome termini of varicella-zoster virus. Journal of General Virology 65, 1969-1977.

DAVISON, A.J. (1991). Varicella-zoster virus. (The Fourteenth Fleming Lecture). Journal of General Virology, in press.

DAVISON, A.J., EDSON, C.M., ELLIS, R.W., FORGHANI, B., GILDEN, D., GROSE, C., KELLER, P.M., VAFAI, A., WROBLEWSKA, Z., & YAMANISHI, K. (1986). New common nomenclature for glycoprotein genes of varicella-zoster virus and their glycosylated products. Journal of Virology 57, 1195-1197.

DAVISON, A.J., & SCOTT, J.E. (1983). Molecular cloning of the varicella-zoster virus genome and derivation of six restriction endonuclease maps. Journal of General Virology 64, 1811-1814.

DAVISON, A.J., & SCOTT, J.E. (1986). The complete DNA sequence of varicella zoster virus. Journal of General Virology 67, 1759-1816.

DAVISON, A.J., WATERS, D.J., & EDSON, C.M. (1985). Identification of a varicella-zoster glycoprotein gene. Journal of General Virology 66, 2237-2242.

DAVISON, A.J., & WILKIE, N.M. (1981). Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. Journal of General Virology 55, 315-331.

DAVISON, A.J., & WILKIE, N.M. (1983). Location and orientation of homologous sequences in the genomes of five herpesviruses. Journal of General Virology 64, 1927-1942.

DAVISON, B.L., EGLY, J-M., MULVIHILL, E.R., & CHAMBON, P. (1983). Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter seqeunces. Nature 301, 680-686.

DAVISON, M-J., PRESTON, V.G., & McGEOCH, D.J. (1984). Determination of the sequence alteration in the DNA of the herpes simplex virus type 1 temperature-sensitive mutant *ts*K. Journal of General Virology 65, 859-863.

DEATLY, A.M., SPIVACK, J.G., LAVI, E., & FRASER, N.W. (1987). RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. Proceedings of the National Academy of Sciences (USA) 84, 3204-3208.

DeLUCA, N.A., COURTNEY, M.A., & SCHAFFER, P.A. (1984). Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. Journal of Virology 52, 767-776.

DeLUCA, N.A., & SCHAFFER, P.A. (1985). Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Molecular and Cellular Biology 5, 1997-2008.

DeLUCA, N.A., & SCHAFFER, P.A. (1987). Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides. Nucleic Acids Research 11, 4491-4511.

DeLUCA, N.A., & SCHAFFER, P.A. (1988). Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. Journal of Virology 62, 732-743.

DiDONATO, J.A., & MULLER, M.T. (1989). DNA binding and gene regulation by the herpes simplex virus type 1 protein ICP4 and involvement of the TATA element. Journal of Virology 63, 3737-3747.

DIGNAM, J.D., LEBOVITZ, R.M., & ROEDER, R.G. (1983). Accurate transcription initiation by RNA polymerse II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Research 11, 1475-1489.

DIXON, R.A.F., & SCHAFFER, P.A. (1980). Fine structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding herpes simplex virus type 1 immediate early protein VP175. Journal of Virology 36, 189-203.

DOBERSON, M.J., JERKOFSKY, M., & GREER, S. (1976). Enzymatic basis for the selective inhibition of varicella-zoster virus by 5-halogenated analogues of deoxycytidine. Journal of Virology 20, 478-486.

DOLIN, R., REICHMAN, R.C., MAZUR, M.H., & WHITLEY, R.J. (1978). Herpes zoster-varicella infections in immunosuppressed patients. Annals of Internal Medicine **89**, 375-388.

DUMAS, A.M., GEELEN, J.L.M.C., MARIS, W., & VAN DER NOORDA, J. (1980). Infectivity and molecular weight of varicella-zoster virus DNA. Journal of General Virology **47**, 233-235.

DUMAS, A.M., GEELEN, J.L.M.C., WESTSTRATE, M.W., WERTHERM, P., & VAN DER NOORDA, J. (1981). *Xba*I, *Pst*I, and *Bgl*II restriction maps of the two orientations of the varicella-zoster virus genome. Journal of Virology **39**, 390-400.

DUSH, M.K., SIKELA, J.M., KHAN, S.A., TISCHFIELD, J.A., & STAMBROOK, P.J. (1986). Nucleotide sequence and organization of the mouse adenine phosphoribosyltransferase gene: Presence of a coding region common to animal and bacterial phosphoribosyltransferases that has a variable intron/exon arrangement. Proceedings of the National Academy of Sciences (USA) **82**, 2731-2735.

DYNAN, W.S., & TJIAN, R. (1985). Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature **316**, 774-778.

ECKER, J.R., & HYMAN, R.W. (1982). Varicella-zoster virus DNA exists as two isomers. Proceedings of the National Academy of Sciences (USA) **79**, 156-160.

EISENBERG, S.P., COEN, D.M., & McKNIGHT, S.L. (1985). Promoter domains required for expression of plasmid-borne copies of the herpes simplex virus thymidine kinase gene in virus-infected mouse fibroblasts and microinjected frog oocytes. Molecular and Cellular Biology 5, 1940-1947.

EISENMANN, D.M., DOLLARD, C., & WINSTON, F. (1989). *SPT15*, the gene encoding the yeast TATA binding factor TFIID, is required for normal transcription initiation in vivo. Cell **58**, 1183-1191.

ElKAREH, A., MURPHY, A.J.M., FICHTER, T., ESTRATIADIS, A., & SILVERSTEIN, S. (1985). "Transactivation" control signals in the promoter of the herpesvirus thymidine kinase gene. Proceedings of the National Academy of Sciences (USA) **82**, 1002-1006.

ELLIS, R.W., KELLER, P.M., LOWE, R.S., ZIVIN, R.A. (1985). Use of a bacterial expression vector to map the varicella-zoster virus major glycoprotein gene, gC. Journal of Virology **53**, 81-88.

EPSTEIN, M.A. (1962). Observations on the fine structure of mature herpes simplex virus and on the composition of its nucleoid. Journal of Experimental Medicine **115**, 1-9.

EVERETT, R.D. (1983). DNA sequence elements required for regulated expression of the HSV-1 glycoprotein D gene lie within 83bp of the RNA capsites. Nucleic Acids Research **11**, 6647-6666.

EVERETT, R.D. (1984a). A detailed analysis of an HSV-1 early promoter: Sequences involved in trans-induction by viral immediate-early gene products are not early-gene specific. Nucleic Acids Research **12**, 3037-3056.

EVERETT, R.D. (1984b). Transactivation of transcription by herpesvirus products. Requirements for two HSV-1 immediate-early polypeptides for maximum activity. The EMBO Journal 3, 3135-3141.

EVERETT, R.D. (1985). Activation of cellular promoters during herpes virus infection of biochemically transformed cells. EMBO Journal **4**, 1973-1980.

EVERETT, R.D. (1986). The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1,2 and 3 can activate HSV-1 gene expression in *trans*. Journal of General Virology **67**, 2507-2513.

EVERETT, R.D. (1987). A detailed analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. The EMBO Journal **6**, 2069-2076.

EVERETT, R.D. (1988). Promoter sequence and cell type can dramatically affect the efficiency of transcriptional activation induced by herpes simplex virus type 1 and its immediate-early gene products Vmw175 and Vmw110. Journal of Molecular Biology **203**, 739-751.

EVERETT, R.D. (1989). Construction and characterisation of herpes simplex virus type 1 mutants with defined lesions in immediate-early gene 1. Journal of General Virology **70**, 1185-1202.

EVERETT, R.D., BATY, D., & CHAMBON, P. (1983). The repeated GC-rich motifs upstream from the TATA box are important elements of the SV40 early promoter. Nucleic Acids Research **11**, 2447-2464.

EVERETT, R.D., & DUNLOP,M. (1984). Transactivation of plasmid borne promoters by adenovirus and several herpes group viruses. Nucleic Acids Research **12**, 5969-5978.

EVERETT, R.D., & ORR, A. (1991). The Vmw175 binding site in the IE-1 promoter has no apparent role in the expression of Vmw110 during herpes simplex virus type 1 infection. Virology, in press.

EVERETT, R.D., PATERSON, T., & ELLIOT, M. (1990). The major transcriptional regulatory protein of herpes simplex virus type 1 includes a protease resistant DNA binding domain. Nucleic Acids Research **18**, 4579-4585.

FABER, S.W., & WILCOX, K.W. (1986a). Characterization of a herpes simplex virus regulatory protein: Aggregation and phosphorylation of a temperature sensitive variant of ICP4. Archives of Virology **91**, 297-312.

FABER, S.W., & WILCOX, K.W. (1986b). Association of the herpes simplex regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Research **14**, 6067-6083.

FABER, S.W., & WILCOX, K.W. (1988). Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. Nucleic Acids Research **16**, 555-570.

FELDMAN, L., RIXON, F.J., JEAN, J-H., BEN-PORAT, T., & KAPLAN, A.S. (1979). Transcription of the genome of pseudorabies virus (a herpesvirus) is strictly controlled. Virology **97**, 316-327.

FELSER, J.M., STRAUS, S.E., & OSTROVE, J.M. (1987). Varicella zoster virus complements herpes simplex virus type 1 temperature sensitive mutants. Journal of Virology **61**, 225-228.

FELSER, J.M., KINCHINGTON, P.R., INCHAUSPE, G., STRAUS, S.E., & OSTROVE, J.M. (1988). Cell lines containing varicella zoster virus open reading frame 62 and expressing the "IE" 175 protein complement ICP4 mutants of herpes simplex virus type 1. Journal of Virology **62**, 2076-2082.

FENNER, F. (1948). The pathogenesis of acute exanthums: An interpretation based on experimental investigations with mousepox (infectious ectromelia of mice). Lancet **ii**, 915-920.

FENWICK, M.L., & CLARK, J. (1983). The effect of cycloheximide on the accumulation and stability of functional α-mRNA in cells infected with herpes simplex virus. Journal of General Virology **64**, 1955-1963.

FIRE, A., SAMUELS, M., & SHARP, P.A. (1984). Interactions between RNA polymerase II, factors, and template leading to accurate transcription. Journal of Biological Chemistry **259**, 2509-2516.

FLETCHER, C., HEINTZ, N., & ROEDER, R.G. (1987). Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2B gene. Cell **51**, 773-781.

FLORES, O., HA, I., & REINBERG, D. (1990). Factors involved in specific transcription by mammalian RNA polymerase II. Purification and subunit composition of transcription factor IIF. Journal of Biological Chemistry **265**, 5629-5634.

FLORES, O., MALDONADO, E., & REINBERG. (1988). Factors involved in specific transcription by mammalian RNA polymerase II. RNA polymerase II-associating protein 30 is an essential component of transcription factor IIF. Journal of Biological Chemistry **263**, 10812-10816.

FLORES, O., MALDONADO, E., & REINBERG, D. (1989). Factors involved in spcific transcription by mammalian RNA polymerase II. Factors IIE and IIF independantly interact with RNA polymerase II. Journal of Biological Chemistry **264**, 8913-8921.

FRASER, N.W., LAWRENCE, W.C., WROBLEWSKA, Z., GILDEN, D.H., & KOPROWSKI, H. (1981). Herpes simplex type 1 DNA in human brain tissue. Proceedings of the National Academy of Sciences (USA) **78**, 6461-6465.

FREEMAN, J.M., & POWELL, K.L. (1982). DNA-binding properties of a herpes simplex virus immediate early protein. Journal of Virology **44**, 1084-1087.

FRENKEL, N., SCHIRMER, E.C., WYATT, L.S., KATSAFANAS, G., ROFFMAN, E., DANOVICH, R.M., & JUNE, C.H. (1990). Isolation of a new herpesvirus from human $CD4^+$ T cells. Proceedings of the National Academy of Sciences (USA) **87**, 748-752.

FURLONG, D., SWIFT, H., & ROIZMAN, B. (1972). Arrangement of herpesvirus deoxyribonucleic acid in the core. Journal of Virology **10**, 1071-1074.

GABEL, C., DUBEY, L., STEINBERG, S., GERSHON, M., & GERSHON, A. (1988). Mannose 6-phosphate (Man 6-P) in an envelope glycoprotein (GP) of varicella-zoster virus (VZV): Possible role in infectivity. Journal of Cellular Biochemistry [suppl] 12C, 33.

GAFFNEY, D.F., McLAUCHLAN, J., WHITTON, J.L., & CLEMENTS, J.B. (1985). A modular system for the assay of transcription regulatory signals: The sequence TAATGARAT is required for herpes simplex virus immediate-early gene activation. Nucleic Acids Research **13**, 7847-7863.

GELB, L.D. (1990). Varicella-zoster virus. In: "Virology" pp 2011-2054. Ed. B.N. Fields and D.M. Knipe *et al.* Raven press, New York.

GELMAN, I.H., & SILVERSTEIN, S. (1985). Identification of immediate-early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proceedings of the National Academy of Sciences, U.S.A. **82**, 5265-5269.

GELMAN, I.H., & SILVERSTEIN, S. (1986). Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate-early gene products. Journal of Molecular Biology **191**, 395-409.

GELMAN, I.H., & SILVERSTEIN, S. (1987a). Herpes simplex virus immediate-early promoters are responsive to virus and cell *trans*-acting factors. Journal of Virology **61**, 2286-2296.

GELMAN, I.H., & SILVERSTEIN, S. (1987b). Dissection of immediate-early gene promoters from herpes simplex virus: Sequences that respond to the virus transcriptional activators. Journal of Virology **61**, 3167-3172.

GERSHON, A., COSIO, L., & BRUNELL, P.A. (1973). Observations on the growth of varicella-zoster virus in human diploid cells. Journal of General Virology **18**, 21-31.

GERSTER, T., & ROEDER, R.G. (1988). A herpesvirus *trans*-acting protein interacts with transcription factor OTF-1 and other cellular proteins. Proceedings of the National Academy of Sciences (USA) **85**, 6347-6351.

GILDEN, D.H., SHTRAM, Y., FRIEDMANN, A., WELLISH, M., DEVLIN, M., FRASER, N., & BECKER, Y. (1982). The internal organization of the varicella-zoster virus genome. Journal of General Virology **60**, 371-374.

GILDEN, D.H., VAFAI, A., SHTRAM, Y., BECKER, Y., DEVLIN, M., & WELLISH, M. (1983). Varicella-zoster virus DNA in human sensory ganglia. Nature **306**, 478-480.

GOLD, E. (1965). Characerization of herpes zoster and varicella viruses propagated *in vitro*. Journal of Immunology **95**, 683-691.

GORDON, J.E. (1962). Chickenpox: an epidemiological review. American Journal of the Medical Sciences **244**, 362-389.

GORMAN, C.M., MOFFAT, L.F., & HOWARD, B.H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Molecular and Cellular Biology **2**, 1044-1051.

GREAVES, R.F., & O'HARE, P.O. (1989). Separation of requirements for protein-DNA complex assembly from those for functional activity in herpes simplex virus regulatory protein Vmw65. Journal of Virology **63**, 1041-1050.

GREAVES, R.F., & O'HARE, P.O. (1990). Structural requirements in the herpes simplex virus type 1 transactivator Vmw65 for interaction with the cellular octamer-binding protein and target TAATGARAT sequences. Journal of Virology **64**, 2716-2724.

GROSE, C. (1981). Variation on a theme by Fenner: The pathogenesis of chickenpox. Pediatrics **68**, 735-737.

GROSSCHEDL, R., & BIRNSTIEL, M.L. (1980). Identification of regulatory sequences in the prelude sequences of an H2A histone gene by the study of specific deletion mutants *in vivo*. Proceedings of the National Academy of Sciences (USA) **77**, 1432-1436.

GROSVELD, G.C., deBOER, E., SHEWMAKER, C.K., & FLAVELL, R.A. (1982). DNA sequences necessary for transcription of the rabbit β-globin gene *in vivo*. Nature **295**, 120-126.

GROSVELD, G.C., SHEWMAKER, C.K., JAT, P., & FLAVELL, R.A. (1981). Localization of DNA sequences necessary for transcription of the rabbit β-globin gene *in vitro*. Cell **25**, 215-226.

GRUNDY, F.J., BALIMAN, R.P., & O'CALLAGHAN, D.J. (1989). DNA sequence and comparative analyses of the equine herpesvirus type 1 immediate-early gene. Virology **172**, 223-236.

GUESS, H.A., BROUGHTON, D.D., MELTON, L.J. III, & KURLAND, L.T. (1986). Population based studies of varicella complications. Pediatrics **78**, 723-727.

HAHN, S., BURATOWSKI, S., SHARP, P.A., & GUARENTE, L. (1989a). Identification of a yeast protein homologous in function to the mammalian general transcription factor, TFIIA. EMBO Journal **8**, 3379-3382.

HAHN, S., BURATOWSKI, S., SHARP, P.A., & GUARENTE, L. (1989b). Isolation of the gene encoding the yeast TATA binding protein TFIID: A gene identical to the *SPT15* suppressor of Ty element insertions. Cell **58**, 1173-1181.

HAI, T., HORIKOSHI, M., ROEDER, R.G., & GREEN, M.R. (1988). Analysis of the role of the transcription factor ATF in the assembly of a functional preinitiation complex. Cell **54**, 1043-1051.

HAMPL, H., BEN-PORAT, T., ERLICHER, C., HABERMEHL, K-O, & KAPLAN, A. (1984). Characterization of the envelope proteins of pseudorabies virus. Journal of Virology **52**, 583-590.

HARRIS, R.A., EVERETT, R.D., XHU, X., & SILVERSTEIN, S. (1989). Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. Journal of Virology **63**, 3513-3515.

HARRIS-HAMILTON, E., & BACHENHEIMER, S.L. (1985). Accumulation of herpes simplex virus type 1 RNAs of different kinetic classes in the cytoplasm of infected cells. Journal of Virology **53**, 144-151.

HAY, R.T., & HAY, J. (1980). Properties of herpesvirus-induced "immediate early" polypeptides. Virology **104**, 230-234.

HAYAKAWA, Y., & HYMAN, R.W. (1987). Isomerization of the U_L region of varicella-zoster virus DNA. Virus Research **8**, 25-31.

HAYAKAWA, Y., YAMAMOTO, T., YAMANISHI, K., & TAKAHAHSI, M. (1986). Analysis of varicella-zoster virus DNAs of clinical isolates by endonuclease *Hpa*I. Journal of General Virology **67**, 1817-1829.

HEAD, H., & CAMPBELL, A.W. (1900). The pathology of herpes zoster and its bearing on sensory localization. Brain **23**, 353-523.

HO, M. (1982). Human cytomegalovirus infections in immunosuppressed patients. In: "Cytomegaloviruses, Biology and Infection, Current Topics in Infectious Disease." Eds. W.B. Greenough III and T.C. Menigan. Plenum press, New York.

HOEY, T., DYNLACHT, B.D., PETERSON, M.G., PUGH, B.F., & TJIAN, R. (1990). Isolation and characterization of the drosophila gene encoding the TATA box binding protein, TFIID. Cell **61**, 1179-1186.

HOFFMAN, A., HORIKOSHI, M., WANG, C.K., SCHROEDER, S., WEIL, P.A., & ROEDER, R.G. (1990). Cloning of the *schizosaccharomyces pombe* TFIID gene reveals a strong conservation of functional domains in *saccharomyces cerevisiae* TFIID. Genes and Development **4**, 1141-1148.

HOLLAND, L.E., ANDERSON, K.P., SHIPMAN, C. Jr., & WAGNER, E.K. (1980). Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. Virology **101**, 10-24.

HOLMES, S.M. & QUIGLEY, M. (1981). A rapid boiling method for the for the preparation of bacterial plasmids. Annals of Biochemistry **114**, 193-197.

HOMA, F.L., OTAL, T.M., GLORIOSO, J.C., & LEVINE, M. (1986). Transcription control signals of a herpes simplex virus type 1 late (gamma 2) gene lie within bases -34 to +124 relative to the 5' terminus of the mRNA. Molecular and Cellular Biology 6, 3652-3666.

HONESS, R.W., BODEMER, W., CAMERON, K.R., NILLER, H.H., FLECKENSTEIN, B., & RANDALL, R.E. (1986). The A+T rich genome of herpesvirus saimiri contains a highly conserved gene for thymidylate synthetase. Proceedings of the National Academy of Sciences (USA) 83, 3604-3608.

HONESS, R.W., & ROIZMAN, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. Journal of Virology 14, 8-19.

HOPE, I.A., & STRUHL, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell 46, 885-894.

HOPE, I.A., MAHADEVAN, S., & STRUHL, K. (1988). Structural and functional characterization of the short acidic transcriptional activation region of yeast GCN4 protein. Nature 333, 635-640.

HOPE-SIMPSON, R.E. (1965). The nature of herpes zoster: a long term study and a new hypothesis. Proceedings of the Royal Society of Medicine 58, 9-20.

HORIKOSHI, M., CAREY, M.F., KAKIDANI, H., & ROEDER, R.G. (1988a). Mechanism of action of a yeast activator: Direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. Cell 54, 665-669.

HORIKOSHI, M., HAI. T., LIN, Y-S., GREEN, M.R., & ROEDER, R.G. (1988b). Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell 54, 1033-1042.

HORIKOSHI, M., WANG, C.K., FUJII, H., CROMLISH, J.A., WEIL, P.A., & ROEDER, R.G. (1989). Cloning and structure of a yeast gene encoding a general transcription initiation factor TFIID that binds to the TATA box. Nature 341, 299-303.

HORIKOSHI, M., YAMAMOTO, T., OKUMA, Y., WEIL, P.A., & ROEDER, R.G. (1990). Analysis and structure-function relationships of yeast TATA box binding factor TFIID. Cell 61, 1171-1178.

HYMAN, R.W., ECKER, J.R., & TENSER, R.B. (1983). Varicella-zoster virus RNA in human trigeminal ganglia. Lancet ii, 814-816.

IHARA, S., FELDMAN, L., WATANABE, S., & BEN-PORAT, T. (1983). Characterization of the immediate-early functions of pseudorabies virus. Virology 131, 437-454.

IMBALZANO, A.N., SHEPARD, A.A., DeLUCA, N.A. (1989). Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promoter-regulatory domain of the viral thymidine kinase gene. Journal of Virology **64**, 2620-2631.

INCHAUSPE, G., NAGPAL, S., & OSTROVE, J.M. (1989). Mapping of two varicella zoster virus-encoded genes that activate the expression of viral early and late genes. Virology **173**, 700-709.

JEAN, J-H., BEN-PORAT, T., & KAPLAN, S. (1974). Early functions of the genome of herpesvirus. III. Inhibition of the transcription of the viral genome in cells treated with cycloheximide early during the infective process. Virology **59**, 516-523.

JOHNSON, P.A., & EVERETT, R.D. (1986a). DNA replication is required for abundant expression of a plasmid-borne late US11 gene of herpes simplex virus type 1. Nucleic Acids Research **14**, 3609-3625.

JOHNSON, P.A., & EVERETT, R.D. (1986b). The control of herpes simplex virus type 1 late gene transcription: a 'TATA-box'/capsite region is sufficient for fully efficient regulated activity. Nucleic Acids Research **14**, 8247-8264.

JONES, K.A., & TJIAN, R. (1985). Sp1 binds to promoter sequences and activates herpes simplex virus immediate-early gene transcription *in vitro*. Nature **317**, 179-182.

JONES, K.A., YAMAMOTO, K.R., & TJIAN, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42**, 559-572.

JONES, P.C., & ROIZMAN, B. (1979). Regulation of herpesvirus macromolecular synthesis. VIII. The transcription programme consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. Journal of Virology **31**, 299-314.

KAKIDANI, H., & PTASHNE, M. (1988). GAL4 activates gene expression in mammalian cells. Cell **52**, 161-167.

KALDERON, D., ROBERTS, B.L., RICHARDSON, W.D., & SMITH, A.E. (1984). A short amino acid sequence able to specify nuclear location. Cell **39**, 499-509.

KAO, C.C., LEIBERMAN, P.M., SCHMIDT, M.C., ZHOU, Q., PEI, R., & BERK, A.J. (1990). Cloning of a transcriptionally active human TATA binding factor. Science **248**, 1646-1650.

KAPLAN, A., ACHORD, D.T., & SLY, W.S. (1977). Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. Proceedings of the National Academy of Sciences 74, 2026-2030.

KATTAR-COOLEY, P., & WILCOX, K.W. (1989). Characterization of the DNA-binding properties of herpes simplex virus regulatory protein ICP4. Journal of Virology 63, 696-704.

KELLEHER III, R.J., FLANAGAN, P.M., & KORNBERG, R.D. (1990). A novel mediator between activator proteins and the RNA polymerase II transcriptional apparatus. Cell 61, 1209-1215.

KELLER, P.M., DAVISON, A.J., LOWE, R.S., BENNET, C.D., & ELLIS, R.W. (1986). Identification and structure of the gene encoding gpII, a major glycoprotein of varicella-zoster virus. Virology 152, 181-191.

KELLER, P.M., DAVISON, A.J., LOWE, R.S., REIMAN, M.W., & ELLIS, R.W. (1987). Identification and sequence of the gene encoding gpIII, a major glycoprotein of varicella-zoster virus. Virology 157, 526-533.

KEMP, L.M., & LATCHMAN, D.S. (1988a). Differential regulation of octamer-containing cellular genes by the herpes simplex virus virion protein Vmw65 is mediated by sequence differences in the octamer element. EMBO Journal 7, 4239-4244.

KEMP, L.M., & LATCHMAN, D.S. (1988b). Induction and repression of cellular gene transcription during herpes simplex virus infection is mediated by different viral immediate-early gene products. European Journal of Biochemistry 174, 443-449.

KENNEDY, P.G.E. (1987). Neurological complications of varicella-zoster virus. In: "Infections of the Nervous System", pp 177-208. Eds. P.G.E. Kennedy and R.J. Johnson. Butterworths.

KINCHINGTON, P.R., INCHAUSPE, G., SUBAK-SHARPE, J.H., ROBEY, F., HAY, J., & RUYECHAN, W.T. (1988). Identification and characterization of a varicella-zoster virus DNA-binding protein by using antisera directed against a predicted synthetic oligopeptide. Journal of Virology 62, 802-809.

KINCHINGTON, P.R., REINHOLD, W.C., CASEY, T.A., STRAUS, S.E., HAY, J., & RUYECHAN, W.T. (1985). Inversion and circularization of the varicella-zoster virus genome. Journal of Virology 56, 194-200.

KINCHINGTON, P.R., REMENICK, J., OSTROVE, J.M., STRAUS, S.E., RUYECHAN, W.T., & HAY, J. (1986). Putative glycoprotein gene of varicella-zoster virus with variable copy numbers of a 42 bp repeat sequence has homology to herpes simplex virus glycoprotein C. Journal of Virology 59, 660-668.

KINOSHITA, H., HONDO, R., TAGUCHI, F., & YOGO, Y. (1988). Variation of R1 repeated sequence present in open reading frame 11 of varicella-zoster virus strains. Journal of Virology 62, 1097-1100.

KISHI, M., HARADA, H., TAKAHASHI, M., TANAKA, A., HAYASHI, M., NONOYAMA, M., JOSEPHS, S.F., BUCHNINDER, A., SCHACHTER, F., ABLASHI, D.V., WONG-STAHL, F., SALAHUDDIN, S.K., & GALLO, R.C. (1988). A repeat sequence, GGGTTA, is shared by DNA of human herpesvirus 6 and Marek's disease virus. Journal of Virology 62, 4284-4287.

KNIPE, D.M., BATTERSON, W., NOSAL, C., ROIZMAN, B., & BUCHAN, A. (1981). Molecular genetics of herpes simplex virus. VI. Characterization of a temperature-sensitive mutant defective in the expression of all early viral gene products. Journal of Virology 38, 539-547.

KNIPE, D.M., SENECHEK, D., RICE, S.A., & SMITH, J.L. (1987). Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. Journal of Virology 61, 276-284.

KNIPE, D.M., & SMITH, J.L. (1986). A mutant herpesvirus protein leads to a block in nuclear localization of other viral proteins. Molecular and Cellular Biology 6, 2371-2381.

KRISTIE, T.M., LeBOWITTZ, J.H., & SHARP, P.A. (1989). The octamer-binding proteins form multi-protein-DNA complexes with the HSV α-TIF regulatory protein.

KRISTIE, T.M., & ROIZMAN, B. (1984). Separation of sequences defining basal expression from those conferring α gene recognition within the regulatory domains of herpes simplex virus 1 α genes. Proceedings of the National Academy of Sciences (USA) 81, 4065-4069.

KRISTIE, T.M., & ROIZMAN, B. (1986a). α4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of α genes and of selected other viral genes. Proceedings of the National Academy of Sciences (USA) 83, 3218-3222.

KRISTIE, T.M., & ROIZMAN, B. (1986b). DNA-binding site of major regulatory protein α4 specifically associated with promoter-regulatory domains of genes of herpes simplex virus type 1. Proceedings of the National Academy of Sciences (USA) 83, 4700-4704.

KWONG, A.D., KRUPER, J.A., & FRENKEL, N. (1988). HSV virion host shutoff function. Journal of Virology 62, 912-921.

LaMARCO, K.L., & McKNIGHT, S.L. (1989). Purification of a set of cellular polypeptides that bind to the purine-rich *cis*-regulatory element of herpes simplex virus immediate-early genes. Genes and Development 3, 1372-1383.

LANDAU, N.R., St.JOHN, T.P., WEISSMAN, I.L., WOLF, S.C., SILVERSTONE, A.E., & BALTIMORE, D. (1984). Cloning of terminal transferase by antibody screening. Proceedings of the National Academy of Sciences (USA) 81, 5836-5840.

LANG, J.C., SPANDIDOS, D.A., & WILKIE, N.M. (1984). Transcriptional regulation of a herpes simplex virus immediate-early gene is mediated through an enhancer-type sequence. EMBO Journal 3, 389-395.

LATCHMAN, D.S., ESTRIDGE, J.K., & KEMP, L.M. (1987). Transcriptional induction of the ubiquitin gene during herpes simplex virus infection is dependant upon the viral immediate-early protein ICP4. Nucleic Acids Research 15, 7283-7293.

LAWRENCE, G.L., CHEE, M., CRAXTON, M.A., GOMPELS, U.A., HONESS, R.W., & BARRELL, B.G. (1990). Human herpes virus 6 is closely related to human cytomegalovirus. Journal of Virology 64, 287-299.

LECH, K., ANDERSON, K., & BRENT, R. (1988). DNA-bound fos proteins activate transcription in yeast. Cell 52, 179-184.

LEWIS, M.K., & BURGESS, R.R. (1982). Eukaryotic RNA polymerases. In: The Enzymes, vol XV, pp 109-153. Ed. P.D. Boyer. Academic Press, New York.

LIN, Y-S., CAREY, M.F., PTASHNE, M., & GREEN, M.R. (1988). GAL4 derivatives function alone and in synergy with mammalian activators in vitro. Cell 54, 659-664.

LONGNECKER, R., & ROIZMAN, B. (1986). Generation of an inverting herpes simplex virus 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α47 gene. Journal of Virology 58, 583-591.

LOPETEGUI, P., COMPO-VERA, H., & YAMANICHI, K. (1985). Varicella-zoster virus (VZV)-specific polypeptides detected in cells treated with metabolic inhibitors. Micobiology and Immunology **29**, 569-575.

LOPEZ, C., PELLET, P., STEWART, J., GOLDSMITH, C., SANDERLIN, K., BLACK, J., WARFIELD, D., & FEORINO, P. (1988). Characteristics of human herpesvirus-6. Journal of Infectious Diseases **157**, 1271-1273.

LUBY, J.P., RAMIREZ-RONDA, C., RINNER, S., HULL, A., & VERGNE-MARINI, P. (1977). A longitudinal study of varicella-zoster virus infections in renal transplant recipients. Journal of Infectious Diseases **135**, 659-663.

LUDWIG, H., HAINES, H.G., BISWAL, N., & BENYESH-MELNICK, M. (1972). The characterization of varicella-zoster virus DNA. Journal of General Virology **14**, 111-114.

MacPHERSON, I., & STOKER, M. (1962). Polyoma transformation of hamster cell clones-an investigation of genetic factors affecting cell competence. Virology **16**, 147-151.

McCARTHY, A.M., McMAHAN, L., & SCHAFFER, P.A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. Journal of Virology **63**, 18-27.

McGEOCH, D.J., DALRYMPLE, M.A., DAVISON, A.J., DOLAN, A., FRAME, M.C., McNAB, D., PERRY, L.J., SCOTT, J.E., & TAYLOR, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. Journal of General Virology **69**, 1531-1574.

McGEOCH, D.J., DOLAN. J.A., DONALD, S., & BRAUER, D.H.K. (1986). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Research **14**, 1727-1764.

McKEE, T.A., DISNEY, G.H., EVERETT, R.D., & PRESTON, C.M. (1990). Control of expression of the varicella-zoster virus major immediate early gene. Journal of General Virology **71**, 897-906.

McKNIGHT, J.L.C., KRISTIE, T.M., & ROIZMAN, B. (1987). Binding of the virion protein mediating alpha gene induction in herpes simplex 1-infected cells to its *cis* site requires cellular proteins. Proceedings of the National Academy of Sciences (USA) **84**, 7061-7065.

McKNIGHT, S.L., GAVIS, E.R., KINGSBURY, R., & AXEL, R. (1981). Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell **25**, 385-398.

McKNIGHT, S.L., & KINGSBURY, R. (1982). Transcription control signals of a eukaryotic protein coding gene. Science **217**, 316-324.

MA, J. & PTASHNE, M. (1987a). Deletion analysis of GAL4 defines two transcriptional activating segments. Cell **48**, 847-853.

MA, J., & PTASHNE, M. (1987b). A new class of yeast transcriptional activators. Cell **51**, 113-119.

MACKEM, S., & ROIZMAN, B. (1982a). Differentiation between α promoter and regulatory regions of herpes simplex virus 1: The functional domains and sequence of a movable α regulator. Proceedings of the National Academy of Sciences (USA) **79**, 4917-21.

MACKEM, S., & ROIZMAN, B. (1982b). Regulation of α genes of herpes simplex virus: The α27 gene promoter-thymidine kinase chimera is positively regulated in converted L cells. Journal of Virology **43**, 1015-1023.

MACKEM, S., & ROIZMAN, B. (1982c). Structural features of herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. Journal of Virology **44**, 939-949.

MAGUIRE, H.F., & HYMAN, R.W. (1986). Polyadenylated cytoplasmic transcripts of varicella-zoster virus. Intervirology **26**, 181-191.

MANIATIS, T., GOODBOURN, S., & FISCHER, J.A. (1987). Regulation of inducible and tissue-specific gene expression. Science **236**, 1237-1245.

MAR, E-C., HUANG, Y-S., HUANG, E-S. (1978). Purification and characterization of varicella-zoster virus-induced DNA polymerase. Journal of Virology **26**, 249-256.

MARSDEN, H.S., CAMPBELL, M.E.M., HAARR, L., FRAME, M.C., PARRIS, P.S., MURPHY, M., HOPE, R.G., MULLER, M.T., & PRESTON, C.M. (1987). The 65,000-M_r DNA binding and *trans*-inducing proteins of herpes simplex virus type 1. Journal of Virology **61**, 2428-2437.

MARSDEN, H.S., CROMBIE, I.K., & SUBAK-SHARPE, J.H. (1976). Control of protein synthesis in herpes virus-infected cells: analysis of the polypeptides induced by wild-type and sixteen temperature-sensitive mutants of strain 17. Journal of General Virology **31**, 347-372.

MARSDEN, H.S., STOW, N.D., PRESTON, V.G., TIMBURY, M.C., & WILKIE, N.M. (1978). Physical mapping of herpes simplex virus-induced polypeptides. Journal of Virology **28**, 624-642.

MARTIN, J.H., DOHNER, D.E., WELLINGHOF, W.J., & GELB, L.D. (1982). Restriction endonuclease analysis of varicella-zoster vaccine virus and wild type DNAs. Journal of Medical Virology 9, 69-76.

MATHIS, D.J., & CHAMBON, P. (1981). The SV40 early region TATA box is required for accurate *in vitro* initiation of transcription. Nature **290**, 310-315.

MATSUI, T., SEGALL, J., WEIL, P.A., & ROEDER, R.G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. Journal of Biological Chemistry **255**, 11992-11996.

MATTHEWS, R.E.F. (1982). Classification and nomenclature of viruses. Fourth report of the International Comittee on Taxonomy of Viruses. Intervirology **17**, 1-199.

MAVROMARA-NAZOS, P., & ROIZMAN, B. (1987). Activation of herpes simplex virus 1 gamma 2 genes by viral DNA replication. Virology **161**, 593-598.

MAVROMARA-NAZOS, P., SILVER, S., HUBENTHALL-VOSS, J., McKNIGHT, J.L.C., & ROIZMAN, B. (1986). Regulation of herpes simplex virus 1 genes: α gene sequence requirements for transient induction of indicator genes regulated by β or late (gamma 2) promoters. Virology **149**, 152-164.

MAXAM, A.M., & GILBERT, W. (1977). A new method for sequencing DNA. Proceedings of the National Academy of Sciences (USA) **74**, 560-564.

MAXAM, A.M., & GILBERT, W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. Methods in Enzymology 65, 499-560.

MESSING, J. (1979). A multipurpose cloning system based on a single stranded DNA bacteriophage M13. Recombinant DNA Technical Bulletin 2, 43-48.

METZLER, D.W., & WILCOX, K.W. (1985). Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. Journal of Virology 55, 329-337.

MICHAEL, N., & ROIZMAN, B. (1989). Binding of the herpes simplex virus major regulatory protein to viral DNA. Proceedings of the National Academy of Sciences (USA) **86**, 9808-9812.

MICHAEL, N., SPECTOR, D., MAVROMARA-NAZOS, P., KRISTIE, P.M., & ROIZMAN, B. (1988). The DNA binding properties of the major regulatory protein alpha 4 of herpes simplex virus. Science 239, 1531-1534.

MILLER, G. (1985). Epstein-Barr virus. In: "Virology". Ed. B.N. Fields. Raven press, New York.

MILLER, R.L., & RAPP, F. (1977). Varicella-zoster virus-induced DNA polymerase. Journal of General Virology **36**, 515-524.

MISHRA, L., DOHNER, D.E., WELLINGHOFF, W.J., & GELB, L.D. (1984). Physical maps of varicella-zoster virus DNA derived with 11 restriction enzymes. Journal of Virology **50**, 615-618.

MITTNACHT, S., STRAUB, P., KIRCHNER, H., & JACOBSEN, H. (1988). Interferon treatment inhibits onset of herpes simplex virus immediate-early transcription. Virology **164**, 201-210.

MONTALVO, E.A., & GROSE, C. (1987). Assembly and processing of the disulfide linked varicella-zoster virus glycoprotein gpII (140). Journal of Virology **61**, 2877-2884.

MOCSCA, J.D., REYES, G.R., PITHA, P.M., & HAYWARD, G.S. (1985). Differential activation of hybrid genes containing herpes simplex virus immediate-early or delayed-early promoters after superinfection of stable DNA-transfected cell lines. Journal of Virology **56**, 867-78.

MULLANEY, J., McL.MOSS, H.W., & McGEOCH, D.J. (1989). Gene U_L 2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. Journal of General Virology **70**, 449-452.

MULLER, M.T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. Journal of Virology **61**, 858-865.

MYERS, M.G., DUER, H.L., & HAUSLER, C.K. (1980). Experimental infection of guinea pigs with varicella zoster virus. Journal of Infectious Diseases **142**, 414-420.

NAKAJIMA, N., HORIKOSHI, M., & ROEDER, R.G. (1988). Factors involved in specific transcription by mammalian RNA polymerase II: Purification, genetic specificity, and TATA box-promoter interactions of TFIID. Molecular and Cellular Biology **8**, 4028-4040.

NALBANTOGLU, J., PHEAR, G.A., & MEUTH, M. (1986). Nucleotide sequence of hamster adenine phosphoribosyl transferase (aprt) gene. Nucleic Acids Research **14**, 1914.

NAZERIAN, K. (1974). DNA configuration in the core of Marek's Disease virus. Journal of Virology **13**, 1148-1150.

NEIDERMAN, J., MILLER, G., PEARSON, H., & PAGANO, J. (1976). Patterns of excretion of Epstein-Barr virus in saliva and other oropharyngeal sites during infectious mononucleosis. New England Journal of Medicine 294, 1335-1359.

NORRANDER, J., KEMPE, T.E., & MESSING, J. (1983). Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene 26, 101-106.

NORRIS, K., NORRIS, F., CHRISTIANSEN, L., & FIIL, N. (1983). Efficient site-directed mutagenesis by simultaneous use of two primers. Nucleic Acids Research 11, 5103-5112.

O'HARE, P., & GODING, C.R. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell 52, 435-445.

O'HARE, P., GODING, C.R., & HAIGH, A. (1988). Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of immediate-early gene expression. EMBO Journal 7, 4231-4238.

O'HARE, P., & HAYWARD, G.S. (1984). Expression of recombinant genes containing herpes simplex virus delayed-early and immediate-early regulatory regions and *trans*-activation by herpesvirus infection. Journal of Virology 52, 522-531.

O'HARE, P., & HAYWARD, G.S. (1985a). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway invoving positive and negative feedback regulation. Journal of Virology 56, 723-733.

O'HARE, P., & HAYWARD, G.S. (1985b). Evidence for a direct role for both the 175,000 and 110,000 molecular weight immediate early proteins of herpes simplex virus in the transactivation of delayed early promoters. Journal of Virology 53, 751-760.

O'HARE, P., & HAYWARD, G.S. (1987). Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus encoded trans-acting factors. Journal of Virology 61, 190-199.

OSTROVE, J.M., REINHOLD, W., FAN, C., ZORN, S., HAY, J., & STRAUS, S.E. (1985). Transcription mapping of the varicella-zoster virus genome. Journal of Virology 56, 600-606.

PANNING, B., & SMILEY, J.R. (1989). Regulation of cellular genes transduced by herpes simplex virus. Journal of Virology 63, 1929-1937.

PATERSON, T., & EVERETT, R.D. (1988a). Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. Virology **156**, 186-196.

PATERSON, T., & EVERETT, R.D. (1988b). The regions of the herpes simplex virus type 1 immediate-early protein Vmw175 required for the site specific DNA binding closely correspond to those involved in transcriptional regulation. Nucleic Acids Research **16**, 11005-11025.

PATERSON, T., & EVERETT, R.D. (1990). A prominent serine-rich region in Vmw175, the major transcriptional regulator of herpes simplex virus type 1, is not essential for virus growth in tissue culture. Journal of General Virology **71**, 1775-1783.

PATERSON, T., PRESTON, V.G., & EVERETT, R.D. (1990). A mutant of herpes simplex virus type 1 immediate early polypeptide Vmw175 binds to the cap site of its own promoter *in vitro* but fails to autoregulate *in vivo*. Journal of General Virology **71**, 851-861.

PEDERSDEN, M., TALLEY-BROWN, S., & MILLETTE, R.L. (1981). Gene expression of herpes simplex virus. III. Effect of arabinosyladenine on viral polypeptide synthesis. Journal of Virology **38**, 712-719.

PELLET, P.E., McKNIGHT, J.L.C., JENKINS, F.J., & ROIZMAN, B. (1985). Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of *trans*-inducing α-genes. Proceedings of the National Academy of Sciences (USA) **82**, 5870-5874.

PEREIRA, L., WOLFF, M.H., FENWICK, M., & ROIZMAN, B. (1977). Regulation of herpesvirus macromolecular synthesis. V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**, 733-749.

PERRY, L.J., RIXON, F.J., EVERETT, R.D., FRAME, M.C., & McGEOCH, D.J. (1986). Characterisation of the IE 110 gene of herpes simplex virus type 1. Journal of General Virology **67**, 2365-2380.

PERSSON, R.H., BACCHETTI, S., & SMILEY, J.R. (1985). Cells that constitutively express the herpes simplex virus immediate early protein ICP4 allow efficient activation of viral delayed-early genes in *trans*. Journal of Virology **54**, 414-421.

PETERSON, M.G., TANESE, N., PUGH, F., & TJIAN, R. (1990). Functional domains and upstream activation properties of cloned human TATA binding protein. Science **248**, 1625-1630.

PIZER, L.I., TEDDER, D.G., BETZ, J.L., WILCOX, K.W., & BEARD, P. (1986). Regulation of transcription in vitro from herpes simplex virus genes . Journal of Virology **60**, 950-959.

POST, L.E., MACKEM, S., & ROIZMAN, B. (1981). Regulation of α genes of herpes simplex virus: Expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell **24**, 555-565.

POST, L.E., & ROIZMAN, B. (1981). A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25**, 277-232.

POWELL, K.L., & PURIFOY, D.J.M. (1976). DNA-binding proteins of cells infected by herpes simplex virus type 1 and type 2. Intervirology **7**, 225-239.

POWELL, K.L., PURIFOY, D.J.M., & COURTNEY, R.J. (1975). The synthesis of herpes simplex virus proteins in the absence of viral DNA synthesis. Biochemistry and Biophysics Research Communications **66**, 262-271.

PRESTON, C.M. (1979a). Control of herpes simplex type 1 mRNA synthesis in cells infected with wild-type virus or the temperature sensitive mutant *ts*K. Journal of Virology **29**, 275-284.

PRESTON, C.M. (1979b). Abnormal properties of an immediate-early polypeptide in cells infected with the herpes simplex virus type 1 mutant *ts*K. Journal of Virology **32**, 357-369.

PRESTON, C.M., CORDINGLY, M.G., & STOW, N.D. (1984). Analyses of DNA sequences which regulate the transcription of a herpes simplex virus immediate-early gene. Journal of Virology **50**, 708-716.

PRESTON, C.M., FRAME, M.C., & CAMPBELL, M.E.M. (1988). A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate-early gene regulatory DNA sequence. Cell **52**, 425-434.

PRESTON, C.M., & NOTARIANNI, E.L. (1983). Poly(ADP-ribosyl)ation of a herpes simplex virus immediate early polypeptide. Virology **131**, 492-501.

PRESTON, C.M., & TANNAHILL, D. (1984). Effects of orientation and position on the activity of a herpes simplex virus immediate-early gene far-upstream region. Virology **137**, 439-444.

PRESTON, V.G., DAVISON, A.J., MARSDEN, H.S., TIMBURY, M.C., SUBAK-SHARPE, J.H., & WILKIE, N.M. (1978). Recombinants between herpes simplex virus types 1 and 2: analyses of genome structures and expression of immediate early polypeptides. Journal of Virology **28**, 499-517.

PRESTON, V.G., & FISHER, F.B. (1984). Identification of the herpes simplex virus type 1 gene encoding the dUTPase. Virology **138**, 58-68.

PROVOST, P.J., KELLER, P.M., BANKER, F.S., KEECH, B.J., KLEIN, H.J., LOWE, R.S., MORTON, P.H., PHELPS, A.H., McALEER, W.J., & ELLIS, R.W. (1987). Successful infection of the common marmoset (*Callitrix jaccus*) with human varicella-zoster virus. Journal of Virology **61**, 2951-2955.

PRUIJN, G.J.M., VAN DRIEL, W., & VAN DER VLIET, D.C. (1986). Nuclear factor III, a novel sequence specific DNA-binding protein from HeLa cells stimulating adenovirus DNA replication. Nature **322**, 656-659.

PTASHNE, M. (1988). How eukaryotic transcriptional activators work. Nature **335**, 683-689.

PUGH, B.F., & TJIAN, R. (1990). Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell **61**, 1187-1197.

QUINLAN, M.P., CHEN, L.B., & KNIPE, D.M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36**, 857-868.

QUINLAN, M.P., & KNIPE, D.M. (1985). Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Molecular and Cellular Biology **5**, 957-963.

RAKUSANOVA, T., BEN-PORAT, T., HIMENO, M., & KAPLAN, A.S. (1971). Early functions of the genome of herpesvirus. I. Characterization of the RNA synthesized in cycloheximide-treated, infected cells. Virology **46**, 877-889.

RANDALL, R.E., & DINWOODIE, N. (1986). Intranuclear localization of herpes simplex virus immediate-early proteins: Evidence that ICP4 is associated with progeny virus DNA. Journal of General Virology **67**, 2163-2177.

RAPP, F., & VANDERSLICE, D. (1964). Spread of zoster virus in human embryonic lung cells and the inhibitory effect of iododeoxyuridine. Virology **22**, 321-330.

RAWLS, W.E., COHEN, R.A., & HERRMANN, E.C. Jr. (1964). Inhibition of varicella-zoster virus by 5-iodo-2-deoxyuridine. Proceedings of the Society for Experimental Biology **115**, 123-127.

RAYCHAUDHURI, P., BAGCHI, S., & NEVINS, J.R. (1989). DNA-binding of the adenovirus-induced E4F transcription factor is regulated by phosphorylation. Genes and Development **3**, 620-627.

REINBERG, D., HORIKOSHI, M., & ROEDER, R.G. (1987). Factors involved in specific transcription by mammalian RNA polymerase II. Functional analysis of initiation factors IIA and IID, and identification of a new factor operating downstream of the initiation site. Journal of Biological Chemistry **262**, 3322-3330.

REINBERG, D., & ROEDER, R.G. (1987). Factors involved in specific transcription by mammalian RNA polymerase II: purification and functional analysis of initiation factors IIB and IIE. Journal of Biological Chemistry **262**, 3310-3321.

REINHOLD, W.C., STRAUS, S.E., & OSTROVE, J.M. (1988). Directionality and further mapping of varicella-zoster virus transcripts. Virus Research **9**, 249-261.

RESNICK, J., BOYD, B.A., & HAFFEY, M.L. (1989). DNA binding by the herpes simplex virus type 1 ICP4 protein is necessary for efficient down regulation of the ICP0 promoter. Journal of Virology **63**, 2497-2503.

RICE, S.A., & KNIPE, D.M. (1988). Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. Journal of Virology **62**, 3814-3823.

RICHARDS, J.C., HYMAN, R.W., & RAPP, F. (1979). Analysis of the DNAs from seven varicella-zoster virus isolates. Journal of Virology **32**, 812-821.

RIGBY, P., DIECKMAN, M., RHODES, C., & BERG, P. (1977). Labelling deoxyribonucleic acid to a high specific activity *in vitro* by nick translation with DNA polymerase I. Journal of Molecular Biology **13**, 237-251.

RIXON, F.J., CAMPBELL, M.E., & CLEMENTS, J.B. (1982). The immediate-early mRNA that encodes the regulatory polypeptide Vmw175 of herpes simplex virus type 1 is unspliced. The EMBO Journal **1**, 1273-1277.

RIXON, F.J., & McLAUCLAN, J. (1990). Insertion of DNA sequences at a unique restriction enzyme site engineered for vector purposes into the genome of herpes simplex virus type 1. Journal of General Virology **71**, 2931-2939.

ROBERTS, M.S., BOUNDY, A., O'HARE, P., PIZZORNO, M.C., CIUFO, D.M., & HAYWARD, G.S. (1988). Direct correlation between a negative autoregulatory response element at the capsite of the herpes simplex virus type 1 IE175 (α4) promoter and a specific site for the IE175 (ICP4) protein. Journal of Virology **62**, 4307-4320.

ROCK, D.L., NESBURN, A.B., GHIASI, H., ONG, J., LEWIS, T.L., LOKENSGARD, J.R., & WESCHLER, S.L. (1987). Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. Journal of Virology 61, 3820-3826.

ROFFMAN, E., ALBERT, J.P., GOFF, J.P., & FRENKEL, N. (1990). Putative site for the acquisition of human herpesvirus 6 virion tegument. Journal of Virology 64, 6308-6313.

ROIZMAN, B. (1979). The structure and isomerization of herpes simplex virus genomes (review). Cell 16, 481-494.

ROIZMAN, B. (1982). The family herpesviridae: general description, taxonomy, and classification. In: "The Herpesviruses" vol. 1, pp1-23. Ed. B. Roizman. Plenum press, New York.

ROIZMAN, B., & BATTERSON, N. (1985). Herpesviruses and their replication. In: "Fundamental Virology", pp 607-636. Eds. B.N. Fields, R.M. Chanock, B. Roizman, D.M. Knipe, J.L. Melnick, and R.E. Shope. Raven press, New York.

ROIZMAN, B., CARMICHAEL, L.E., DEINHARDT, F., De THÉ, G., NAHMIAS, A.J., PLOWRIGHT, F., SHELDRICK, P., TAKAHASHI, M., & WOLF, K. (1981). Herpesviridae: Definition, provisional nomenclature, and taxonomy. Intervirology 16, 201-217.

ROIZMAN, B., & FURLONG, D. (1974). The replication of herpesviruses. In: "Comprehensive Virology" vol. 3, pp 229-403. Eds. H. Fraenkel-Conrat and R.R. Wagner. Plenum press, New York.

RUSSELL, J., STOW, N.D., STOW, E.C., & PRESTON, C.M. (1987). Herpes simplex virus genes involved in latency *in vitro*. Journal of General Virology 68, 3009-3018.

RUYECHAN, W.T., CASEY, T.A., REINHOLD, W., WEIR, A.F., WELLMAN, M., STRAUS, S.E., & HAY, J. (1985). Distribution of G+C regions in varicella-zoster virus DNA. Journal of General Virology 66, 43-54.

SACKS, W.R., GREENE, C.C., ASCHMAN, D.P., & SCHAFFER, P.A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. Journal of Virology 55, 796-805.

SACKS, W.R., & SCHAFFER, P.A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. Journal of Virology 61, 829-839.

SADOWSKI, I., MA, J., TRIEZENBERG, S., & PTASHNE, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. Nature **335**, 563-564.

SAFFER, J.D., JACKSON, S.P., & THURSTON, S.J. (1990). SV40 stimulates expression of the *trans*-acting factor Sp1 at the mRNA level. Genes and Development **4**, 659-666.

SAMUELS, M., FIRE, A., & SHARP, P.A. (1982). Separation and characterization of factors mediating accurate transcription by RNA polymerase II. Journal of Biological Chemistry **257**, 14419-14427.

SANGER, F., NICKLEN, S., & COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences (USA) **74**, 5463-5467.

SAWADOGO, M., & ROEDER, R.G. (1985). Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative *in vitro* assay. Proceedings of the National Academy of Sciences (USA) **82**, 4394-4398.

SAWYER, M.H., OSTROVE, J.M., FELSER, J.M., & STRAUS, S.E. (1986). Mapping of the varicella zoster virus deoxypyrimidine kinase gene and preliminary identification of its transcript. Virology **149**, 1-9.

SCHMIDT, M.C., KAO, C.C., PEI, R., & BERK, A.J. (1989). Yeast TATA-box transcription factor gene. Proceedings of the National Academy of Sciences (USA) **86**, 7785-7789.

SCHMIDT, N.J., & LENNETTE, E.H. (1976). Improved yields of cell-free varicella-zoster virus. Infection and Immunity **14**, 709-715.

SEARS, A.E., HALLIBURTON, I.W., MEIGNIER, B., SILVER, S., & ROIZMAN, B. (1985). Herpes simplex virus 1 mutant deleted in the α22 gene: Growth and gene expression in permissive and restrictive cells and establishment of latency in mice. Journal of Virology **55**, 338-346.

SEKULOVICH, R.E., LEARY, K., & SANDRI-GOLDIN, R. (1988). The herpes simplex virus type 1 α protein ICP27 can act as *trans*-repressor or *trans*-activator in combination with ICP4 and ICP0. Journal of Virology **62**, 4510-4522.

SHEMER, Y., LEVENTON-KRISS, S., & SAROV, I. (1980). Isolation and polypeptide characterization of varicella-zoster virus. Virology **106**, 133-140.

SHEPARD, A.A., & DeLUCA, N.A. (1989). Intragenic complementation among partial polypeptides of herpes simplex virus regulatory protein ICP4. Journal of Virology **63**, 1203-1211.

SHEPARD, A.A., IMBALZANO, A.N., & DeLUCA, N.A. (1989). Separation of primary structural components conferring autoregulation, transactivation, and DNA binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. Journal of Virology 63, 3714-3728.

SHIRAKI, K., & HYMAN, R.W. (1987). The immediate-early proteins of varicella zoster virus. Virology 156, 423-426.

SHIRAKI, K., OKUNO, T., YAMANISHI, K., & TAKAHASHI, M. (1982). Polypeptides of varicella-zoster virus (VZV) and immunological relationships of VZV and herpes simplex virus (HSV). Journal of General Virology 61, 255-269.

SILVER, S., & ROIZMAN, B. (1985). Gamma-2-thymidine kinase chimeras are identically transcribed but regulated as gamma-2 genes in herpes simplex virus genomes and as β genes in cell genomes. Molecular and Cellular Biology 5, 518-528.

SIMON, M.C., FISCH, T.M., BENECKE, B.J., NEVINS, J.R., & HEINTZ, N. (1988). Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1a regulation. Cell 52, 723-729.

SMALE, S.T., & BALTIMORE, D. (1989). The "initiator" as a transcription control element. Cell 57, 103-113.

SMILEY, J.R., SMIBERT, C., & EVERETT, R.D. (1987). Expression of a cellular gene cloned in herpes simplex virus: Rabbit β-globin is regulated as an early viral gene in infected fibroblasts. Journal of Virology 61, 2368-2377.

SMITH, C.A., & SCHAFFER, P.A. (1987a). Mutants defective in herpes simplex virus type 2 ICP4: Isolation and preliminary characterization. Journal of Virology 61, 1092-1097.

SMITH, C.A., & SCHAFFER, P.A. (1987b). Intertypic recombinants of herpes simplex virus types 1 and 2 infected cell polypeptide 4. Virology 160, 176-182

SMITH, I.L., & SANDRI-GOLDIN, R.M. (1988). Evidence that transcriptional control is the major mechanism of regulation for the glycoprotein D gene in herpes simplex virus type 1-infected cells. Journal of Virology 62, 1474-1477.

SMITH, R.F., & SMITH, T.F. (1989). Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus, and Epstein-Barr virus. Journal of Virology 63, 450-455.

SOPTA, M., BURTON, Z.F., & GREENBLATT, J. (1989). Structure and associated DNA-helicase activity of a general transcription initiation factor that binds RNA polymerase II. Nature **341**, 410-414.

SOUTHERN, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology **98**, 503-517.

SPEAR, P.G., & ROIZMAN, B. (1972). Proteins specified by herpes simplex virus. V. purification and structural components of the herpes virion. Journal of Virology **9**, 143-159.

SPIVACK, J.G., & FRASER, N.W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection of mice. Journal of Virology **61**, 3841-3847.

STERN. S., TANAKA, M., & HERR, W. (1989). The Oct-1 homeodomain directs formation of a multiprotein complex with the HSV transactivator VP16. Nature **341**, 624-630.

STEVENS, J.G., & COOK, M.L. (1971). Latent herpes simplex virus in spinal ganglia of mice. Science **173**, 843-845.

STEVENS, J.G., WAGNER, E.K., DEVI-RAO, G.B., COOK, M.L., & FELDMAN, L.T. (1987). RNA complementary to a herpesvirus α gene mRNA is prominent in latently infected neurones. Science **235**, 1056-1059.

STOW, N.D., McMONAGLE, E.C., & DAVISON, A.J. (1983). Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Research **11**, 8205-8220.

STOW, N.D., MURRAY, M.D., & STOW, E.C. (1986). *Cis*-acting signals involved in the replication and packaging of herpes simplex virus type 1 DNA. In: CANCER CELLS 4/ DNA tumor viruses, pp 497-507. Eds Botchan, M., Grodzicker, T. & Sharp, P.A. Cold Spring Harbor Lab., New York.

STOW, N.D., & STOW, E.C. (1986). Isolation and charaterisation of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate-early polypeptide Vmw110. Journal of General Virology **67**, 2571-2585.

STOW, N.D., & WILKIE, N.M. (1976). An improved technique for enhancing infectivity with herpes simplex virus type 1 DNA. Journal of General Virology **33**, 447-458.

STRAUS, S.E., ANLAKH, H.S., RUYECHAN, W.T., HAY, J., CASEY, T.A., VAN DE WOUDE, G.F., OWENS, J., & SMITH, H.A. (1981). Structure of varicella-zoster virus DNA. Journal of Virology **40**, 516-525.

STRAUS, S.E., HAY, J., SMITH, H., & OWENS, J. (1983). Genomic differences among varicella-zoster virus isolates. Journal of General Virology **64**, 1031-1041.

STRAUS, S.E., OWENS, J., RUYECHAN, W.T., TAKIFF, H.E., CASEY, T.A., VAN DE WOUDE, G.F., & HAY, J. (1982). Molecular cloning and physical mapping of varicella-zoster virus DNA. Proceedings of the National Academy of Sciences (USA) **79**, 993-997.

STRAUS, S.E., REINHOLD, W., SMITH, H.A., RUYECHAN, W.T., HENDERSON, D.K., BLAESE, R.M., & HAY, J. (1984). Endonuclease analysis of viral DNA from varicella and subsequent zoster infections in the same patient. New England Journal of Medicine **311**, 1362-1364.

STRINGER, K.F., INGLES, C.J., & GREENBLATT, J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature **345**, 783-786.

TACKNEY, C., CACHIANES, G., & SIVERSTEIN, S. (1984). Transduction of the chinese hamster ovary *aprt* gene by herpes simplex virus. Journal of Virology **52**, 606-614.

TEDDER, D.G., EVERETT, R.D., WILCOX, K.W., BEARD, P., & PIZER, L. (1989). ICP4-binding sites in the promoter and coding regions of the herpes simplex virus gD gene contribute to activation of in vitro transcription by ICP4. Journal of Virology **63**, 2510-2520.

TEDDER, D.G., & PIZER, L.I. (1988). Role for DNA-protein interaction in activation of the herpes simplex virus glycoprotein D gene. Journal of Virology **62**, 4661-4672.

THOMPSON, R., HONESS, R.W., TAYLOR, L., MORRAN, J., & DAVISON, A.J. (1987). Varicella-zoster virus specifies a thymidylate synthetase. Journal of General Virology **68**, 1449-1455.

TRIEZENBERG, S.J., KINGSBURG, R.C., & McKNIGHT, S.L. (1988a). Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression. Genes and Development **2**, 718-729.

TRIEZENBERG, S.J., LaMARCO, K.L., McKNIGHT, S.L. (1988b). Evidence of protein interactions that mediate HSV-1 immediate-early gene activation by VP16. Genes and Development **2**, 730-742.

ULLRICH, K., MERSMAN, G., WEBER, E., & VON FIGURA, K. (1978). Evidence for lysosomal enzyme recognition by human fibroblasts via a phosphorylated carbohydrate moiety. Biochemical Journal **170**, 643-650.

UMENE, K. (1986). Conversion of a fraction of the unique sequence to part of the S component of the herpes simplex virus type 1 genome. Journal of General Virology **67**, 1035-1048.

VAN DYKE, M.W., ROEDER, R.G., & SAWADOGO, M. (1988). Physical analysis of transcription preinitiation complex assembly on a class II gene promoter. Science **241**, 1335-1338.

VAN DYKE, M.W., & SAWADOGO, M. (1990). DNA-binding and transcriptional properties of human transcription factor TFIID after mild proteolysis. Molecular and Cellular Biology **10**, 3415-3420.

VLCEK, C., PACES, V., & SCHWYZER, M. (1989). Nucleotide sequence of the pseudorabies virus immediate early gene, encoding a strong trans-activator protein. Virus Genes **2**, 335-346.

VON FIGURA, K., & WEBER, E. (1978). An alternative hypothesis of cellular transport of lysosomal enzymes in fibroblasts. Biochemical Journal **176**, 943-950.

WAGNER, E.K., DEVI-RAO, G., FELDMAN, L.T., DOBSON, A.T., ZHANG, Y., FLANAGAN, M., & STEVENS, J.G. (1988). Physical charaterization of the herpes simplex virus latency-associated transcripts in neurons. Journal of Virology **62**, 1194-1202.

WAGNER, E.K., SWANSTROM, R.I., & STAFFORD, M.G. (1972). Transcription of the herpes simplex virus genome in human cells. Journal of Virology **10**, 675-682.

WARD, R.L., & STEVENS, J.G. (1975). Effect of cytosine arabinoside on viral-specific protein synthesis in cells infected with herpes simplex virus. Journal of Virology 15, 71-80.

WASYLYK, B., DERBYSHIRE, R., GUY, A., MOLKO, D., ROGET, A., TEOULE, R., & CHAMBON, P. (1980). Specific *in vitro* transcription of conalbumin gene is drastically decreased by single-point mutation in T-A-T-A box homology sequence. Proceedings of the National Academy of Sciences (USA) **77**, 7024-7028.

WATSON, R.J., & CLEMENTS, J.B. (1978). Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. Virology 91, 364-379.

WATSON, R.J., & CLEMENTS, J.B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature 285, 329-330.

WEINHEIMER, S.P., & McKNIGHT, S.L. (1987). Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. Journal of Molecular Biology 5, 819-833.

WELLER, T.H., WITTON, H.M., & BELL, E.J. (1958). The etiological agents of varicella and herpes zoster: isolation, propagation, and cultural characteristics *in vitro*. Journal of Experimental Medicine **108**, 843-868.

WELLER, T.H., & WITTON, H.M. (1958). The etiological agents of varicella and herpes zoster. Serological studies with the viruses as propagated *in vitro*. Journal of Experimental Medicine **108**, 869-890.

WHITLEY, R.J. (1985). Epidemiology of herpes simplex viruses. In: "The Herpesviruses" volume 3, pp1-44. Ed. B. Roizman. Plenum press, New York.

WILCOX, K.W., KOHN, A., SKLYANSKAY, E., & ROIZMAN, B. (1980). Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. Journal of Virology **33**, 167-182.

WILDY, P., RUSSELL, W.C., & HORNE, R.W. (1960). The morphology of herpes virus. Virology **12**, 204-222.

WILDY, P., & WATSON, D.H. (1963). Electron microscopic studies on the architecture of animal viruses. Cold Spring Harbor Symposia on Quantitative Biology **27**, 25-47.

WILLIAMS, G.T., McCLANAHAN, T.K., & MORIMOTO, R.I. (1989). E1a transactivation of the human *HSP70* promoter is mediated through the basal transcription complex. Molecular and Cellular Biology **9**, 2574-2587.

WOLFF, M.H. (1978). The proteins of varicella-zoster virus. Medical Microbiology and Immunology **166**, 21-28.

WORKMAN, J.L., ABMAYR, S.M., CROMLISH, W.A., & ROEDER, R.G. (1988). Transcriptional regulation by the immediate early protein of pseudorabies virus during in vitro nucleosome assembly. Cell **55**, 211-219.

WORRAD, D.M., & CARRADONA, S. (1988). Identification of the coding sequence for herpes simplex virus uracil-DNA glycosylase. Journal of Virology **62**, 4774-4777.

WU, C-L., & WILCOX, K.W. (1990). Codons 262 to 490 from the herpes simplex virus ICP4 gene are sufficient to encode a sequence-specific DNA binding protein. Nucleic Acids Research **18**, 531-538.

WU, L., & BERK, A.J. (1988). Transcriptional activation by the pseudorabies virus immediate-early protein requires the TATA box element in the adenovirus 2 E1B promoter. Virology **167**, 318-322.

WU, L., ROSSER, D.S.E., SCHMIDT, M.C., & BERK, A. (1987). A TATA box implicated in E1A transcriptional activation of a simple adenovirus 2 promoter. Nature **326**, 512-515.

YAMANISHI, K., SHIRAKI, K., KONDO, T., OKUNA, T., TAKAHASHI, M., ASANO, Y., & KURATA, T. (1988). Identification of human-herpes-virus 6 as a causal agent for exanthum subitum. Lancet ii, 1065-1067.

ZEHRING, W.A., LEE, J.M., WEEKS, J.R., JOKERST, R.S., & GREENLEAF, A.L. (1988). The C-terminal repeat domain of RNA polymerase II largest subunit is essential *in vivo* but is not required for accurate transcription initiation *in vitro*. Proceedings of the National Academy of Sciences (USA) 85, 3698-3702.

ZHANG, Y.F., & WAGNER, E.K. (1987). The kinetics of expression of individual herpes simplex virus type 1 transcripts. Virus Genes 1, 49-60.

ZHENG, X-M., MONCOLLIN, V., EGLY, J.M., & CHAMBON, P. (1987). A general transcription factor forms a stable complex with RNA polymerase B (II). Cell 50, 361-368.

ZOLLER, M.J., & SMITH, M. (1987). Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single-stranded DNA template. Methods in Enzymology 154, 329-350.

ZWEERINK, H.J., MORTON, D.H., STANTON, L.W., & NEFF, B.J. (1981). Restriction endonuclease analysis of the DNA from varicella-zoster virus: stability of the DNA after passsage *in vitro*. Journal of General Virology 55, 207-211.

Journal of General Virology (1990), 71, 2681–2689. *Printed in Great Britain*

A herpes simplex virus type 1 recombinant with both copies of the Vmw175 coding sequences replaced by the homologous varicella-zoster virus open reading frame

Graham H. Disney and Roger D. Everett*

MRC Virology Unit, Church Street, Glasgow G11 5JR, U.K.

Varicella-zoster virus (VZV) gene 62 encodes a protein with a predicted M_r of 140000 (VZV 140K) that shares considerable amino acid homology with the immediate early (IE) regulatory protein Vmw175 of herpes simplex virus type 1 (HSV-1) and is believed to be its functional equivalent. We have tested this hypothesis by insertion of VZV gene 62 (expressed from the HSV-1 IE3 promoter) into both IE3 gene loci in the short region repeats of the HSV-1 genome. The parent virus used for this manipulation was D30EBA, which is a variant of HSV-1 from which the majority of the Vmw175 coding sequences have been deleted. Like other HSV-1 viruses lacking Vmw175 function, D30EBA is able to grow only in cell lines which express Vmw175 constitutively. The resulting recombinant virus, HSV-140, is able to propagate (but unable to form obvious plaques) on normal cell lines. The properties of HSV-140 were studied by monitoring the time course of polypeptide expression and DNA replication during normal infection. We found that at high multiplicity HSV-140 synthesized apparently normal amounts of many viral polypeptides but that the expression of certain late genes was reduced; this slight defect may be related to less efficient DNA replication by HSV-140. At low multiplicity HSV-140 expressed viral proteins inefficiently. Surprisingly, VZV 140K was produced in large amounts at later times of a normal infection, indicating that the polypeptide fails to autoregulate the IE3 promoter. The results strongly suggest that VZV 140K is able to perform most of the functions of Vmw175 during growth of HSV-1, but that differences in detail lead to less efficient virus growth.

Introduction

Varicella-zoster virus (VZV) is a common human pathogen which causes chickenpox on initial exposure and shingles on subsequent reactivation from latency. It is a member of the neurotropic alphaherpesvirinae subfamily and is genetically closely related to herpes simplex virus type 1 (HSV-1) (Davison & Scott, 1986; McGeoch *et al.*, 1988). At present, in contrast to the increasingly sophisticated knowledge of the molecular biology of HSV, studies on the mechanisms of gene regulation during VZV infection are at an early stage. This is principally a result of the growth properties of VZV in tissue culture, which make the isolation of single plaques and establishment of high titre stocks difficult. However, the availability of the complete DNA sequence of VZV (Davison & Scott, 1986) and the use of transfection and expression systems allow individual genes, selected from the 71 predicted major open reading frames (ORFs), to be studied. This paper concerns the product of VZV gene 62, whose predicted product (VZV 140K) is closely related to the regulatory polypeptide Vmw175 (or ICP4) of HSV-1 (McGeoch *et al.*, 1986).

Gene regulation during HSV-1 infection in tissue culture has been extensively studied (for reviews see Wagner, 1985; Everett, 1987*a*). The viral genes are expressed in three broad classes termed immediate early (IE), early and late. The IE genes are transcribed by the unmodified host RNA polymerase II at the onset of infection (Costanzo *et al.*, 1977). Of the five IE genes, at least four can function as transcriptional activators of viral (and in some cases cellular) gene expression. The product of IE gene 3, Vmw175, is absolutely required for the expression of early and late genes and is also required for the repression or autoregulation of IE gene expression (Preston, 1979; Watson & Clements, 1980). HSV-1 viruses with temperature-sensitive (ts) lesions in Vmw175 are unable to grow at the non-permissive temperature.

Homologues of Vmw175 have been identified from DNA sequence information in all the other alphaherpesviruses so far analysed, including VZV (Davison &

0000-9664

Wilkie, 1983; McGeoch *et al.*, 1986). The product of VZV genes 62 and 71 (the identical gene is present in both copies of the short region repeats; Fig. 1*b*) has a predicted M_r of 140K and for several reasons is believed to be the VZV equivalent of Vmw175. The genes are located in equivalent positions and there is extensive predicted amino acid sequence conservation between them (McGeoch *et al.*, 1986). Also, VZV 140K has been shown to be a potent activator of gene expression in transient transfection assays (Everett & Dunlop, 1984; Everett, 1984; Inchauspe *et al.*, 1989) and the regions of the Vmw175 polypeptide that have been shown to be most important for its transactivation and repression phenotypes are the most highly conserved in 140K (Paterson & Everett, 1988*a*; DeLuca & Schaffer, 1988). These same regions are also highly conserved in the other alphaherpesvirus homologues of Vmw175 (Grundy *et al.*, 1989; Cheung, 1989; Vleck *et al.*, 1989). Four IE proteins have been identified in VZV-infected cells using cycloheximide reversal experiments, the largest of which has an apparent molecular weight of 185000 and has been proposed to be the product of gene 62 (Shiraki & Hyman, 1987). Perhaps the most compelling evidence that VZV 140K and HSV-1 Vmw175 are functionally homologous is that HSV-1 mutants with lesions in Vmw175 can be complemented by either transfected plasmids or transformed cell lines which express VZV 140K (Felser *et al.*, 1987, 1988).

In this paper we have extended such studies by construction of a virus (HSV-140) which contains VZV 140K in place of the coding sequences for Vmw175 in the HSV-1 genome. HSV-140 was constructed from D30EBA (Paterson & Everett, 1990), an HSV-1 derivative that has the majority of the Vmw175 coding sequences deleted, and a plasmid in which 140K is expressed from the IE3 transcription regulatory signals. Unlike D30EBA, HSV-140 is able to grow in normal tissue culture cells. We have further investigated the properties of VZV 140K and its complementation of Vmw175 by studies on the growth, polypeptide expression and DNA replication of HSV-140. We found that although VZV 140K is able to complement for the absence of Vmw175, HSV-140 is partially defective in comparison to HSV-1.

Methods

Plasmids. Plasmid pGD140, which contains the VZV 140K coding sequences in an HSV-1 IE3 transcription unit (Fig. 1*a*) was constructed from fragments derived from pGX161, pI38 and p140BTE. Plasmid pGX161 (kindly provided by Dr C. M. Preston) has been described previously (Preston & Fisher, 1984) and contains the promoter and upstream regulatory sequences of the HSV-1 IE3 gene (nucleotides −331 to +27) between the *Hin*dIII and *Bam*HI sites of pAT153. Plasmid pI38 (kindly provided by Dr T. Paterson) is a derivative of p175 (Perry *et al.*, 1986) created by insertion of a 12 bp *Eco*RI linker oligonucleotide into a *Nae*I site within the Vmw175 coding sequences, resulting in an in-frame insertion of four amino acids into the proline codon at position 1236 (Paterson & Everett, 1988*a*). Plasmid p140 contains VZV gene 62 in a fragment from the *Cla*I site at position −1146 to the terminal *Pst*I site of pVZVSstf (Everett, 1984) inserted between the *Acc*I and *Pst*I sites of a pUC9 plasmid from which the *Eco*RI site had been removed by cutting and filling in (McKee *et al.*, 1990). Plasmid p140BTE is a derivative of p140 which has a unique *Bgl*II site at position +57 (relative to the transcription start site at +1) and a unique *Eco*RI site four nucleotides beyond the 3′ end of the ORF. Plasmid p140 was subjected to site-directed mutagenesis to create a unique *Eco*RI site at +57 (p140ET; McKee *et al.*, 1990), which was subsequently converted to a *Bgl*II site by insertion of an eight nucleotide *Bgl*II linker to create p140BT. Plasmid p140BT was linearized by digestion with *Tth*111I, which cuts two nucleotides beyond the end of the gene 62 reading frame, and *Eco*RI linkers were inserted after filling in the ends to yield p140BTE. Plasmid pGD140 was constructed in a tripartite ligation using the *Bgl*II–*Eco*RI fragment from p140BTE containing the VZV 140K coding sequences, the smaller *Pst*I–*Bam*HI fragment of pGX161 containing the IE3 promoter (and part of the vector sequences) and the smaller *Pst*I–*Eco*RI fragment of pI38 which provides the rest of the vector sequences, the C-terminal 62 codons of Vmw175, the 3′ end of the IE3 transcription unit and subsequent HSV-1 DNA through to the *Sst*I site in TR_L (Fig. 1*a*). The C-terminal portion of Vmw175 in this construct follows the TGA stop codon of VZV gene 62. Plasmid p111 (Everett, 1987*b*) was derived from p110del7 (Perry *et al.*, 1986) by filling in the *Eco*RI site in the polylinker sequences.

Cells. Baby hamster kidney cells (BHK-21 clone 13; Macpherson & Stoker, 1962), were grown in Glasgow Modified Eagle's medium (GMEM, Gibco) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 10% tryptose phosphate and 10% newborn calf serum. Human foetal lung (HFL) cells were grown in GMEM supplemented with 10% foetal calf serum, penicillin and streptomycin as above.

Viruses. Throughout this paper wild-type (wt) virus refers to HSV-1 Glasgow strain 17 *syn*$^+$ (Brown *et al.*, 1973). The HSV-1 derivative D30EBA, which lacks codons 84 to 1270 of the Vmw175 coding sequence (Paterson & Everett, 1990), was used as the parent virus for the construction of HSV-140. D30EBA was propagated on M64A cells, a transformed cell line that expresses Vmw175 and which is equivalent to M65 cells as described by Davidson & Stow (1985).

Construction of HSV-140. A mixture containing approximately 1 μg of plasmid pGD140 linearized with *Pst*I, 3 μg of D30EBA viral DNA and 5 μg of calf thymus DNA (as a carrier) were transfected into 2×10^6 BHK cells by the method of Stow & Wilkie (1976). Cells were also transfected with viral or plasmid DNA alone as a control. Following transfection, cells were incubated at 37 °C and when c.p.e. was apparent (after 3 to 4 days), the cells and medium from each plate were harvested, sonicated and stored as a virus stock. The presence of the desired recombinant virus was detected by slot blot analysis of total cellular DNA prepared from BHK cells infected with the initial virus stocks. The structure of the recombinant virus genome was analysed by Southern blotting. Finally, HSV-140 was purified from these initial isolates by limiting dilution.

Preparation and analysis of viral DNA. Viral DNA was prepared as total cellular DNA from BHK cell monolayers infected with HSV-140 or wt virus (as detailed in the text) by the method of Stow *et al.* (1983) and analysed after digestion with *Bam*HI by Southern blotting (Southern, 1975) using nick-translated p140 as a probe (Rigby *et al.*, 1977).

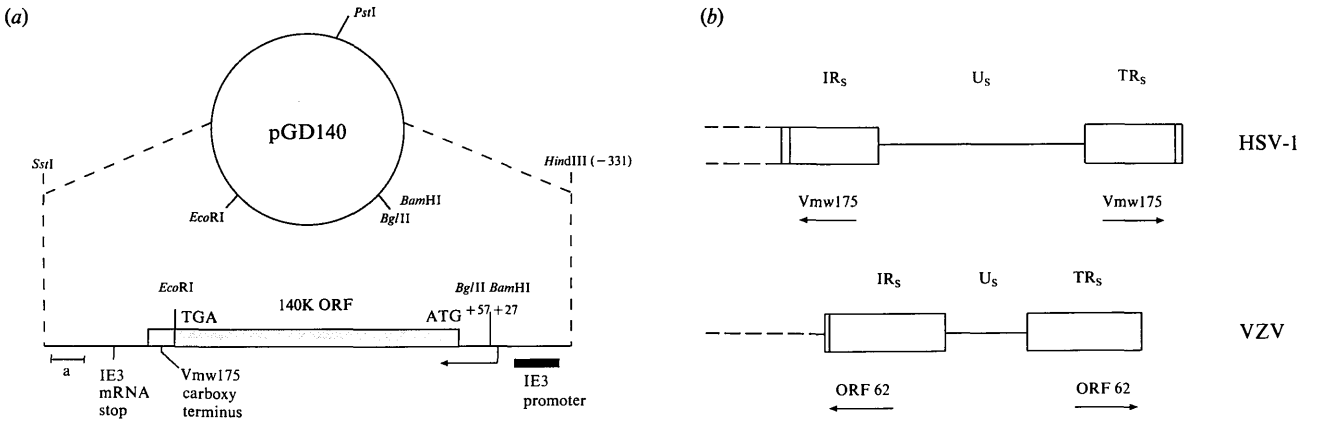


Fig. 1. The structure of plasmid pGD140 and a summary of the structure of virus HSV-140. (*a*) Plasmid pGD140 with the VZV gene 62 coding region flanked by the HSV-1 IE3 transcriptional control sequences. The vector sequences extend from the *Pvu*II site of pBR322 (which had been altered to an *Sst*I site) through the ampicillin resistance gene (with its *Pst*I site) to the *Hin*dIII site. The *Hin*dIII site is located at the junction of HSV-1 and pAT153 sequences and is joined to the *Sma*I site at position −331 of the IE3 promoter. The VZV gene 62 sequences from position +57 to an introduced *Eco*RI site just after the VZV 140K stop codon are shown, with the coding sequence in a stippled box. Sequences downstream of this include the 3′ portion of IE3, the 'a' sequence and part of TR_L as far as the *Sst*I site as shown (not to scale). The plasmid was constructed by ligation of the *Pst*I–*Bam*HI fragment of pGX161, the *Bgl*II–*Eco*RI fragment of p140BTE and the *Eco*RI–*Sst*I fragment of pI38 as detailed in Methods. (*b*) The locations of the Vmw175 and VZV gene 62 coding regions (indicated by arrows) in the genomes of HSV-1 and VZV respectively. The short unique region (U_S) and flanking repeat sequences (IR_S and TR_S; open boxes) of the genomes are shown (not to scale).

Labelling and analysis of viral polypeptides. Examination of polypeptide synthesis throughout the growth cycle was performed at 37 °C using a 2 h labelling period. Single wells of 24-well Linbro blocks were infected at various multiplicities (as described in the text) and at various times thereafter, washed with phosphate-buffered saline (PBS) and incubated with 15 μCi [^{35}S]methionine (Amersham, >800 Ci/mmol) in 200 μl of PBS. After labelling, the cells were washed and harvested and the labelled proteins analysed on discontinuous 7·5% SDS–polyacrylamide gels as described by Marsden *et al.* (1978).

Assay of DNA replication. BHK cells in Linbro wells were infected with HSV-140 or wt virus and total cellular DNA was prepared at various times post-infection (as described in the text). DNA samples were treated with RNase (100 μg/ml, 65 °C for 1 h) prior to application to a nitrocellulose membrane using a slot blot apparatus (Schleicher and Schuell, Minifold II). Blots were probed with nick-translated p111 (Rigby *et al.*, 1977).

Results

Construction of a virus expressing VZV 140K in place of Vmw175

Virus D30EBA lacks the majority of the Vmw175 coding sequences and is unable to grow in normal cell lines (Paterson & Everett, 1990). Since VZV and plasmids expressing the VZV 140K polypeptide complement HSV-1 viruses with ts mutations in Vmw175 (Felser *et al.*, 1987, 1988), we expected to be able to rescue D30EBA by the introduction of gene 62 of VZV. Hence plasmid pGD140, which contains the VZV gene 62 coding region inserted into an IE3 transcription unit (Fig. 1 *a*), was constructed. The IE3 sequences 5′ and 3′ of the VZV 140K coding region allow recombinational insertion into the normal location of IE3 in the short region repeats.

D30EBA viral DNA was cotransfected into BHK cells with linearized pGD140. We expected that any recombinants that contained ORF62 would be able to grow, whereas input viral DNA alone would not lead to virus production. After 4 days c.p.e. was observed in cells cotransfected with viral and plasmid DNA, whereas no c.p.e. was observed in the control transfection with viral DNA alone. Cells and medium combined from both the control and cotransfected plates were sonicated and retained as virus stocks. These stocks were used to infect further BHK cells and total infected cell DNA was prepared. This was initially analysed by DNA slot blots using nick-translated p140 (McKee *et al.*, 1990) as a probe, which showed that only DNA prepared from the cotransfected cells hybridized to VZV sequences (results not shown).

The recombinant viral DNA was further analysed by Southern blotting (Fig. 3). The controls show that neither mock- nor wt HSV-1-infected cell DNA samples hybridize to the p140 probe. The *Bam*HI restriction map for the expected recombinant virus (Fig. 2) predicts that the probe will hybridize to the following fragments on the blot: *Bam*HI [k′] (6·0 kb) and *Bam*HI [y′ + n] (7·1 kb) derived from IR_s; *Bam*HI [x + y′] (4·1 kb); *Bam*HI [q′] (3·0 kb) derived from TR_s. Fragments corresponding in size to the three larger predicted *Bam*HI restriction fragments are clearly observed on the blot. However, the

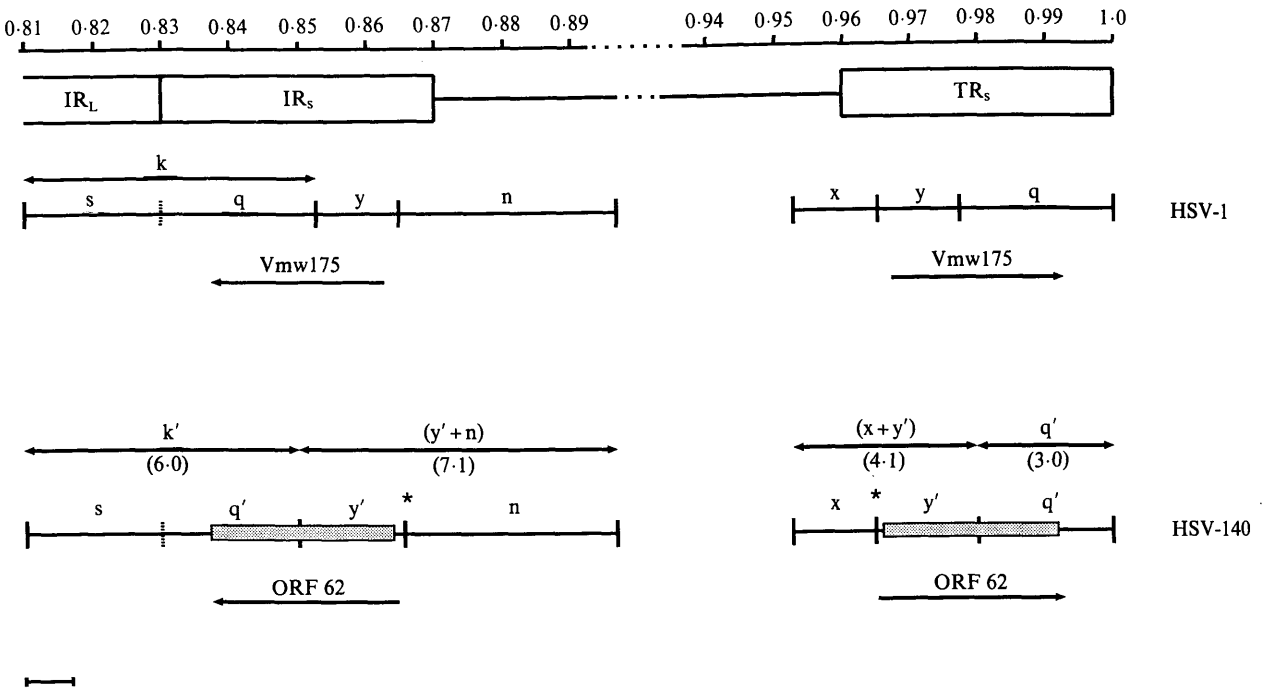


Fig. 2. The predicted *Bam*HI restriction maps for the short regions of the wt HSV-1 and HSV-140 genomes. VZV gene 62 coding sequences replace both copies of Vmw175 in the short region repeats of the HSV-1 genome in HSV-140; VZV sequences are represented by the stippled box and the limits of the ORFs shown by arrows. The Vmw175 coding sequences in HSV-1 are contained within *Bam*HI q and *Bam*HI y; their replacement by the corresponding gene 62 sequences in HSV-140 results in restriction fragments designated *Bam*HI q′ and *Bam*HI y′. *Bam*HI k′ represents (q′+s) derived from the IR_L/IR_s junction. The *Bam*HI site at the cap site of the IE3 gene (present at map units 0·865 and 0·965 approximately in HSV-1) was destroyed during the construction of pGD140 (marked * in HSV-140, see Fig. 1). Therefore *Bam*HI digestion of HSV-140 DNA produces fragments (y′+n) and (x+y′). The sizes (kb) of these four novel fragments containing portions of the VZV gene 62 sequence are shown.

*Bam*HI [q′] fragment, which represents sequences from the genome terminus, was not observed in this experiment. A weakly hybridizing fragment of 3 kb was once observed during the isolation and purification of HSV-140, which probably indicates that viral DNA prepared from infected cells is highly concatemerized with consequent under-representation of the terminal fragment. The simplest interpretation of these results is that an intact copy of VZV gene 62 is present in both IE3 gene loci in the HSV-1 genome in our primary isolate of the recombinant virus HSV-140.

The initial isolate was amplified and plaque-purification under agar was attempted, but despite several attempts no plaques were observed (see below). Virus HSV-140 was subsequently purified by limiting dilution in Linbro wells, such that c.p.e. was observed in 30% or less of wells. Medium was retained from wells that displayed c.p.e. and used to infect cells for total cellular DNA preparation. Southern blot analysis of the resulting DNAs showed all eight stocks of virus to be of the predicted structure with no evidence of contamination. Recombinant viruses carrying only one copy of gene 62 were not detected at any stage, suggesting that HSV-140, with two copies of gene 62, has a strong growth advantage. One of the small scale stocks was selected for a large scale virus preparation which was again checked by Southern blotting. The isolation of HSV-140 on normal BHK cells shows that VZV-140K can functionally replace Vmw175.

Growth properties of HSV-140

HSV-140 was compared to wt virus in a standard plaque assay in BHK cells to assess the extent to which VZV 140K can complement for loss of Vmw175 function. Cells were infected with increasing dilutions of each virus and fixed and stained after 2 days. At lower dilutions both viruses produced extensive c.p.e. although the appearance of c.p.e. during HSV-140 infections was delayed approximately 12 h compared to that during wt virus infections. At increasing dilutions single plaques were obtained with wt virus as normal, but no clear plaques were produced by HSV-140. The assay was repeated several times with independent stocks of HSV-140 and in no case were distinct plaques formed. Similar results were obtained using HFL cells. The results suggest that although HSV-140 is able to grow in the

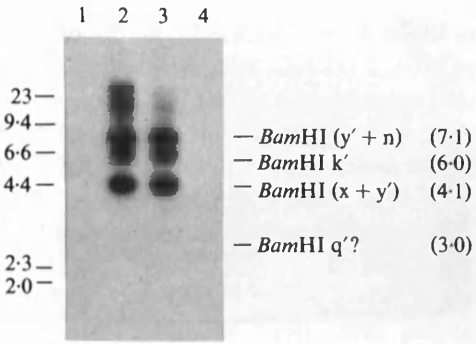


Fig. 3. Southern blot analysis of DNA prepared from HSV-140-infected cells. DNA was prepared from infected cells when extensive c.p.e. was apparent. The DNA was digested with *Bam*HI and subjected to Southern blot analysis using a nick-translated plasmid p140 probe which contains VZV gene 62 but no HSV-1 sequences. Lane 1, mock-infected cell DNA; lanes 2 and 3, HSV-140-infected cell DNA using two different virus stocks; lane 4, HSV-1-infected (strain 17) cell DNA. The positions of λ *Hin*dIII size markers (kb) are shown on the left. The identities of fragments hybridizing to the probe and their predicted sizes (kb) are shown on the right. They were assigned by comparison of their estimated sizes to those predicted from the maps in Fig. 2. The larger fragments in lane 2 represent partial digestion products.

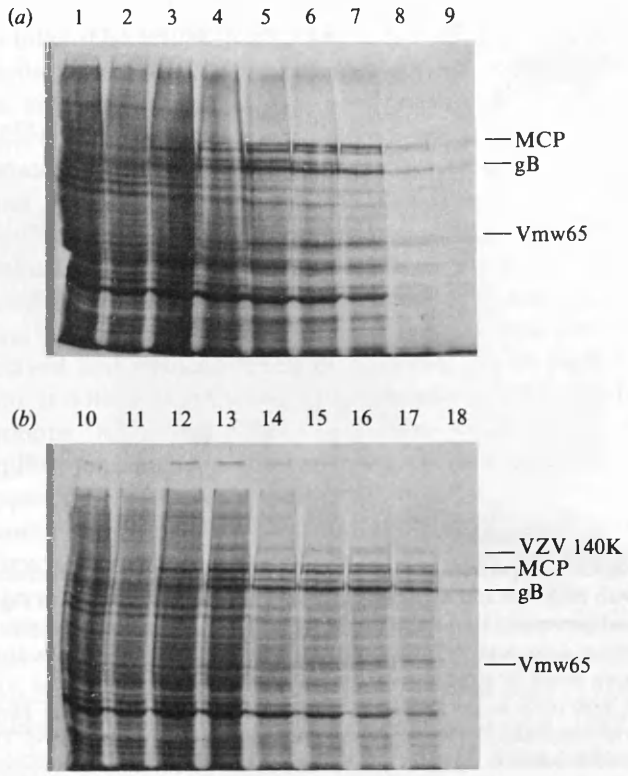


Fig. 4. The time course of viral protein expression in BHK cells infected with HSV-140 and wt virus. Linbro wells containing 4×10^5 cells were infected with (*a*) 10 p.f.u./cell wt HSV-1 (strain 17) or (*b*) 100 p.p.c. HSV-140. Proteins were labelled with [^{35}S]methionine at: lane 2, 0 to 2 h; lane 3, 2 to 4 h; lane 4, 4 to 6 h; lane 5, 6 to 8 h; lane 6, 8 to 10 h; lane 7, 10 to 12 h; lane 8, 16 to 18 h; lane 9, 22 to 24 h p.a. and analysed by SDS–PAGE. The samples in lane 1 are from mock-infected cells. Some of the major viral proteins are indicated: major capsid protein, MCP; glycoprotein B, gB; Vmw65 and VZV 140K (HSV-140-infected cells only). Less intense labelling in the later time point samples is due to loss of terminally infected cells.

absence of Vmw175 expression, plaque formation is impaired, at least in the cell types used in these studies.

The numbers of virus particles in the large scale stocks of cell-associated and supernatant HSV-140 virus were determined. HSV-140 cell-associated stocks were routinely around 3×10^{10} particles/ml, which was equivalent to the corresponding particle counts of stocks of wt virus produced in parallel. The supernatant virus particle count of HSV-140 was reduced by a factor of 10 compared to the cell-associated virus and the wt supernatant virus stocks. If this reflects a slower rate of virus assembly or release then it may in part explain the lack of plaque formation by HSV-140.

The time course of HSV-140 viral polypeptide synthesis

As titres could not be determined by plaque assay for HSV-140 virus stocks, viral polypeptide synthesis in HSV-140- and wt virus-infected cells were compared using 10 p.f.u./cell of wt virus and increasing dilutions of HSV-140. Cells were labelled with [^{35}S]methionine from 3 to 6 h post-adsorption (p.a.) and proteins analysed by SDS–PAGE. Infection with dilutions of HSV-140 corresponding to multiplicities of 2500, 500 and 100 particles per cell (p.p.c.) resulted in levels of viral gene expression that were equivalent to the wt virus infection (results not shown). Since an amount of VZV inoculum corresponding to 100 p.p.c. gave viral polypeptide expression similar to 10 p.f.u. wt of virus/cell in this single experiment, this amount of HSV-140 was used in a subsequent time course experiment.

Cells were mock-infected or infected with wt and HSV-140 viruses and labelled between 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 10, 10 to 12, 16 to 18 and 22 to 24 p.a. These results are shown in Fig. 4. The protein expression profiles of the two viruses were similar, but a significant difference was the kinetics of expression of a high M_r protein in HSV-140-infected cells. On the basis of its mobility, expression by HSV-140 and Western blotting using a polyclonal rabbit antiserum directed against a fusion protein including part of VZV 140K (data not shown; D. Stephenson & A. Davison, personal communication), the most likely interpretation is that this high M_r band is the VZV 140K polypeptide. It is notable that the kinetics of expression of VZV 140K in HSV-140-infected cells is very different compared to Vmw175 in wt virus-infected cells. Strikingly, VZV 140K was expressed as an early protein and was abundant late in infection. Thus VZV 140K is apparently not subject to autoregulation in HSV-140, which suggests that the

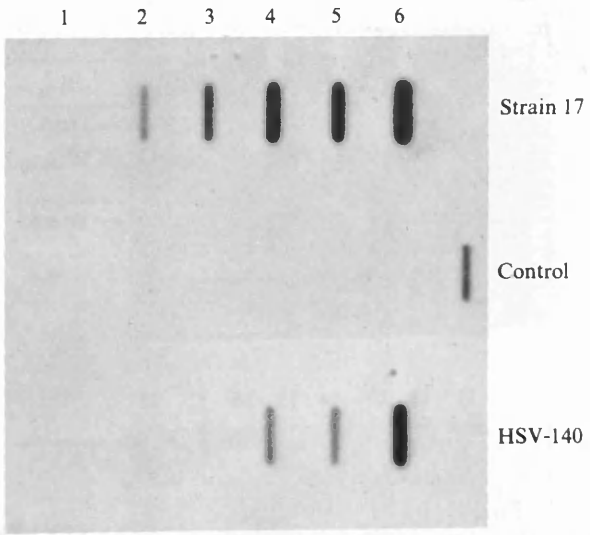


Fig. 5. Comparison of viral DNA replication in BHK cells infected with HSV-140 and wt virus. Cells were infected as described in Fig. 4 and harvested at: lane 2, 4 h; lane 3, 6 h; lane 4, 8 h; lane 5, 10 h; lane 6, 16 h p.a. for total cellular DNA preparations. DNA was also isolated from mock-infected cells (lane 1). The samples were treated with RNase prior to slot blot analysis using a nick-translated p111 probe which contains IE1 sequences common to HSV-140 and wt HSV-1. The control contains 10 ng of p111.

normal repression of IE gene expression may be defective in this virus. It is not possible to determine from these data whether Vmw110, Vmw68 or Vmw63 are also overexpressed during HSV-140 infection.

Most other clearly visible viral proteins are expressed in HSV-140- and wt virus-infected cells in similar amounts and with similar kinetics. However, the expression of certain late gene products (for example, Vmw65 and the major capsid protein) appears to be lower in HSV-140-infected cells. This may be the result of less efficient replication of viral DNA during HSV-140 infection (see below).

Comparison of viral DNA replication during strain 17 and HSV-140 infections

BHK cells were infected with HSV-140 and wt virus and total cellular DNA was prepared at 4, 6, 8, 10 and 16 h p.a. The DNA samples were treated with RNase and analysed in a slot blot experiment to compare the amounts of viral DNA present in the two infections. Nick-translated p111 (which contains HSV-1 IE gene 1 sequences; Everett, 1987*b*) was used as a probe as it contains sequences common to both viruses. In cells infected with wt virus, DNA accumulation began prior to the earliest time point of 4 h p.a. and continued through to 16 h p.a. (Fig. 5). The onset of HSV-140 DNA replication was delayed 2 to 4 h relative to that of wt virus and the amounts of viral DNA produced were generally reduced; no accumulation of DNA was seen at 4 h p.a. and only very low levels were seen at 6 h p.a. (Fig. 5).

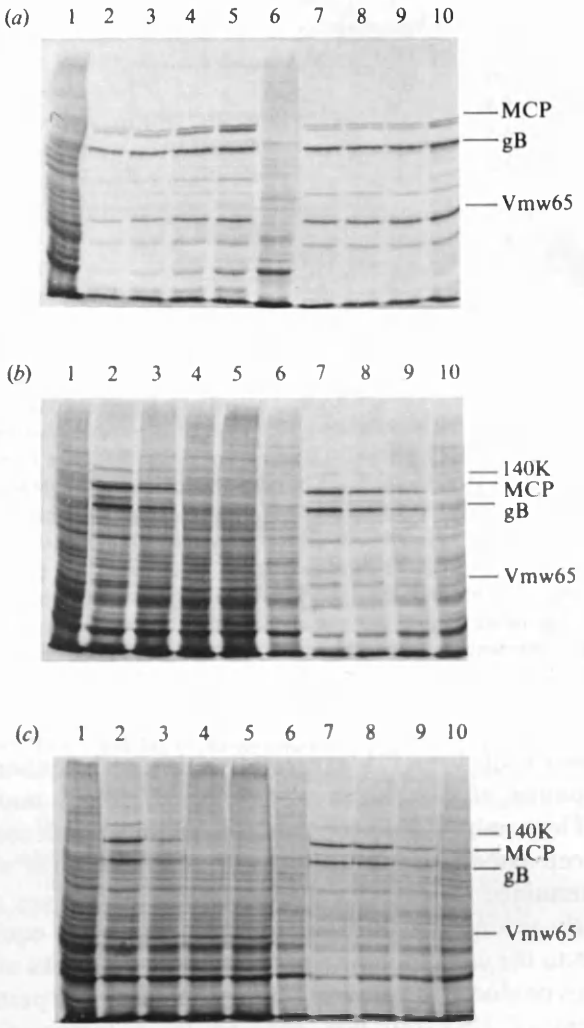


Fig. 6. HSV-140 viral gene expression is impaired at lower multiplicities of infection. BHK or HFL cells in Linbro wells were infected with (*a*) wt HSV-1 (strain 17) and (*b*) and (*c*), two separate stocks of HSV-140 prepared in parallel at multiplicities corresponding to: lanes 2 and 7, 50 p.p.c.; lanes 3 and 8, 30 p.p.c.; lanes 4 and 9, 10 p.p.c.; lanes 5 and 10, 3 p.p.c. Cells were labelled with [^{35}S]methionine from 10 to 12 h p.a. and proteins analysed by SDS-PAGE. Lanes 1 to 5 of each gel are samples prepared from BHK cells, lanes 6 to 10 are samples from HFL cells. Lanes 1 and 6 of each gel show mock-infected samples. Some major viral proteins are indicated, as described in the legend to Fig. 4.

Viral gene expression during infection with HSV-140 is multiplicity-dependent

Several mutants of HSV-1 containing mutations in regulatory functions have been shown to have defects in viral gene expression at low multiplicities in certain cell types (Stow & Stow, 1986; Sacks & Schaffer, 1987; Ace *et al.*, 1989; Everett, 1989). We decided to investigate the

synthesis of viral polypeptides during high and low multiplicity infections of BHK and HFL cells by HSV-140. In an attempt to minimize experimental variation, the virus stocks used in this study were all prepared in parallel from the same batch of cells. BHK or HFL cells were infected at 50, 30, 10 and 3 p.p.c. with HSV-140 or wt virus and labelled with [^{35}S]methionine at 10 to 12 h p.a. (Fig. 6). In agreement with the time-course experiment (Fig. 4) at higher multiplicities both viruses expressed similar levels of most viral polypeptides, although the expression of certain late proteins (for example Vmw65 and the major capsid protein) was again reduced in HSV-140 infections. At the lower multiplicities of 10 and 3 p.p.c., HSV-140 expressed reduced amounts of all viral polypeptides compared to wt virus in both BHK and HFL cells. This finding was consistent with several different stocks of HSV-140. Therefore HSV-140 exhibits a multiplicity-dependent gene expression defect which, in the cases examined, is not cell type-dependent.

In addition, the results in Fig. 6 suggest that the ability of HSV-140 to shut off host protein synthesis is significantly impaired. These results cannot be considered conclusive because labelling at late times means that secondary infection by the faster growing wt virus could contribute to the observed shut off of host protein synthesis. However, an experiment in which cells were infected with equal particle numbers of the two viruses (1000 particles per cell) in the presence of actinomycin D showed that virion-associated shut off by HSV-140 was significantly reduced compared to that of wt virus (results not shown). This could be due to inefficient presentation by HSV-140 of the virion host shutoff function encoded by gene UL41 (Kwong *et al.*, 1988; McGeoch *et al.*, 1988), or slight alterations in the composition or structure of HSV-140 virus particles might reduce their efficiency of cellular adsorption or penetration.

Discussion

The predicted amino acid sequence of VZV gene 62, which encodes VZV 140K, shows strong homology to Vmw175 of HSV-1 (McGeoch *et al.*, 1986). Like Vmw175, VZV 140K is a potent trans-activator of gene expression in transfection assays (Everett & Dunlop, 1984; Everett, 1984; Inchauspe *et al.*, 1989). Previous studies have shown that VZV 140K can complement the growth of HSV-1 viruses with ts and deletion mutations in Vmw175 (Felser *et al.*, 1987, 1988). By construction of a virus in which the VZV 140K coding sequences replace those of Vmw175 we have confirmed and extended the previous observations on the relatedness of the two proteins. The results graphically illustrate the functional similarities of Vmw175 and VZV 140K since recombinant virus HSV-140 propagates in normal cell lines. Its failure to form clearly defined plaques may be a consequence of slight alterations in the balance of viral gene expression which would be difficult to define. Similarly, if one of the proteins involved in viral DNA replication were aberrantly expressed during HSV-140 infection it would not be easily detected by analysis of viral polypeptide expression, yet could account for the delayed and reduced levels of HSV-140 DNA replication. It is likely that the reduced expression of certain late proteins is a consequence of less efficient DNA replication and this in turn may explain the slower appearance of c.p.e. in HSV-140-infected cells. These results show that the properties of VZV 140K and Vmw175 are not indistinguishable but they are largely interchangeable.

The multiplicity dependence of HSV-140 gene expression is reminiscent of other HSV-1 variants with mutations in Vmw65 and Vmw110 (Stow & Stow, 1986; Sacks & Schaffer, 1987; Ace *et al.*, 1989; Everett, 1989). Since one of the phenotypes of the Vmw65 virus mutant is synthesis of reduced amounts of Vmw110 and since the synergistic activation of gene expression by the combination of Vmw110 and Vmw175 observed in transfection assays (Everett, 1984; Quinlan & Knipe, 1985; Gelman & Silverstein, 1985) does not appear to occur with VZV 140K and Vmw110 (our unpublished data), it is intriguing to speculate that the multiplicity dependence of HSV-140 gene expression might be a reflection of this lack of synergy.

The regulation of VZV 140K expression during HSV-140 infection is also of interest since it accumulates to high levels as infection proceeds, which is in contrast to the relatively low levels of accumulation of Vmw175 during wt virus infection. Therefore, VZV 140K is apparently unable to effect the repression of IE3 gene expression which is normally a function of Vmw175. Repression of the IE3 promoter by Vmw175, which requires the presence of a specific Vmw175 DNA-binding sequence at the cap site of the IE3 promoter (Muller, 1987; Roberts *et al.*, 1988), can be reproduced in transfection assays (O'Hare & Hayward, 1985, 1987). Mutants of the Vmw175 protein that fail to bind to an IE3 cap site probe are unable to repress the IE3 promoter (DeLuca & Schaffer, 1988; Paterson & Everett, 1988*b*). The failure of VZV 140K to repress the IE3 promoter during HSV-140 infection is consistent with data which show that VZV 140K does not repress the IE3 promoter in transfection assays (Disney *et al.*, 1990) and does not bind to the IE3 cap site region (our unpublished data). We do not yet know whether the other IE genes are also overexpressed in HSV-140 infections, but if not it

suggests that the mechanism of their repression may differ from that of IE3. If this is the case, then HSV-140 may be a useful tool with which to study the mechanism of IE gene repression.

In conclusion, our results demonstrate that VZV 140K is able to complement, to a great extent, for loss of Vmw175 function in HSV-1. The high levels of expression of VZV 140K in HSV-140-infected cells should permit a more detailed analysis of the biochemical properties of the protein.

The authors would like to thank Professor J. H. Subak-Sharpe and Dr C. M. Preston for helpful criticism of the manuscript. We also thank Dr J. McLauchlan for synthesis of oligonucleotides and Mr J. Aitken for performing particle counts on virus stocks. G.D. is supported by an MRC postgraduate studentship.

References

ACE, C. I., MCKEE, T. A., RYAN, M. J., CAMERON, J. M. & PRESTON, C. M. (1989). Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate early gene expression. *Journal of Virology* **63**, 2260–2269.

BROWN, S. M., RITCHIE, D. A. & SUBAK-SHARPE, J. H. (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. *Journal of General Virology* **18**, 329–346.

CHEUNG, A. K. (1989). DNA nucleotide sequence analysis of the immediate-early gene of pseudorabies virus. *Nucleic Acids Research* **17**, 4637–4646.

COSTANZO, F., CAMPADELLI-FIUME, G., FOA-TOMASI, L. & CASAI, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *Journal of Virology* **21**, 996–1001.

DAVIDSON, I. & STOW, N. D. (1985). Expression of an immediate-early polypeptide and activation of a viral origin of DNA replication in cells containing a fragment of herpes simplex virus DNA. *Virology* **141**, 77–88.

DAVISON, A. J. & SCOTT, J. E. (1986). The complete DNA sequence of varicella-zoster virus. *Journal of General Virology* **67**, 1759–1816.

DAVISON, A. J. & WILKIE, N. M. (1983). Location and orientation of homologous sequences in the genomes of five herpesviruses. *Journal of General Virology* **64**, 1927–1942.

DELUCA, N. A. & SCHAFFER, P. A. (1988). Physical and functional domains of the herpes simplex virus transcriptional regulatory protein. ICP4. *Journal of Virology* **62**, 732–743.

DISNEY, G. H., MCKEE, T. A., PRESTON, C. M. & EVERETT, R. D. (1990). The product of varicella-zoster virus gene 62 autoregulates its own promoter. *Journal of General Virology* (in press).

EVERETT, R. D. (1984). Transactivation of transcription by herpesvirus products. Requirements for two HSV-1 immediate-early polypeptides for maximum activity. *EMBO Journal* **3**, 3135–3141.

EVERETT, R. D. (1987*a*). The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products. *Anticancer Research* **7**, 589–604.

EVERETT, R. D. (1987*b*). A detailed analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. *EMBO Journal* **6**, 2069–2076.

EVERETT, R. D. (1989). Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *Journal of General Virology* **70**, 1185–1202.

EVERETT, R. D. & DUNLOP, M. (1984). Transactivation of plasmid borne promoters by adenovirus and several herpes group viruses. *Nucleic Acids Research* **12**, 5969–5978.

FELSER, J. M., STRAUS, S. E. & OSTROVE, J. M. (1987). Varicella-zoster virus complements herpes simplex virus type 1 temperature sensitive mutants. *Journal of Virology* **61**, 225–228.

FELSER, J. M., KINCHINGTON, P. R., INCHAUSPE, G., STRAUS, S. E. & OSTROVE, J. M. (1988). Cell lines containing varicella-zoster virus open reading frame 62 and expressing the "IE" 175 protein complement ICP4 mutants of herpes simplex virus type 1. *Journal of Virology* **62**, 2076–2082.

GELMAN, I. H. & SILVERSTEIN, S. (1985). Identification of immediate-early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proceedings of the National Academy of Sciences, U.S.A.* **82**, 5265–5269.

GRUNDY, F. J., BALIMAN, R. P. & O'CALLAGHAN, D. J. (1989). DNA sequence and comparative analyses of the equine herpesvirus type 1 immediate-early gene. *Virology* **172**, 223–236.

INCHAUSPE, G., NAGPAL, S. & OSTROVE, J. M. (1989). Mapping of two varicella-zoster virus-encoded genes that activate the expression of viral early and late genes. *Virology* **173**, 700–709.

KWONG, A. D., KRUPER, J. A. & FRENKEL, N. (1988). HSV virion host shutoff function. *Journal of Virology* **62**, 912–921.

MCGEOCH, D. J., DOLAN , J. A., DONALD, S. & BRAUER, D. H. K. (1986). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. *Nucleic Acids Research* **14**, 1727–1764.

MCGEOCH, D. J., DALRYMPLE, M. A., DAVISON, A. J., DOLAN, A., FRAME, M. C., MCNAB, D., PERRY, L. J., SCOTT, J. E. & TAYLOR, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *Journal of General Virology* **69**, 1531–1574.

MCKEE, T. A., DISNEY, G. H., EVERETT, R. D. & PRESTON, C. M. (1990). Control of expression of the varicella-zoster virus major immediate early gene. *Journal of General Virology* **71**, 897–906.

MACPHERSON, I. & STOKER, M. (1962). Polyoma transformation of hamster cell clones–an investigation of genetic factors affecting cell competence. *Virology* **16**, 147–151.

MARSDEN, H. S., STOW, N. D., PRESTON, V. G., TIMBURY, M. C. & WILKIE, N. M. (1978). Physical mapping of herpes simplex virus-induced polypeptides. *Journal of Virology* **28**, 624–642.

MULLER, M. T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. *Journal of Virology* **61**, 858–865.

O'HARE, P. & HAYWARD, G. S. (1985). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. *Journal of Virology* **56**, 723–733.

O'HARE, P. & HAYWARD, G. S. (1987). Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus encoded trans-acting factors. *Journal of Virology* **61**, 190–199.

PATERSON, T. & EVERETT, R. D. (1988*a*). Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. *Virology* **156**, 186–196.

PATERSON, T. & EVERETT, R. D. (1988*b*). The regions of the herpes simplex virus type 1 immediate-early protein Vmw175 required for the site specific DNA binding closely correspond to those involved in transcriptional regulation. *Nucleic Acids Research* **16**, 11005–11025.

PATERSON, T. & EVERETT, R. D. (1990). A predominant serine-rich region in Vmw175, the major transcriptional regulator protein of herpes simplex virus type 1, is not essential for virus growth in tissue culture. *Journal of General Virology* **71**, 1775–1783.

PERRY, L. J., RIXON, F. J., EVERETT, R. D., FRAME, M. C. & MCGEOCH, D. J. (1986). Characterization of the IE110 gene of herpes simplex virus type 1. *Journal of General Virology* **67**, 2365–2380.

PRESTON, C. M. (1979). Control of herpes simplex type 1 mRNA synthesis in cells infected with wild-type or the temperature sensitive mutant tsK. *Journal of Virology* **29**, 275–284.

PRESTON, V. G. & FISHER, F. B. (1984). Identification of the herpes simplex virus type 1 gene encoding the dUTPase. *Virology* **138**, 58–68.

QUINLAN, M. P. & KNIPE, D. M. (1985). Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. *Molecular and Cellular Biology* **5**, 957–963.

RIGBY, P. W. J., DIECKMANN, M., RHODES, C. & BERG, P. (1977). Labelling deoxyribonucleic acid to a high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237–251.

ROBERTS, M. S., BOUNDY, A., O'HARE, P., PIZZORNO, M. C., CIUFO, D. M. & HAYWARD, G. S. (1988). Direct correlation between a negative autoregulatory response element at the capsite of the herpes simplex virus type 1 IE175 (α4) alpha promoter and a specific site for the IE175 (ICP4) protein. *Journal of Virology* **62**, 4307–4320.

SACKS, W. R. & SCHAFFER, P. A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. *Journal of Virology* **61**, 829–839.

SHIRAKI, K. & HYMAN, R. W. (1987). The immediate-early proteins of varicella-zoster virus. *Virology* **156**, 423–426.

SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

STOW, N. D. & STOW, E .C. (1986). Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *Journal of General Virology* **67**, 2571–2585.

STOW, N. D. & WILKIE, N. M. (1976). An improved technique for enhanced infectivity with herpes simplex virus type 1 DNA. *Journal of General Virology* **33**, 447–458.

STOW, N. D., MCMONAGLE, E. C. & DAVISON, A. J. (1983). Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. *Nucleic Acids Research* **11**, 8205–8220.

VLECK, C., PACES, V. & SCHWYZER, M. (1989). Nucleotide sequence of the pseudorabies virus immediate early gene, encoding a strong trans-activator protein. *Virus Genes* **2**, 335–346.

WAGNER, E. K. (1985). Individual HSV transcripts: characterisation of specific genes. In *The Herpesviruses*, vol. 3, pp. 45–104. Edited by B. Roizman. New York & London: Plenum Press.

WATSON, R. J. & CLEMENTS, J. B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. *Nature, London* **285**, 329–330.

(Received 10 May 1990; Accepted 20 July 1990)

Journal of General Virology (1990), **71**, 2999–3003. *Printed in Great Britain*

The product of varicella-zoster virus gene 62 autoregulates its own promoter

Graham H. Disney, Tom A. McKee,† Chris M. Preston and Roger D. Everett*

MRC Virology Unit, Church Street, Glasgow G11 5JR, U.K.

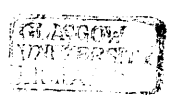
Varicella-zoster virus (VZV) gene 62 encodes a protein with a predicted M_r of 140000 (140K) which has considerable amino acid identity with the major immediate early (IE) protein Vmw175 (ICP4) of herpes simplex virus type 1 (HSV-1). Vmw175 is an essential virus polypeptide with a pivotal role in the activation of early and late viral gene expression and also in the repression of IE gene expression. The VZV 140K protein has been shown to function as a strong transcriptional activator in transfection assays and largely complements for the loss of Vmw175 function in HSV-1. We report the results of cotransfection experiments which demonstrate that the 140K protein strongly represses expression from its own promoter, that of gene 62, thus establishing further functional similarity between it and Vmw175. However, whereas Vmw175 can substitute for the 140K protein in repression of the gene 62 promoter, the 140K protein does not repress the HSV-1 IE3 promoter in the reciprocal experiment. The integrity of a domain of Vmw175 (designated region 2), previously shown to be crucial for repression of the HSV-1 IE3 promoter, is also required for repression of the gene 62 promoter. Moreover, a similar requirement for the highly similar region 2 of the 140K protein for repression is demonstrated, suggesting that VZV 140K protein and HSV-1 Vmw175 autoregulate IE gene expression by a related mechanism.

Varicella-zoster virus (VZV), a member of the neurotropic alphaherpesvirus subfamily, is the causative agent of two human diseases. Primary exposure to the virus results in chickenpox and reactivation following a period of latency gives rise to shingles. Despite being highly infectious in the human population, VZV grows poorly in the available tissue culture systems; the virus is predominantly cell-associated and does not readily yield stable, high titre stocks. Consequently, the identification and mapping of specific VZV functions has been limited. The determination of the complete VZV DNA sequence identified 71 major open reading frames (ORFs) (Davison & Scott, 1986). Furthermore, similarity comparisons between VZV and herpes simplex virus type 1 (HSV-1) sequences has allowed potential functions to be assigned to some of the VZV ORFs. The products of these ORFs can now be studied in more detail using transfection and expression systems.

Gene expression following HSV-1 infection of tissue culture cells is well characterized (for reviews see Wagner, 1985; Everett, 1987). The first class of polypeptides to be expressed during infection has been designated immediate early (IE). Of the five IE polypeptides, four can function as transcriptional regulators and have a key role in mediating control of viral gene expression. They each participate, to varying degrees, in the induction of early and late gene expression, while protein Vmw175 (and possibly protein Vmw63) is also involved in the repression of IE gene expression. Analysis of viruses with temperature-sensitive lesions has demonstrated that two of the IE polypeptides, Vmw175 and Vmw63, are essential for growth in tissue culture. VZV genes 4 and 62 encode products which share similarity with Vmw63 and Vmw175, respectively (Davison & Scott, 1986; McGeoch *et al.*, 1986), suggesting that they encode VZV regulatory polypeptides. This study is concerned with the product of VZV gene 62, which has a predicted M_r of 140000 (140K). Considerable functional similarity between Vmw175 and the VZV 140K protein has previously been demonstrated. The 140K protein functions as a powerful transcriptional trans-activator in transfection assays (Everett & Dunlop, 1984; Everett, 1984; Inchauspe *et al.*, 1989) and, furthermore, largely complements the growth defects that result from loss of Vmw175 function in HSV-1 (Felser *et al.*, 1987, 1988; Disney & Everett, 1990).

As noted above, during HSV-1 infection functional Vmw175 significantly reduces the levels of IE gene expression from those observed in its absence (Honess &

† Present address: Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037, U.S.A.



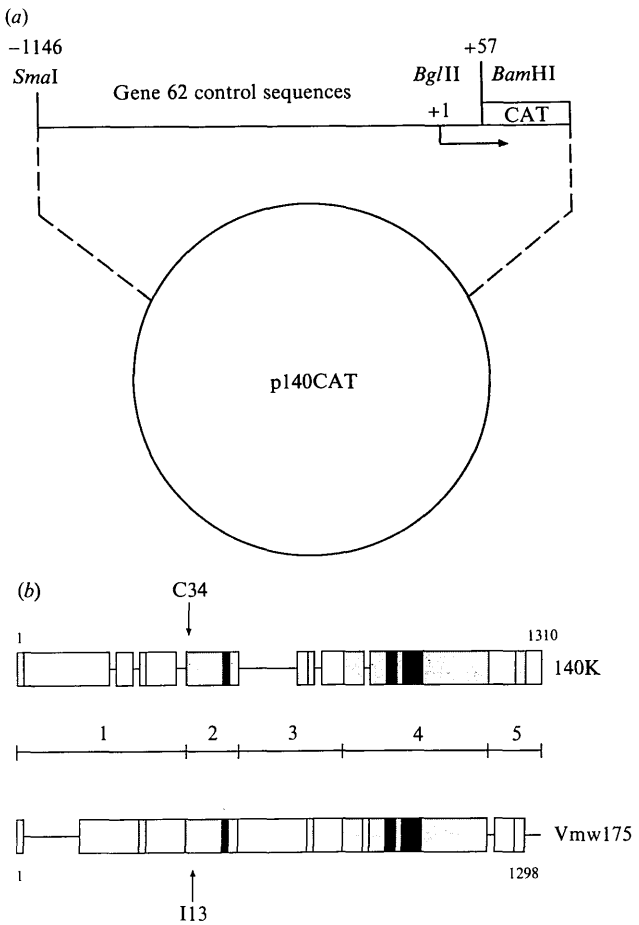


Fig. 1. (*a*) Structure of plasmid p140CAT (McKee *et al.*, 1990). A *Sma*I/*Bgl*II fragment representing VZV gene 62 sequences from −1146 (*Cla*I) to +57 (and containing the complete promoter of the gene) was isolated from plasmid p140BT (McKee *et al.*, 1990) and inserted between the *Sma*I and *Bam*HI sites of the vector pCAT, upstream of the CAT coding sequences, to generate p140CAT. The transcription start site (+1) is indicated. The *Sma*I site is derived from pUC9 polylinker sequences located 5′ to the VZV insert in p140BT. The *Bgl*II site is the result of a two-step mutagenesis protocol in which nucleotide +57 (of gene 62) was first mutated, changing the sequence from AAATTC to GAATTC; the resulting *Eco*RI site was subsequently modified by linker insertion to create the unique *Bgl*II site in p140BT. (*b*) Sequence alignment of the VZV 140K protein and HSV-1 Vmw175. The coding sequences are divided into five regions (1 to 5) on the basis of sequence similarity (McGeoch *et al.*, 1986). Portions of the coding sequences are represented by blocks, the solid lines being spacers introduced to optimize the alignment. The extent of similarity represented by the different shadings is □, low; ▩, 30 to 50% similarity; ■, over 80% similarity. The positions of the insertion mutations in pC34 and pI13 (used in this study) are shown.

Roizman, 1975; Preston *et al.*, 1978; Preston, 1979; Watson & Clements, 1980). Since repression of the HSV-1 IE-3 promoter by Vmw175 has been reproduced in transfection assays (O'Hare & Hayward, 1985), it was of interest to determine whether the VZV 140K protein exhibited similar autoregulation of its own promoter. Therefore, plasmid p140CAT was constructed in which the promoter–leader sequences of VZV gene 62 (which encodes the VZV 140K protein) drive expression of the chloramphenicol acetyltransferase (CAT) gene (Fig. 1*a*). We have previously reported that gene 62 promoter activity is increased about 15-fold by cotransfection with a plasmid expressing Vmw65, the HSV-1 virion-associated IE promoter transactivator (McKee *et al.*, 1990). The ability of the VZV 140K protein to repress the gene 62 promoter was therefore determined both in the absence and in the presence of stimulation by Vmw65. Plasmid p140SV, which contains the VZV gene 62 transcription unit (without its own promoter) linked to the simian virus 40 (SV40) early promoter and enhancer region, was used to express VZV 140K protein. The use of p140SV (rather than a plasmid which expresses VZV 140K protein from the gene 62 promoter) reduces difficulties in the interpretation of the results which would arise from promoter competition effects and through repression of the gene 62 promoter in the VZV 140K protein expression plasmid.

BHK cells, at a density of 10^6 cells per 50 mm dish, were transfected (by calcium phosphate precipitation; Corsalo & Pearson, 1981) with 4 μg p140CAT in the presence or absence of pMC1 (which expresses Vmw65) and increasing amounts of p140SV. The total amount of plasmid DNA in each transfection was equalized to 14 μg by the addition of pUC9. CAT assay extracts were prepared after 48 h and CAT activities determined as described by Gorman *et al.* (1982). The protein concentrations of the extracts were determined by the method of Bradford (1976) and the percentage conversion from substrate to product per microgram protein was calculated. The CAT activities of extracts prepared from cells transfected with test plasmids were expressed as a percentage of the control value (no test plasmid). The results, depicted graphically in Fig. 2(*a*), are from a typical repression experiment. Transfection of p140CAT alone into cells resulted in 4% conversion per microgram protein (representing a total conversion of 36% in the assay) and the inclusion of pMC1 in the transfection increased CAT activity 30-fold (determined using suitable extract dilutions). The results clearly show that both the basal and Vmw65-stimulated levels of CAT activity from p140CAT were repressed up to 50-fold by the addition of increasing amounts of p140SV. This repression is not due to promoter competition effects because transfection with equivalent molar amounts of pSVEB (which contains the SV40 promoter region present in p140SV but lacks coding sequences) resulted in levels of repression which were insignificant compared to those induced by p140SV (Fig. 2*a*). Similar results were obtained using Vero cells (data not shown).

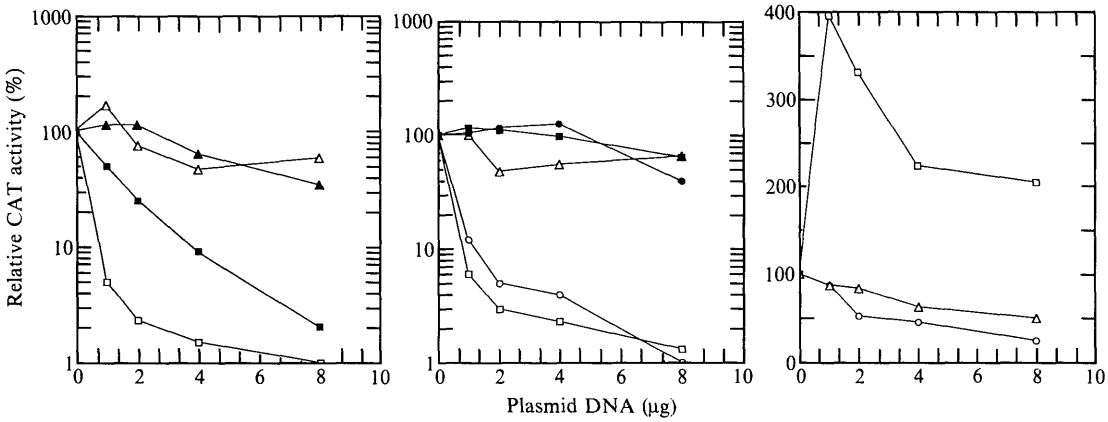


Fig. 2. Graphs showing repression of expression from VZV gene 62 promoter-leader sequences (in p140CAT) by VZV 140K protein and Vmw175 (and their mutant derivatives), and activation (rather than repression) of the HSV-1 IE3 promoter (in pIE3CAT) by the VZV 140K protein. BHK cells were transfected with 4 μg of p140CAT (or pIE3CAT) and increasing amounts of test plasmids in the presence or absence of pMC1 (which expresses Vmw65). The test plasmids used express the following polypeptides: VZV 140K protein (p140SV); Vmw175 (p175); insertion mutant derivatives of the 140K protein and Vmw175, respectively (pC34 and pI13). The negative control plasmid, pSVEB, lacks coding sequences present in the test plasmids and is consequently approximately half the size. Hence the actual amounts used in transfections were 0·5, 1, 2 and 4 μg to maintain a molar equivalence of common sequences. (*a*) Repression of the gene 62 promoter by 140K protein in the absence and presence of pMC1 (■, p140SV; ▲, pSVEB; □, p140SV + pMC1; △, pSVEB + pMC1). (*b*) Repression of the Vmw65 (pMC1)-stimulated gene 62 promoter by 140K protein, Vmw175 and their insertion mutant derivatives (□, p140SV; ○, p175; △, pSVEB; ■, pC34; ●, pI13). (*c*) Activation of the Vmw65 (pMC1)-stimulated IE3 promoter by 140K protein. Plasmid pIE3CAT (in which the HSV-1 IE3 promoter drives expression of the CAT gene) replaced p140CAT in this experiment (□, p140; ○, p175; △, SVEB). The data shown in (*a*) and (*b*) are derived from a typical titration experiment. The degree of repression observed in titration experiments was variable and almost certainly reflects the variation in levels of expression from the gene 62 promoter noted in the text. However, these experiments have been repeated a minimum of three times [with the exception of titration in the absence of pMC1 in (*a*), which was performed twice] and in all cases the pattern of results was consistent with those above. The data in (*c*) are the result of a single titration experiment.

It should be noted that repression of the gene 62 promoter by p140SV was most clearly observed when the levels of CAT activity in control transfections were high (about 50 to 200-fold above background). If the cells transfected poorly then the degree of repression was correspondingly reduced (as might be expected). Since repression was always most clearly observed with high level expression from the gene 62 promoter, most experiments included pMC1.

The results presented above clearly demonstrate that cotransfection of p140SV represses the gene 62 promoter. Given the functional similarities of VZV 140K protein and Vmw175 in trans-activation transfection assays (Everett, 1984), it was of interest to determine whether the two proteins were functionally interchangeable in repression transfection assays. Therefore, p140CAT was contransfected with increasing amounts of p175. The results show that Vmw175 represses the gene 62 promoter with an efficiency similar to that of the VZV 140K protein (Fig. 2*b*). The converse experiment to determine whether the 140K protein is able to repress the HSV-1 IE-3 promoter was performed in Vero cells, the cell type in which repression of the IE3 promoter by Vmw175 was first demonstrated (O'Hare & Hayward, 1985). (As noted above, the 140K protein efficiently represses the gene 62 promoter in Vero cells as well as BHK cells.) Plasmid pIE3CAT (in which the HSV-1 IE3 promoter drives expression of the CAT gene) replaced p140CAT as the reporter plasmid in these experiments. Fig. 2(*c*) shows the results of a single experiment in which pIE3CAT was contransfected into cells with pMC1 and increasing amounts of test plasmids. The pMC1-stimulated level of expression from pIE3CAT was of the same order of magnitude as that from pMC1-stimulated p140CAT. IE3 promoter activity showed a small reduction in the presence of pSVEB, a maximum of twofold reduction being observed with the largest amount of plasmid. Titration of p175 led to a greater reduction in activity, fourfold in the presence of 8 μg p175. Although this was less efficient than in previous assays (Paterson & Everett, 1988), it does confirm repression of the IE3 promoter by Vmw175. In marked contrast, titration of p140SV resulted in an increase in IE3 promoter activity of two- to fourfold. In agreement with this, it was found that in BHK cells p140SV strongly trans-activated the basal level of expression from the IE-3 promoter (in the absence of pMC1) approximately 40-fold (data not shown). Therefore, although both VZV

140K protein and Vmw175 repress their respective promoters, the mechanisms by which this occurs are not entirely interchangeable. This failure of the VZV 140K protein to repress the IE-3 promoter is consistent with the properties of a recombinant virus (HSV140) in which the Vmw175-encoding sequences have been replaced by those of the VZV 140K protein in a transcription unit with the IE-3 regulatory sequences; infection by HSV140 leads to high levels of VZV 140K protein expression (Disney & Everett, 1990).

The integrity of homology region 2 of Vmw175 (amino acid residues 315 to 484; Fig. 1*b*) has been shown to be of crucial importance for repression of the IE-3 promoter (Paterson & Everett, 1988; DeLuca & Schaffer, 1988; Shepard *et al.*, 1989). To determine whether region 2 of Vmw175 was also involved in the repression of the gene 62 promoter, plasmid pI13 (Paterson & Everett, 1988) was cotransfected in increasing amounts with p140CAT. Plasmid pI13 contains an in-frame insertion of four amino acids in the proline codon at position 324 of Vmw175 (region 2; see Fig. 1*b*). The effect of the mutation is essentially to eliminate repression of the IE-3 promoter by Vmw175 (Paterson & Everett, 1988). The same effect was observed in cotransfections of p140CAT and pI13 (Fig. 2*b*). Therefore the same region of Vmw175 is involved in the repression of both the gene 62 and IE-3 promoters.

Plasmid pC34 encodes a mutant form of the VZV 140K protein which is analogous to the protein expressed by pI13. It was derived from p140 (McKee *et al.*, 1990) by the method of Paterson & Everett (1988) and contains a mutation resulting in the insertion of four amino acids into the glycine codon at position 471 of the 140K protein, which lies within region 2 (amino acid residues 467 to 641; see Fig. 1*b*). Like pI13, pC34 was unable to repress p140CAT (Fig. 2*b*). The interpretation of this result is potentially complicated because pC34 uses the gene 62 promoter instead of the SV40 early promoter in p140SV. This raises the possibility that the lack of repression by pC34 is not due to the mutation in region 2 of the VZV 140K protein but that autoregulation of C34 polypeptide expression from pC34 results in insufficient protein to repress the gene 62 promoter in p140CAT as well. However, it is difficult to envisage how the C34 polypeptide might repress its own expression from pC34 without also reducing gene 62 promoter activity in p140CAT. In addition, in trans-activation transfection assays the C34 mutation, like that of pI13 (Paterson & Everett, 1988), eliminates the ability of plasmid pC34 to trans-activate the HSV-1 glycoprotein D (gD) promoter (whose activity is increased approximately 100-fold by the 140K protein; results not shown). Therefore it is most likely that, as with the mutations in region 2 of Vmw175 (Paterson & Everett, 1988; DeLuca & Schaffer, 1988; Shepard *et al.*, 1989), the C34 mutation results in an inactive polypeptide. The experiment therefore demonstrates that the same similar regions of Vmw175 and the VZV 140K protein are involved in the repression of their respective promoters.

The mechanism of repression of the IE-3 promoter by Vmw175 requires a functional Vmw175 DNA-binding domain (Paterson & Everett, 1988; Shepard *et al.*, 1989) and the presence of a cognate DNA-binding site at the cap site (Roberts *et al.*, 1988). This binding site includes a consensus sequence (ATCGTC) common to many, but not all, sequences with which Vmw175 can associate (Muller, 1987; Faber & Wilcox, 1986; Michael *et al.*, 1988). We attempted to determine whether analogous sequences in the region of the gene 62 cap site were required for repression by the VZV 140K protein by constructing deletion variants of p140CAT in which promoter sequences upstream of the TATA box remained intact but leader or cap site–leader sequences were deleted. It was found, however, that the intrinsic activities of both deleted promoters were consistently reduced by at least 10-fold relative to p140CAT, which implies that sequences in the gene 62 leader region contribute to the efficiency of its expression. The activity of both promoters was increased 15- to 30-fold when pMC1 was present in transfections (as with the intact promoter) but promoter activity was still at least 10-fold less than that of the pMC1-activated gene 62 promoter. As noted above, low levels of promoter activity result in relatively poor levels of repression and therefore it was not possible to draw unequivocal conclusions from the results obtained with these constructs.

In conclusion, we have shown that the functional similarity of Vmw175 of HSV-1 and the VZV 140K protein extends to the repression of their respective promoters and that Vmw175 is also able to repress the VZV gene 62 promoter. Since the C34 mutation, which inactivates repression by the VZV 140K protein, is located within a region similar to that of the DNA-binding domain of Vmw175, we speculate that the mechanism of repression used by the VZV 140K protein might involve sequence-specific DNA binding. However, our attempts to identify a 140K protein DNA recognition element by deletion of cap site or leader sequences from p140CAT were inconclusive. We have also carried out preliminary studies to investigate the DNA-binding properties of the 140K protein by using nuclear extracts from cells infected with HSV140 (which contain 140K protein in abundance) in gel retardation DNA-binding assays with probes encompassing the entire gene 62 promoter region in p140CAT. Although we have been able to show that the VZV 140K protein binds to DNA, as yet we have been unable to define any specific VZV 140K protein recognition sequence in the

gene 62 promoter region. Further elucidation of the properties of VZV 140K protein, and the comparison of its functions and mechanisms of action with those of Vmw175, will be facilitated by the availability of more purified preparations of the protein. These studies are in progress.

The authors wish to thank Dr J. McLauchlan for synthesis of oligonucleotides. T.A.M. was supported by an MRC fellowship and G.H.D. by an MRC postgraduate studentship.

References

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

CORSALO, C. M. & PERSON, M. L. (1981). Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. *Somatic Cell Genetics* 7, 603–616.

DAVISON, A. J. & SCOTT, J. E. (1986). The complete DNA sequence of varicella-zoster virus. *Journal of General Virology* **67**, 1759–1816.

DELUCA, N. A. & SCHAFFER, P. A. (1988). Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. *Journal of Virology* **62**, 732–743.

DISNEY, G. H. & EVERETT, R. D. (1990). A herpes simplex virus type 1 recombinant with both copies of the Vmw175 coding sequences replaced by the homologous varicella-zoster virus open reading frame. *Journal of General Virology* **71**, 2681–2689.

EVERETT, R. D. (1984). Transactivation of transcription by herpesvirus products. Requirements for two HSV-1 immediate-early polypeptides for maximum activity. *EMBO Journal* **3**, 3135–3141.

EVERETT, R. D. (1987). The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products. *Anticancer Research* 7, 589–604.

EVERETT, R. D. & DUNLOP, M. (1984). Transactivation of plasmid borne promoters by adenovirus and several herpes group viruses. *Nucleic Acids Research* **12**, 5969–5978.

FABER, S. W. & WILCOX, K. W. (1986). Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. *Nucleic Acids Research* **14**, 6067–6083.

FELSER, J. M., STRAUS, S. E. & OSTROVE, J. M. (1987). Varicella-zoster virus complements herpes simplex virus type 1 temperature-sensitive mutants. *Journal of Virology* **61**, 225–228.

FELSER, J. M., KINCHINGTON, P. R., INCHAUSPE, G., STRAUS, S. E. & OSTROVE, J. M. (1988). Cell lines containing varicella-zoster virus open reading frame 62 and expressing the "IE" 175 protein complement ICP4 mutants of herpes simplex virus type 1. *Journal of Virology* **62**, 2076–2082.

GORMAN, C. M., MOFFAT, L. F. & HOWARD, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Molecular and Cellular Biology* **2**, 1044–1051.

HONESS, R. W. & ROIZMAN, B. (1975). Regulation of herpes virus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proceedings of the National Academy of Sciences, U.S.A.* **72**, 1276–1280.

INCHAUSPE, G., NAGPAL, S. & OSTROVE, J. M. (1989). Mapping of two varicella-zoster virus-encoded genes that activate the expression of viral early and late genes. *Virology* **173**, 700–709.

MCGEOCH, D. J., DOLAN, J. A., DONALD, S. & BRAUER, D. H. K. (1986). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. *Nucleic Acids Research* **14**, 1727–1764.

MCKEE, T. A., DISNEY, G. H., EVERETT, R. D. & PRESTON, C. M. (1990). Control of expression of the varicella-zoster virus major immediate early gene. *Journal of General Virology* **71**, 897–906.

MICHAEL, N., SPECTOR, D., MAVROMARA-NAZOS, P., KRISTIE, P. M. & ROIZMAN, B. (1988). The DNA binding properties of the major regulatory protein alpha 4 of herpes simplex virus. *Science* **239**, 1531–1534.

MULLER, M. T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. *Journal of Virology* **61**, 858–865.

O'HARE, P. & HAYWARD, G. S. (1985). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. *Journal of Virology* **56**, 723–733.

PATERSON, T. & EVERETT, R. D. (1988). Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. *Virology* **156**, 186–196.

PRESTON, C. M. (1979). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type or the temperature sensitive mutant *ts*K. *Journal of Virology* **29**, 275–284.

PRESTON, V. G., DAVISON, A. J., MARSDEN, H. S., TIMBURY, M. C., SUBAK-SHARPE, J. H. & WILKIE, N. M. (1978). Recombinants between herpes simplex virus types 1 and 2: analyses of genome structures and expression of immediate early polypeptides. *Journal of Virology* **28**, 499–517.

ROBERTS, M. S., BOUNDY, A., O'HARE, P., PIZZORNO, M. C., CIUFO, D. M. & HAYWARD, G. S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific site for the IE175 (ICP4) protein. *Journal of Virology* **62**, 4307–4320.

SHEPARD, A. A., IMBALZANO, A. N. & DELUCA, N. A. (1989). Separation of primary structural components conferring autoregulation, transactivation and DNA binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. *Journal of Virology* **63**, 3714–3728.

WAGNER, E. K. (1985). Individual HSV transcripts: characterisation of specific genes. In *The Herpesviruses*, vol. 3, pp. 45–104. Edited by B. Roizman. New York & London: Plenum Press.

WATSON, R. J. & CLEMENTS, J. B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. *Nature, London* **285**, 329–330.

(Received 29 June 1990; Accepted 31 August 1990)

Journal of General Virology (1990), **71**, 897–906. *Printed in Great Britain*

Control of expression of the varicella-zoster virus major immediate early gene

Tom A. McKee, Graham H. Disney, Roger D. Everett and Chris M. Preston*

Medical Research Council Virology Unit, Church Street, Glasgow, G11 5JR, U.K.

The cis-acting DNA sequences and trans-acting proteins that control the expression of the major immediate early (IE) gene of varicella-zoster virus (VZV) were investigated. The location of the IE mRNA 5′ terminus was determined by primer extension and S1 nuclease analyses and the functional activities of DNA sequences upstream of this site were analysed by a transfection assay. The VZV IE promoter exhibited low activity in BHK and HeLa cells, but was trans-activated by the herpes simplex virus type 1 (HSV-1) virion protein Vmw65. DNA sequences between positions −131 and +57 were responsible for promoter activity, whereas sequences between −410 and −131 mediated the response to Vmw65. Two short elements in the −410 to −131 region formed protein–DNA complexes with HeLa cell nuclear proteins and formed a ternary complex when Vmw65 was added. One of the elements, ATGTAAATGAAAT, possessed a strong similarity to the HSV-1 TAATGARAT. The VZV homologue of Vmw65, encoded by open reading frame (ORF) 10, failed to trans-activate expression from HSV-1 or VZV IE promoters and did not form a ternary complex with functional TAATGARAT elements and HeLa cell proteins. Therefore, stimulation of VZV IE transcription by Vmw65 can occur by a mechanism similar to that employed by HSV-1, but VZV ORF 10 does not function as a trans-activator of IE gene expression.

Introduction

Varicella-zoster virus (VZV), an alphaherpesvirus, is the causative agent of two human diseases, chicken pox and shingles. Primary exposure to VZV results in chicken pox and, after an initial infection, the virus becomes latent in the dorsal root ganglia. Reactivation of latent virus results in shingles, a painful vesicular rash which is usually confined to the distribution of a single sensory nerve root. Understanding of the biology of VZV has been hindered by difficulties in propagating viable cell-free virus at high titre in tissue culture. The determination of the complete VZV DNA sequence (Davison & Scott, 1986) has enabled the structure and function of the products of the 71 major open reading frames (ORFs) to be investigated, thus bypassing, to some extent, the problem of virus isolation.

Regulation of herpesvirus gene expression has been most intensively studied in the case of herpes simplex virus type 1 (HSV-1), in which the genes can be classified as immediate early (IE), early or late, depending on their kinetics of expression and response to inhibitors of macromolecular synthesis (Honess & Roizman, 1974; Clements *et al.*, 1977). The five IE genes of HSV-1 are transcribed when infection occurs in the absence of *de novo* protein synthesis and at least three of the IE proteins are important trans-activators that are required for maximal expression of early and late genes (Honess & Roizman, 1974; Clements *et al.*, 1977; Preston, 1979; Everett, 1984; O'Hare & Hayward, 1985*a*; Sacks *et al.*, 1985). The HSV-1 IE genes possess one or more copies of the element TAATGARAT (where R is a purine), a cis-acting motif that mediates stimulation of IE transcription by the virion protein Vmw65 (otherwise named VP16 or α-TIF), between 100 and 600 base pairs (bp) upstream of the mRNA initiation sites (Post *et al.*, 1981; Mackem & Roizman, 1982; Campbell *et al.*, 1984; Preston *et al.*, 1984). Vmw65 does not bind directly to DNA (Marsden *et al.*, 1987), but interacts with one or more cellular factors to form a ternary complex, IEC, that binds to TAATGARAT (McKnight *et al.*, 1987; Preston *et al.*, 1988; O'Hare *et al.*, 1988). One of the cellular components of IEC appears to be a ubiquitous protein with various names, including NFIII (Pruijn *et al.*, 1986), OBP100 (Baumruker *et al.*, 1988; Sturm *et al.*, 1987), OTF-1 (Gerster & Roeder, 1988), TRF (O'Hare & Goding, 1988) and α-H1 (Kristie & Roizman, 1987), that recognizes an octamer element of consensus ATGCAAAT as well as TAATGARAT. Although NFIII binds to a range of target sequences, complex IEC is formed efficiently only when a match to TAATGARAT is present (Gerster & Roeder, 1988; O'Hare *et al.*, 1988;

ApRhys *et al.*, 1989). The formation of IEC upstream of IE mRNA initiation sites enables a strongly acidic activating domain, located within the C-terminal 80 amino acids of Vmw65, to interact with a transcription complex and thereby augment transcription (Dalrymple *et al.*, 1985; Triezenberg *et al.*, 1988; Sadowski *et al.*, 1988; Cousens *et al.*, 1989). At the biological level the stimulation of IE transcription by Vmw65 is important for virus replication after infection at low m.o.i. or *in vivo* (Ace *et al.*, 1989).

Four VZV IE gene products have been detected in infected cells (Shiraki & Hyman, 1987), but little is known about the regulation of their expression. The product of VZV ORF 62 (and ORF 71, since this gene is diploid), a polypeptide of calculated M_r 139989, is thought to be equivalent to HSV-1 Vmw175, which is a major trans-activator of HSV-1 early and late gene transcription. This conclusion is based on their comparable locations in the short repeat region of the genome (Davison & Scott, 1985, 1986), sequence homology (Davison & Scott, 1986) and the ability to stimulate gene expression in transfection assays (Everett & Dunlop, 1984; Everett, 1984). Furthermore, Vero cells that contain integrated copies of ORF 62 (F114 cells) complement the growth of HSV-1 temperature-sensitive mutants with mutations in the coding sequences of Vmw175 (Felser *et al.*, 1988). Interestingly, the transcription of ORF 62 in F114 cells is stimulated by HSV-1 infection and this effect occurs even in the absence of protein synthesis, suggesting that a component of the HSV-1 inoculum, possibly Vmw65, is responsible (Felser *et al.*, 1988). A homologue of Vmw65, ORF 10, has been identified in the VZV genome (Dalrymple *et al.*, 1985). The two proteins share homology over the N-terminal 410 amino acids but, strikingly, ORF 10 is 81 amino acids shorter, lacking the acidic C-terminal activating region of Vmw65 (Dalrymple *et al.*, 1985).

Studies with HSV-1 indicate that the efficiency of IE gene expression has important implications for the biological properties of the virus (Ace *et al.*, 1989). We have therefore investigated the cis-acting sequences that control the expression of VZV ORF 62 and have clarified the functional relationship between Vmw65 and VZV ORF 10.

Methods

Plasmids. Plasmid p140CAT contains the region from −1146 to +57 of the VZV ORF 62 gene inserted into the chloramphenicol acetyltransferase (CAT) vector pCAT, which was derived from pBLW2 (Gaffney *et al.*, 1985) and contains restriction sites for *Hin*dIII, *Pst*I, *Sma*I and *Bam*HI upstream of the CAT coding region. The ORF 62 promoter region in p140CAT was derived from pVZVSstf (kindly provided by Dr A. Davison), which contains the terminal *Sst*I fragment from the short repeat region of VZV cloned into the *Pst*I site of pAT153 by GC tailing, thus effectively creating a *Pst*I site at the genome terminus. Plasmid p140 contains the VZV ORF 62 gene from the *Cla*I site at −1146 to the genome terminus *Pst*I site of pVZVSstf inserted between the *Acc*I and *Pst*I sites of a pUC9 plasmid from which the *Eco*RI site had been removed by cutting and filling in. Plasmid p140ET is a derivative of p140, in which an *Eco*RI site had been created at position +57 by oligonucleotide mutagenesis of nucleotide +57, changing the sequence AAATTC to GAATTC (Fig. 1). The new *Eco*RI site in p140ET was converted to a *Bgl*II site (p140BT) by cleaving with *Eco*RI, filling in and insertion of an oligonucleotide linker (GAGATCTC). Using this novel *Bgl*II site and the *Sma*I site in vector sequences immediately 5′ of the VZV insert, the ORF 62 region from −1146 to +57 of p140BT was cloned between the *Sma*I and *Bam*HI sites of pCAT to give p140CAT. Sequential 5′ deletions of p140CAT were produced by cleaving p140CAT at the unique *Hin*dIII and *Sal*I (−410) or *Xho*I (−131) sites, filling in the 5′ overhangs with T4 DNA polymerase and all four deoxynucleoside triphosphates and religating, to yield p140Δ410CAT and p140Δ131CAT.

The ORF 10 of VZV was cloned by introducing an 8 bp *Eco*RI linker (GGAATTCC) into a *Dra*I site 25 bp upstream of the ORF 10 initiating ATG, then subcloning a 1290 bp *Eco*RI/*Sph*I fragment containing the entire ORF into pTZ18R (Pharmacia), to yield two apparently identical isolates, pTZORF10A and pTZORF10B. The 1290 bp *Eco*RI/*Sph*I fragment from pTZORF10A was recloned between the *Eco*RV and *Sph*I sites of pMC1.in17 (Ace *et al.*, 1988). Plasmid pMCl contains the HSV-1 gene encoding Vmw65 (Campbell *et al.*, 1984) and pMC1.in17 contains a 12 bp insertion, very near to the C terminus of the coding sequences, that does not affect the activity of Vmw65 (Ace *et al.*, 1988). The use of pMC1.in17, as opposed to pMC1, is irrelevant to the work described here. The resultant plasmid, pMCORF10, contains the VZV ORF 10 coding sequences in place of the Vmw65 coding sequences.

Plasmids pGEMTIF and the insertion mutant pGEMTIF.in15, described previously (Ace *et al.*, 1988), were used for *in vitro* transcription and translation. Plasmid pFJ3, containing the β-galactosidase gene controlled by simian virus 40 promoter and enhancer sequences was kindly provided by Dr F. J. Rixon. Plasmid pRR55, containing the CAT gene under the control of human cytomegalovirus (HCMV) strain AD169 IE gene promoter and enhancer sequences (Fickenscher *et al.*, 1989), was kindly provided by Dr R. Rüger. Plasmid pIE3CAT, containing the CAT gene under HSV-1 IE gene 3 control (Stow *et al.*, 1986), was kindly supplied by Dr T. Paterson.

Cells. Baby hamster kidney (BHK) cells were grown in Eagle's medium containing 10% tryptose phosphate and 10% newborn calf serum. Human foetal lung (HFL) cells were grown in Eagle's medium containing 10% foetal calf serum and HeLa cells were grown in Dulbecco's medium containing 2·5% newborn and 2·5% foetal calf serum. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to all media.

Preparation of VZV-infected cell RNA. HFL cells were infected by the addition of a one-sixth quantity of VZV-infected HFL cells showing 70 to 80% c.p.e. When the culture reached 70 to 80% c.p.e. cytoplasmic RNA was extracted as described by Preston (1977).

Primer extension. A 29 base oligonucleotide (5′GGGGTGTA-GAGCGCTGCATCGGCGGCGTA3′), complementary to a region close to the N terminus of VZV ORF 62 (Fig. 1), was 5′ end-labelled with ^{32}P using T4 polynucleotide kinase. Ten μg of cytoplasmic RNA was mixed with radiolabelled oligonucleotide in hybridization buffer (40 mM-KCl and 50 mM-Tris–HCl pH 8·3) and heated to 90 °C. The temperature was decreased to 45 °C over 1 h, $MgCl_2$ was added to a final concentration of 5 mM and the four deoxynucleoside triphosphates were added at a concentration of 0·5 mM. AMV reverse

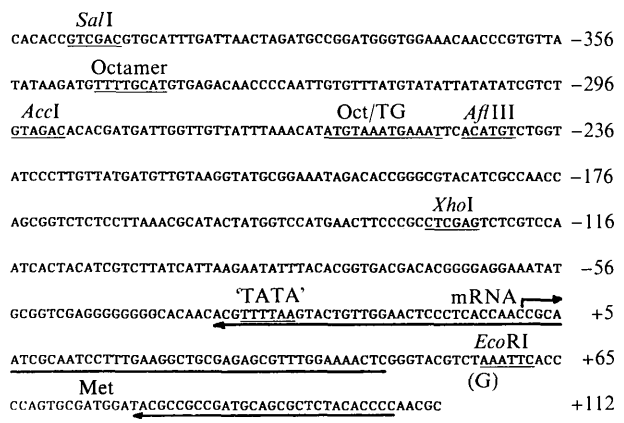


Fig. 1. DNA sequences in the upstream regulatory region of VZV ORF 62. Restriction endonuclease cleavage sites relevant to the results are underlined. The mRNA start site is designated +1 by convention and a TATA box homology TTTTAA is found at positions −25 to −30. A 13 bp sequence centred at −255 contains both octamer and TAATGARAT motifs and a simple octamer motif in inverse orientation is present at nucleotides −346 to −339. Sequences from which oligonucleotides for use in primer extension and S1 nuclease analysis were constructed are underlined. The A residue at position +57 was changed to a G residue during the construction of p140CAT.

transcriptase (10 units) was added and the reaction continued at 45 °C for 30 min. Reaction mixtures were extracted with phenol–chloroform and precipitated with ethanol. Precipitates were dissolved in 90% formamide in 90 mM-Tris–borate pH 8·3 and 2·5 mM-EDTA, and heated at 90 °C for 1 min. Samples were electrophoresed on a 12% sequencing polyacrylamide gel and radiolabelled bands detected by autoradiography.

Nuclease S1 mapping. A 77 base oligonucleotide (5'GAGTTTTCCAAACGCTCTCGCACCTTCAAAGGATTGCGATTGCGTTGGTGAGGGAGTTCCAACAGTACTTAAAACGT3') was synthesized and 5' end-labelled with ^{32}P using T4 polynucleotide kinase. The oligonucleotide was designed to overlap the VZV major IE RNA 5' terminus predicted by primer extension analysis (Fig. 1). Radiolabelled oligonucleotide was hybridized with 10 μg of VZV-infected cell RNA at 42 °C for 16 h, the reaction mixture treated with nuclease S1 and the products were electrophoresed on denaturing 12% polyacrylamide sequencing gels, as described previously (Rixon & Clements, 1982; Preston *et al.*, 1984).

Transfections. Monolayers of 3×10^6 BHK cells in 50 mm diameter Petri dishes were transfected with calcium phosphate-precipitated DNA, as described by Cordingley *et al.* (1983). Precipitates contained 5 μg of CAT-containing plasmid and 0·5 μg of pFJ3. Three μg of pMC1 was added, where appropriate, and the total amount of DNA was adjusted to 9 μg by the addition of pUC18 DNA. Cells were harvested after 18 h at 37 °C.

Monolayers of 10^6 HeLa cells in 50 mm diameter Petri dishes were transfected with calcium phosphate-precipitated DNA, as described by Corsalo & Pearson (1981). Precipitates contained 8 μg of CAT-containing plasmid, 1 μg of pFJ3, 5 μg of pMC1, where appropriate, and pUC18 DNA to give a total of 16 μg. HeLa cells were harvested after incubation at 37 °C for 40 h.

CAT and β-galactosidase assays. Cell extracts were prepared and assayed for CAT activity as described by Gorman *et al.* (1982). β-Galactosidase was assayed as described by Spaete & Mocarski (1985). Before conducting CAT assays, extracts were adjusted to contain equal β-galatosidase activities, to correct for variations in transfection efficiencies. In practice this resulted in dilutions never greater than threefold for BHK cell extracts and fivefold for HeLa cell extracts. To quantify CAT assays radioactive spots were cut out and the percentage of substrate acetylated was calculated after scintillation counting. The amount of extract was varied to ensure that the linear response range of the assay was used.

In vitro *transcription and translation.* Plasmids were cleaved with appropriate restriction enzymes and *in vitro* transcription and translation was carried out as described by Ace *et al.* (1988). Duplicate translations were performed in either the presence or absence of [^{35}S]methionine. Radiolabelled samples were analysed by SDS–PAGE (Preston, 1979) and non-radioactive samples were used in gel retardation assays.

Gel retardation assays. HeLa cell nuclear extracts and extracts of HSV-1 particles were prepared as described previously (Preston *et al.*, 1988). DNA fragments were radiolabelled by incubation with T4 DNA polymerase and two non-radioactive and two ^{32}P-labelled deoxynucleoside triphosphates and purified from polyacrylamide gels. Binding reactions contained 10 mM-HEPES pH 7·9, 0·6 mM-dithiothreitol, 2·3 mM-$MgCl_2$, 0·1 mg/ml bovine serum albumin, 4 μg poly(dI).poly(dC), approximately 0·2 ng radiolabelled DNA fragment, 5 μg HeLa cell nuclear extract and, where appropriate, HSV-1 extract (0·5 μg) or a sample containing Vmw65 or VZV ORF 10 synthesized *in vitro*. After incubation at 25 °C for 30 min reaction mixtures were loaded onto a 3·5% polyacrylamide gel, electrophoresis was carried out for 3·5 h at 160 V and the gel was dried and exposed for autoradiography. If assays involved the use of competitor oligonucleotides then a 100-fold molar excess was incubated with the reaction components for 15 min prior to the addition of a radiolabelled DNA fragment. Competitors used were all 36 bp, one containing a CCAAT sequence (Preston *et al.*, 1988), one the adenovirus major late promoter (MLP) recognition sequence (Preston *et al.*, 1988) and the other two containing ATGCAAATCATGT (octamer) or GCGGTAATGAGAT (TAATGARAT) in an otherwise identical sequence context.

DNase I protection assays. DNA fragments were uniquely 3' end-labelled and used for gel retardation analysis. Protein–DNA complexes were eluted from polyacrylamide gels, and DNase I protected regions were identified as described by Preston *et al.* (1988).

Results

Mapping the 5' terminus of the VZV major IE RNA

To analyse the regulation of VZV IE transcription it was first necessary to determine the location of the mRNA 5' terminus. Two techniques were used for this analysis. First, the length of the 5' untranslated region was determined by primer extension analysis using a 5' ^{32}P-labelled synthetic oligonucleotide complementary to 29 bases of the N-terminal coding sequence of ORF 62. Fig. 2 shows that an extension product of 105 bases was produced from VZV-infected cell RNA, but not mock-infected cell RNA, indicating that the length of the 5' non-coding region is 71 bases. No extension products longer than 105 bases were detected (results not shown). To confirm the primer extension result and to exclude the existence of an intron within the 5' non-coding region,

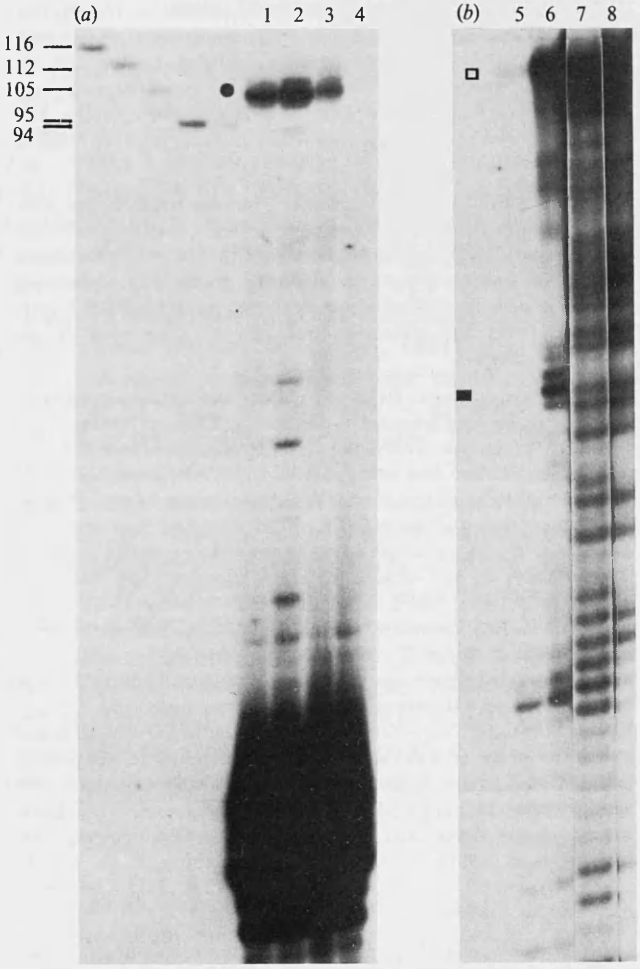


Fig. 2. Mapping the 5′ end of the mRNA. (*a*) Primer extension analysis was performed on three RNA samples prepared from different batches of VZV-infected HFL cells (lanes 1, 2 and 3) and from mock-infected HFL cells (lane 4). Markers of the indicated sizes (bp) were run in parallel and the extended product is labelled (●). (*b*) Nuclease S1 analysis was performed on RNA prepared from VZV-infected HFL cells (lane 5) or mock-infected cells (lane 6). G and G + A lanes of the oligonucleotide used in the assay were included as size markers and appropriate exposures are shown (lanes 7 and 8). Undigested oligonucleotide (□) and digested product (■) are labelled.

nuclease S1 analysis was carried out using a radiolabelled probe that spanned the 5′ terminus predicted by primer extension. As shown in Fig. 2(*b*) a cluster of protected DNA bands of 45, 46 and 47 bases was observed when VZV-infected cell RNA, but not mock-infected cell RNA, was used, in agreement with the result of the primer extension analysis and excluding the possibility of splicing in the 5′ untranslated region.

Inspection of the sequence (Fig. 1) reveals the element TTTTAA to be 25 to 30 bp upstream of the mRNA start site. This probably represents the TATA box, a cis-acting element known to be an important signal for the correct initiation of transcription by RNA polymerase II.

Functional activity of VZV IE gene control sequences

Having ascertained the location of the mRNA 5′ terminus a plasmid, p140CAT, was constructed in which sequences from +57 to −1146 were inserted 5′ to the CAT gene. A comparison of the activity of this plasmid with pIE3CAT and pRR55, in which the HSV IE3 and the HCMV IE control sequences, respectively, direct expression of CAT, is shown in Fig. 3. The activities of

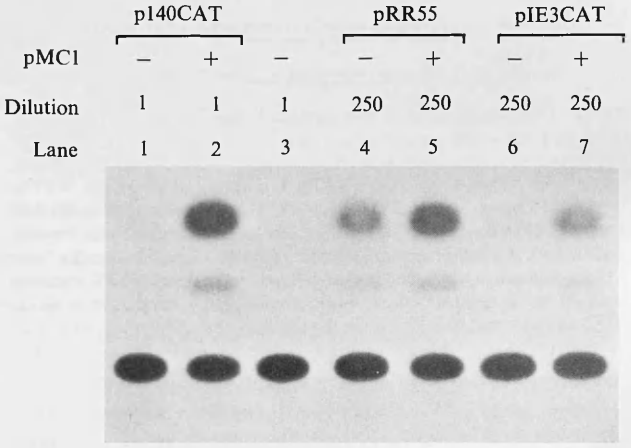


Fig. 3. Comparison of the activities of IE control sequences of VZV (lanes 1 and 2), HCMV (lanes 4 and 5) and HSV-1 (lanes 6 and 7) linked to the CAT gene. Lane 3 represents mock-transfection. In lanes marked + (lanes 2, 5 and 7) reporter plasmids were cotransfected with pMC1, whereas in lanes marked − (lanes 1, 4 and 6) pUC18 was added in order to equalize plasmid quantities.

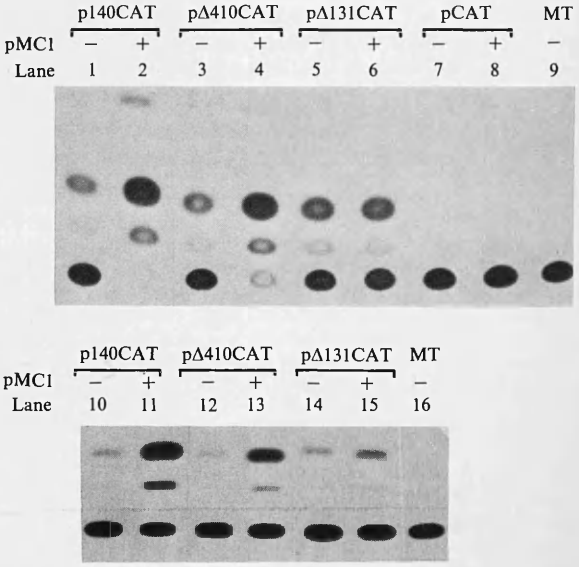


Fig. 4. Activity of p140CAT and deletion mutants in BHK cells (lanes 1 to 9) and HeLa cells (lanes 10 to 16). To demonstrate the responsiveness of these sequences to Vmw65, cells for lanes marked + were cotransfected with pMC1 and those for lanes marked − with pUC18. Samples 10 to 16 were excised from the same autoradiograph and represent equal exposures.

the three plasmids were tested with and without cotransfected pMC1, a plasmid that expresses HSV-1 Vmw65. The VZV upstream sequences were much less active in directing CAT expression, since it was necessary to dilute extracts from cells transfected with pIE3CAT or pRR55 250-fold to obtain comparable enzyme levels. When pMC1 was cotransfected with pIE3CAT a fivefold stimulation of CAT activity was observed, whereas a small stimulation (not greater than twofold) of CAT expression from pRR55 occurred. Cotransfection of pMC1 with p140CAT resulted in a 15-fold stimulation of activity, suggesting that the transcription of the IE gene of VZV is stimulated by Vmw65. Therefore, the major VZV IE gene has a relatively weak promoter, but contains sequences that respond to activation by Vmw65.

To analyse in greater detail the DNA sequences involved in the response to Vmw65, a set of 5′ endpoint deletions of p140CAT was produced. Plasmids were transfected into BHK or HeLa cells, with or without pMC1, and expression of CAT was assessed after correction for different transfection efficiencies by the use of pFJ3, an internal control plasmid that expresses β-galactosidase (Fig. 4). Deletion to −410 or −131 did not affect the expression of CAT in either cell type. When pMC1 was included in transfection mixtures, the expression of p140Δ410CAT was stimulated on average 21-fold in BHK cells and 15-fold in HeLa cells, whereas expression of p140Δ131CAT was not stimulated in either cell type. The apparent small stimulation of p140Δ131CAT in HeLa cells was not reproducible. Therefore, sequences that respond to Vmw65 are located between −410 and −131, but sequences that determine the basal expression of the VZV major IE gene are located within the 131 bp upstream of the mRNA 5′ terminus.

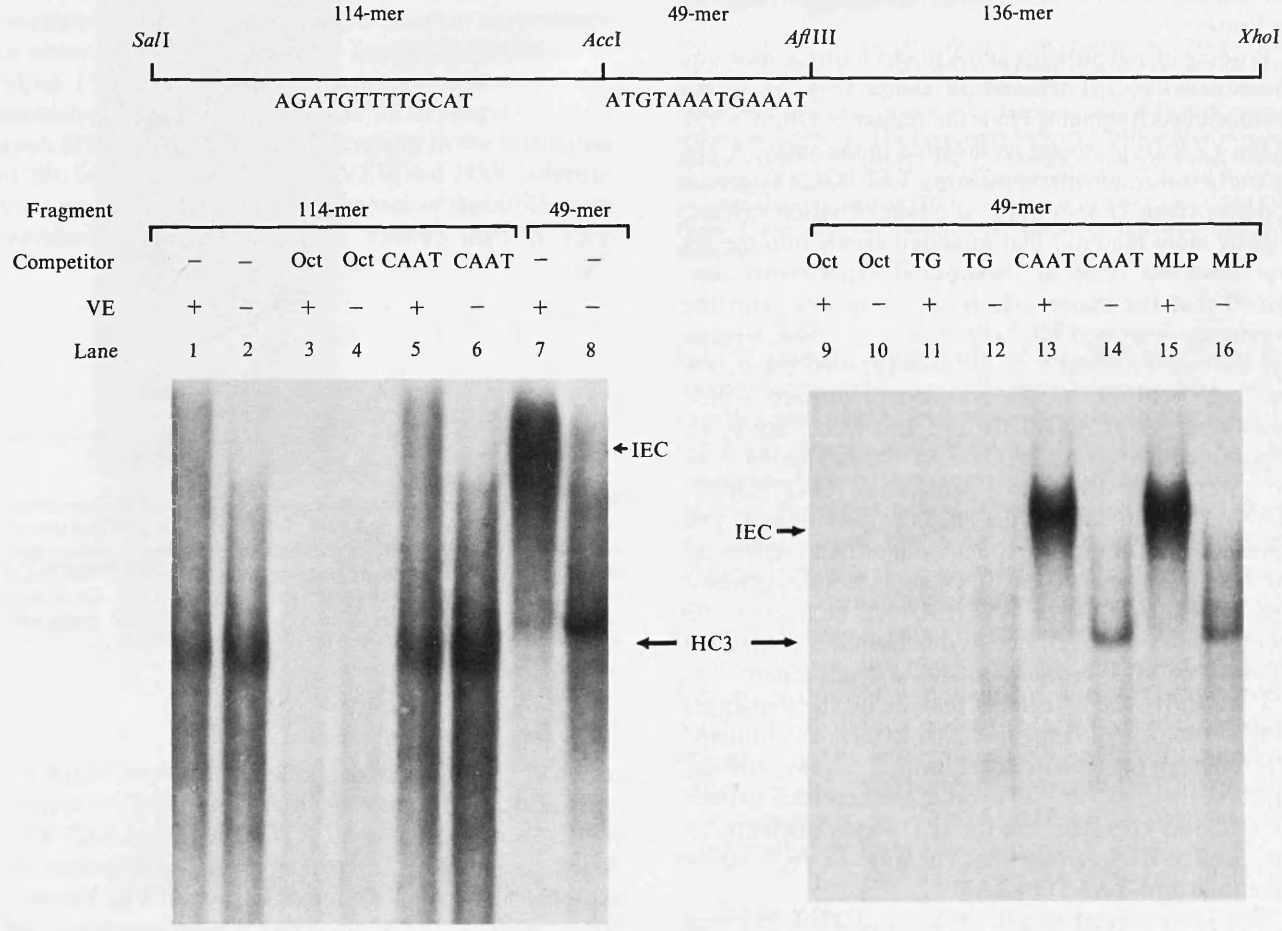


Fig. 5. Gel retardation analysis of the region between −410 and −131. Fragments containing the octamer (Oct) motif (114 bp) and the octamer/TAATGARAT motif (49 bp) were incubated with HeLa cell nuclear extract. HSV-1 virion extract (VE) was present in lanes labelled + and absent from lanes labelled −. A 100-fold molar excess of oligonucleotides containing octamer (lanes 3, 4, 9 and 10), TAATGARAT (lanes 11 and 12), CCAAT box (lanes 5, 6, 13 and 14) or adenovirus MLP binding site (lanes 15 and 16) were added as competitors. Complexes HC3 and IEC are labelled.

Formation of protein–DNA complexes responsible for stimulation by Vmw65

Previous studies have shown that stimulation of transcription by Vmw65 depends on the presence of TAATGARAT in the control sequences of the gene in question. Vmw65 does not bind directly to TAATGARAT, but interacts with proteins that themselves recognize the octamer element ATGCAAAT as well as TAATGARAT. Analysis of the region upstream of the TATA box reveals two sites that match the octamer element closely. Site I (TTTTGCAT) is centred at −345 and matches the octamer element at seven out of eight positions in the opposite orientation. Site II (ATGTAAATGAAAT) is centred at −256 and is a more complicated motif representing a combination of the octamer sequence (seven out of eight matches) and the TAATGARAT motif (eight out of nine matches). Both of these sites are within nucleotides −410 to −131, defined above as the region that determines the response to Vmw65.

Binding of cell proteins and Vmw65 to these sites was investigated by gel retardation assays (Fig. 5). Three radiolabelled fragments from the region −410 to −131 in the VZV IE promoter were tested in the assay. A 114 bp *Sal*I/*Acc*I fragment, containing TTTTGCAT, gave a complex (lane 2) and, upon addition of virion extract, slightly more material that migrated slowly into the gel was observed (lane 1). Additional experiments confirmed that the major complexes comigrated with the previously described HC3 (Preston *et al.*, 1988) (results not shown). Production of the radiolabelled complexes was competed for by the addition of oligonucleotide containing ATGCAAAT (lanes 3 and 4), but not by an oligonucleotide containing CCAAT (lanes 5 and 6). A 49 bp *Acc*I/*Afl*III fragment containing ATGTAAATGAAAT readily formed the HC3 complex (lane 8) and upon addition of virion extract a major band representing IEC was formed (lane 7). Oligonucleotides containing TAATGAGAT or ATGCAAAT competed efficiently (lanes 9 to 12), but oligonucleotides containing CCAAT or MLP recognition sites did not (lanes 13 to 16). An *Afl*III/*Xho*I fragment making up the remainder of the −410 to −131 region failed to form any protein–DNA complexes (results not shown). Thus two subfragments of the −410 to −131 region bind proteins to form an HC3-like complex, and the IEC forms efficiently on the *Acc*I/*Afl*III fragment that contains an eight out of nine match to TAATGARAT.

The protein binding site on an *Acc*I/*Xho*I fragment (Fig. 1) was determined by DNase I protection analysis (Fig. 6). The only footprint detected in the HC3 complex was a disturbance to the digestion pattern at ATGTAAATGAAAT (lane 2) with a slight extension of the region of protection in the presence of Vmw65 (lane 1).

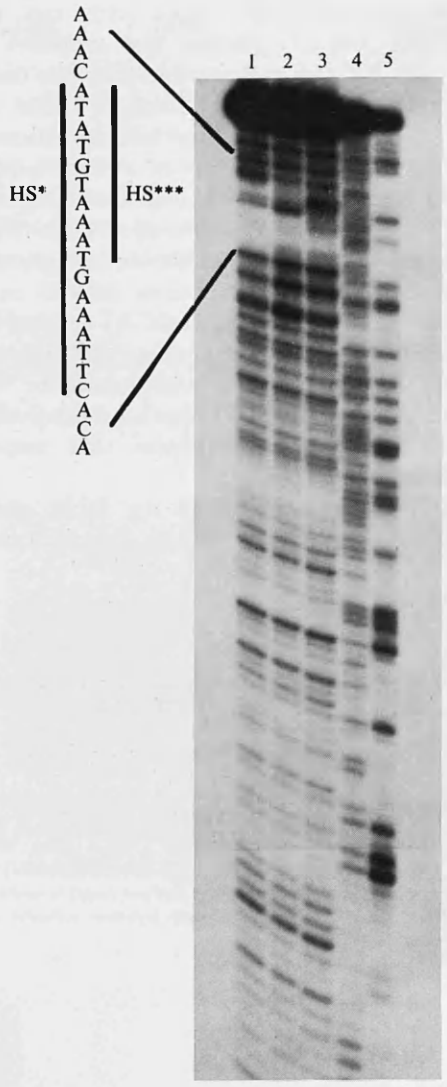


Fig. 6. DNase I footprinting analysis of a 165 bp *Acc*I/*Xho*I (−296 to −131) fragment. Lanes 4 and 5 are G and G + A sequencing tracks. Prior to DNase I treatment, the fragment in lane 1 was incubated with HeLa cell extract and virion extract, the fragment in lane 2 with HeLa extract, whereas lane 3 represents free fragment alone. Dark bars represent the sequences protected in lanes 1 (left) and 2 (right) and hypersensitive sites are labelled HS.

By analogy with the well characterized HSV-1 system it is likely that the element ATGTAAATGAAAT is of major importance for the observed stimulation of expression from the VZV major IE promoter by Vmw65.

Activity of VZV ORF 10

It was of interest to determine whether the VZV homologue of Vmw65, the ORF 10 product, could also

stimulate transcription from the VZV major IE gene. The coding sequences of Vmw65 in the plasmid pMC1 were replaced by those of ORF 10 to form the plasmid pMCORF10. When pMCORF10 was used in short term transfection assays it failed to stimulate expression from either p140CAT or pIE3CAT (results not shown). To determine whether the protein encoded by ORF 10 was able to form a complex analogous to IEC, the coding sequences were inserted into the polylinker of the plasmid pTZ18R, which allows the transcription of ORFs *in vitro*. Two separate clones of this construction, pTZORF10A and pTZORF10B, were isolated and transcribed, together with the plasmid pGEMTIF (Ace *et al.*, 1988), which encodes Vmw65, using T7 RNA polymerase. In addition, pGEMTIF.in15 cleaved with *Bam*HI was transcribed to provide a transcript lacking the sequences encoding the acidic C-terminal tail, but retaining the N-terminal 411 amino acids of Vmw65 that are homologous to ORF 10. The transcripts were then translated using rabbit reticulocyte lysate in the presence or absence of [^{35}S]methionine. Samples of reactions in which [^{35}S]methionine was incorporated revealed the presence of translation products of the expected sizes upon SDS–PAGE (Fig. 7); differences in the intensities of the bands produced by the VZV and HSV polypeptides are largely due to the occurrence of threefold more methionine residues in HSV-1 Vmw65 than in VZV

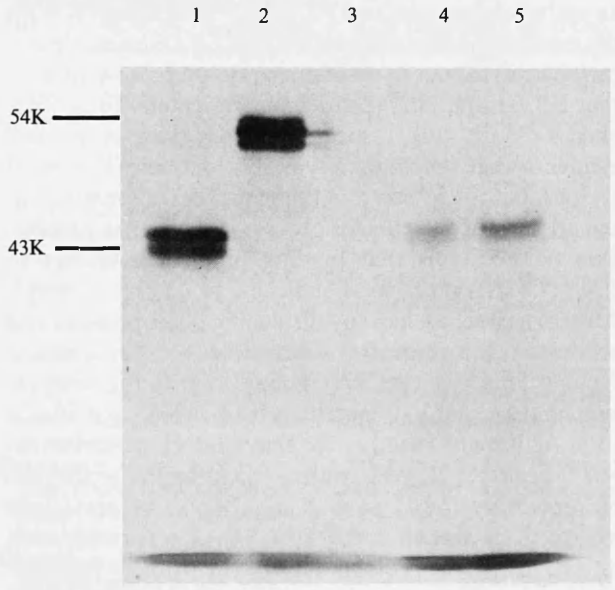


Fig. 7. *In vitro* translation of Vmw65 and ORF 10 polypeptides. RNA transcribed from pGEMTIF.in15 (lane 1), pGEMTIF (lane 2), pGEM2 (lane 3) and pGEMORF10A and -B (lanes 4 and 5) were translated *in vitro* in the presence of [^{35}S]methionine. M_r values of the proteins predicted from the DNA sequence are given. Comparison with M_r standards gives apparent M_r of 63000 (Vmw65) and 54000 (ORF 10).

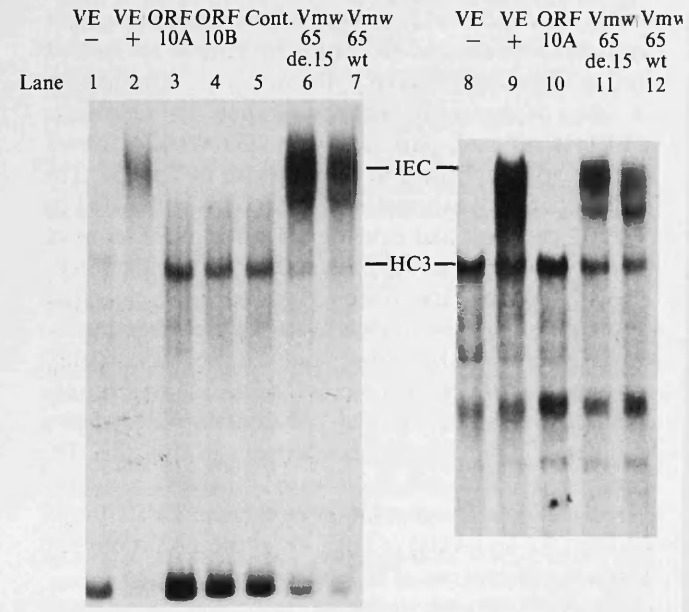


Fig. 8. Gel retardation assay using *in vitro* synthesized Vmw65 and ORF 10 products. Radiolabelled probes used in binding reactions were a 49 bp *Acc*I/*Afl*III fragment from the VZV major IE gene control sequences (lanes 1 to 7) and a 74 bp fragment from HSV-1 IE control sequences (lanes 8 to 12). Fragments were incubated with HeLa cell nuclear extract alone (lanes 1 and 8) and with addition of virion extract (lanes 2 and 9), or translated products of ORF 10 (lanes 3, 4 and 10), pGEM2 (lane 5), pGEMTIF.in15 (lanes 6 and 11), or pGEMTIF (lanes 7 and 12). The positions of complexes IEC and HC3 are indicated.

ORF 10 protein. Samples of reactions lacking [^{35}S]methionine were used for gel retardation assays with radiolabelled DNA fragments containing the TAATGARAT elements of VZV or HSV-1 (Fig. 8). As expected, Vmw65 produced *in vitro* from pGEMTIF directed the formation of IEC when tested with the 49 bp *Acc*I/*Afl*III from the VZV IE gene or the 74 bp oligonucleotide derived from HSV-1 IE gene 4/5 (lanes 7 and 12). Truncated Vmw65 also formed IEC with each fragment (lanes 6 and 11), showing that the acidic C terminus does not participate in the interaction of Vmw65 with cellular proteins to form IEC. However, VZV ORF 10 did not direct the formation of a complex analogous to IEC with either VZV or HSV-1 fragments (lanes 3, 4 and 10). Therefore, although ORF 10 protein is a homologue of Vmw65, it does not activate IE transcription or form IEC.

Discussion

The VZV major IE gene possesses two classes of control sequences, as measured in the transfection assays. A promoter responsible for basal gene expression lies

within the region −131 to +55, whereas sequences that mediate stimulation of expression by Vmw65 are located further upstream, between −410 and −131. The identity of the promoter is unknown, but the elements TTTTGCAT and, particularly, ATGTAAATGAAAT are strongly implicated in the response to Vmw65. The observation that expression of ORF 62 is stimulated by Vmw65 confirms and extends the work of Felser *et al.* (1988), in which the response of ORF 62 to infection by HSV-1 was first described. However, our candidate responding sequences differ from the predicted regulatory elements of the earlier study. Felser *et al.* (1988) assumed that the TATA box is located 374 bp from the first AUG of ORF 62 and consequently identified a number of potential signals upstream of this site. The primer extension and nuclease S1 analyses presented here result in an assignment of the element TTTTAA, 97 bp from the first AUG of ORF 62, as the TATA box and hence a different set of regulatory sequences.

The VZV IE gene upstream region was considerably less effective at directing CAT production than were equivalent control sequences of HSV-1 or HCMV. Thus, VZV does not possess an efficient promoter or enhancer that operates in the systems tested here, in contrast to HSV-1 (Lang *et al.*, 1984; Preston & Tannahill, 1984), HCMV (Boshart *et al.*, 1985) and, presumably, pseudorabies virus (PRV) (Campbell & Preston, 1987). This finding can be viewed in two ways. The alphaherpesviruses HSV-1 and PRV produce only limited amounts of Vmw175 and 180K, the respective IE proteins homologous to the product of VZV ORF 62, during normal infection because autoregulation by the HSV-1 and PRV IE proteins results in a rapid reduction in transcription of their genes (Watson & Clements, 1980; Ihara *et al.*, 1983; O'Hare & Hayward, 1985*b*; Muller, 1987). This homeostatic mechanism stabilizes the levels of Vmw175 and 180K and thus the potential for high level synthesis, which occurs when autoregulation is prevented, is not realized. In the case of VZV, the ORF 62 product may accumulate more slowly, but reach intracellular levels comparable to those of Vmw175 or 180K. Alternatively, the low observed activity of p140CAT may be related to the cell types used in this study. Even the most permissive tissue culture cells, such as HFL cells, human foreskin fibroblasts or Vero cells, give low virus yields and could be viewed as only semi-permissive. Although the activity of p140CAT is low in Vero as well as BHK and HeLa cells (T. A. McKee, unpublished results), the possibility remains that the VZV major IE promoter would be stronger in a natural host cell type. Unfortunately, the tissue culture systems currently available do not accurately reproduce the efficient growth of VZV that occurs during infection *in vivo*.

The failure of VZV ORF 10 protein to stimulate expression from HSV-1 or VZV IE gene promoters was expected, since it lacks the C-terminal acidic region that is crucial for the activity of Vmw65 (Sadowski *et al.*, 1988; Triezenberg *et al.*, 1988; Cousens *et al.*, 1989). It was more surprising to find that ORF 10 did not form a ternary complex with cellular factors and either homologous or heterologous TAATGARAT elements. The possibility that the acidic C terminus is required for formation of IEC was eliminated by the demonstration that the N-terminal 411 amino acids of Vmw65 are fully active in the gel retardation assay, in agreement with the recent report of Greaves & O'Hare (1989). Thus, the observed homology between Vmw65 and VZV ORF 10 protein reflects conservation of features required for assembly of virus particles rather than for structures involved in interaction with cellular factors that bind TAATGARAT. The HSV-1 protein Vmw65 therefore possesses two distinct functional characteristics that are absent from the latter, namely a binding site, as yet undefined, for cellular proteins and an acidic C-terminal activating region.

Functional analyses have now been performed on IE control regions from the alphaherpesviruses HSV-1 (Mackem & Roizman, 1982; Preston *et al.*, 1984; O'Hare & Hayward, 1987), herpes simplex virus type 2 (HSV-2) (Gaffney *et al.*, 1985), PRV (Campbell & Preston, 1987) and VZV. The HSV-1 protein Vmw65 and its HSV-2 homologue stimulate transcription from the IE promoters of all these viruses, but PRV particles do not contain an active counterpart and VZV ORF 10 is inactive. With the reservation that stimulation of VZV IE transcription may be carried out by another virion protein, it appears that HSV-1 and HSV-2 differ fundamentally from PRV and VZV in this respect. All four viruses possess elements that respond to Vmw65 in their IE control regions, but the reason that the sequences are retained in the absence of the effector protein is unclear at present. One possibility is that binding of cellular factors to TAATGARAT *per se* is sufficient to confer IE specificity to a gene, perhaps by displacing other proteins and rendering the promoter accessible to transcription factors. This hypothesis is compatible with the observation that the HSV-1 mutant *in*1814, which specifies a form of Vmw65 inactive for trans-induction, nevertheless transcribes IE genes during infection in the absence of protein synthesis (Ace *et al.*, 1989). As previously suggested (Ace *et al.*, 1989) TAATGARAT, rather than an active effector protein, may be the primary determinant of an IE gene. It is also possible that functional TAATGARAT elements are retained by VZV and PRV because certain cell types contain homologues of Vmw65 that replace its function. This explanation could account for the low activity of the VZV major IE gene promoter in tissue culture cells and suggests that higher activity

could be attained in the appropriate host cells. It is a challenging future prospect to determine whether the intriguing variations in strategies used for the expression of IE genes is related to the biological properties of different herpesviruses.

We thank Professor J. H. Subak-Sharpe for his interest and Dr J. McLauchlan for the synthesis of oligonucleotides. T.A.M. was supported by a Medical Research Council Fellowship and G.H.D. was a recipient of a Medical Research Council Research Training Award.

References

ACE, C. I., DALRYMPLE, M. A., RAMSAY, F. H., PRESTON, V. G. & PRESTON, C. M. (1988). Mutational analysis of the herpes simplex virus type 1 *trans*-inducing factor Vmw65. *Journal of General Virology* **69**, 2595–2605.

ACE, C. I., MCKEE, T. A., RYAN, J. M., CAMERON, J. M. & PRESTON, C. M. (1989). Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. *Journal of Virology* **63**, 2260–2269.

APRHYS, C. M., CUIFO, D. M., O'NEILL, E. A., KELLY, T. K. & HAYWARD, G. S. (1989). Overlapping octamer and TAATGARAT motifs in the VF65-response elements in herpes simplex virus immediate-early promoters represent independent binding sites for cellular nuclear factor III. *Journal of Virology* **63**, 2798–2812.

BAUMRUKER, T., STURM, R. & HERR, W. (1988). OBP100 binds remarkably degenerate octamer motifs through specific interactions with flanking sequences. *Genes and Development* **2**, 1400–1413.

BOSHART, M., WEBER, F., JAHN, G., DORSCH-HASLER, K., FLECKENSTEIN, B. & SCHAFFNER, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41**, 521–530.

CAMPBELL, M. E. M. & PRESTON, C. M. (1987). DNA sequences which regulate the expression of the pseudorabies virus major immediate early gene. *Virology* **157**, 307–316.

CAMPBELL, M. E. M., PALFREYMAN, J. W. & PRESTON, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans acting polypeptide responsible for stimulation of immediate early transcription. *Journal of Molecular Biology* **180**, 1–19.

CLEMENTS, J. B., WATSON, R. J. & WILKIE, N. M. (1977). Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. *Cell* **12**, 275–285.

CORDINGLEY, M. G., CAMPBELL, M. E. M. & PRESTON, C. M. (1983). Functional analysis of a herpes simplex virus type 1 promoter: identification of far-upstream regulatory sequences. *Nucleic Acids Research* **11**, 2347–2365.

CORSALO, C. M. & PEARSON, M. L. (1981). Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. *Somatic Cell Genetics* **7**, 603–616.

COUSENS, D. J., GREAVES, R., GODING, C. R. & O'HARE, P. (1989). The C-terminal 79 amino acids of the herpes simplex virus regulatory protein, Vmw65, efficiently activate transcription in yeast and mammalian cells in chimeric DNA-binding proteins. *EMBO Journal* **8**, 2337–2342.

DALRYMPLE, M. A., MCGEOCH, D. J., DAVISON, A. J. & PRESTON, C. M. (1985). DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate early promoters. *Nucleic Acids Research* **13**, 7865–7879.

DAVISON, A. J. & SCOTT, J. E. (1985). DNA sequence of the major inverted repeat in the varicella-zoster virus genome. *Journal of General Virology* **66**, 207–220.

DAVISON, A. J. & SCOTT, J. E. (1986). The complete DNA sequence of varicella-zoster virus. *Journal of General Virology* **67**, 1759–1816.

EVERETT, R. D. (1984). Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate early polypeptides for maximum activity. *EMBO Journal* **3**, 3135–3141.

EVERETT, R. D. & DUNLOP, M. (1984). Trans activation of plasmid-borne promoters by adenovirus and several herpes group viruses. *Nucleic Acids Research* **12**, 5969–5978.

FELSER, J. M., KINCHINGTON, P. R., INCHAUSPE, G., STRAUS, S. E. & OSTROVE, J. M. (1988). Cell lines containing varicella-zoster virus open reading frame 62 and expressing the 'IE'175 protein complement ICP4 mutants of herpes simplex virus type 1. *Journal of Virology* **62**, 2076–2082.

FICKENSCHER, H., STAMMINGER, T., RÜGER, R. & FLECKENSTEIN, B. (1989). The role of a repetitive palindromic sequence element in the human cytomegalovirus major immediate early enhancer. *Journal of General Virology* **70**, 107–123.

GAFFNEY, D. F., MCLAUGHLAN, J., WHITTON, J. L. & CLEMENTS, J. B. (1985). A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation. *Nucleic Acids Research* **13**, 7847–7863.

GERSTER, T. & ROEDER, R. G. (1988). A herpes virus transactivating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proceedings of the National Academy of Sciences, U.S.A.* **85**, 6347–6351.

GORMAN, C. M., MOFFATT, L. F. & HOWARD, B. H. (1982). Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. *Molecular and Cellular Biology* **2**, 1044–1051.

GREAVES, R. & O'HARE, P. (1989). Separation of requirements for protein–DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. *Journal of Virology* **63**, 1641–1650.

HONESS, R. W. & ROIZMAN, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *Journal of Virology* **14**, 8–19.

IHARA, S., FELDMAN, L., WATANABE, S. & BEN-PORAT, T. (1983). Characterisation of the immediate-early functions of pseudorabies virus. *Virology* **131**, 437–454.

KRISTIE, T. M. & ROIZMAN, B. (1987). Host cell proteins bind to the cis-acting site required for virion mediated induction of herpes simplex virus 1 α genes. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 71–75.

LANG, J. C., SPANDIDOS, D. A. & WILKIE, N. M. (1984). Transcriptional regulation of a herpes simplex virus immediate early gene is mediated through an enhancer type sequence. *EMBO Journal* **3**, 389–395.

MACKEM, S. & ROIZMAN, B. (1982). Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. *Journal of Virology* **44**, 939–949.

MCKNIGHT, J. L. C., KRISTIE, T. M. & ROIZMAN, B. (1987). Binding of the virion protein mediating α gene induction in herpes virus 1-infected cells to its cis site requires cellular proteins. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 7061–7065.

MARSDEN, H. S., CAMPBELL, M. E. M., HAARR, L., FRAME, M. C., PARRIS, D. S., MURPHY, M., HOPE, R. G., MULLER, M. G. & PRESTON, C. M. (1987). The 65,000 M_r DNA-binding and virion trans-inducing proteins of herpes simplex virus type 1. *Journal of Virology* **61**, 2428–2437.

MULLER, M. T. (1987). Binding of the herpes simplex virus immediate early gene product ICP4 to its own transcription start site. *Journal of Virology* **61**, 858–865.

O'HARE, P. & GODING, C. R. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. *Cell* **52**, 453–445.

O'HARE, P. & HAYWARD, G. S. (1985*a*). Evidence for a direct role of both the 175K and 110K immediate-early proteins of herpes simplex virus in the transactivation of the delayed-early promoters. *Journal of Virology* **53**, 752–760.

O'HARE, P. & HAYWARD, G. S. (1985*b*). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. *Journal of Virology* **56**, 723–733.

O'HARE, P. & HAYWARD, G. S. (1987). Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate early gene by three virus-encoded trans-acting factors. *Journal of Virology* **61**, 190–199.

O'HARE, P. C. GODING, C. R. & HAIGH, A. (1988). Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *EMBO Journal* **7**, 4231–4238.

POST, L. E., MACKEM, S. & ROIZMAN, B. E. (1981). Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. *Cell* **24**, 555–565.

PRESTON, C. M. (1977). The cell free synthesis of herpesvirus-induced polypeptides. *Virology* **78**, 349–353.

PRESTON, C. M. (1979). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature sensitive mutant tsK. *Journal of Virology* **29**, 275–284.

PRESTON, C. M. & TANNAHILL, D. J. (1984). Effects of orientation and position on the activity of a herpes simplex virus immediate-early gene far upstream region. *Virology* **137**, 439–444.

PRESTON, C. M., CORDINGLEY, M. G. & STOW, N. D. (1984). Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate early gene. *Journal of Virology* **50**, 708–716.

PRESTON, C. M., FRAME, M. C. & CAMPBELL, M. E. M. (1988). A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. *Cell* **52**, 425–434.

PRUIJN, G. J. M., VAN DRIEL, W. & VAN DER VLIET, P. C. (1986). Nuclear factor III, a novel sequence-specific DNA-binding protein from HeLa cells stimulating adenovirus DNA replication. *Nature, London* **322**, 656–659.

RIXON, F. J. & CLEMENTS, J. B. (1982). Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. *Nucleic Acids Research* **10**, 2241–2256.

SACKS, W. R., GREENE, C. C., ASCHMAN, D. P. & SCHAFFER, P. A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *Journal of Virology* **55**, 796–805.

SADOWSKI, I., MA, J., TRIEZENBERG, S. & PTASHNE, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. *Nature, London* **335**, 563–564.

SHIRAKI, K. & HYMAN, R. W. (1987). The immediate early proteins of varicella zoster virus. *Virology* **156**, 423–426.

SPAETE, R. R. & MOCARSKI, E. S. (1985). Regulation of cytomegalovirus gene expression: α and β promoters are trans activated by viral functions in permissive human fibroblasts. *Journal of Virology* **56**, 135–143.

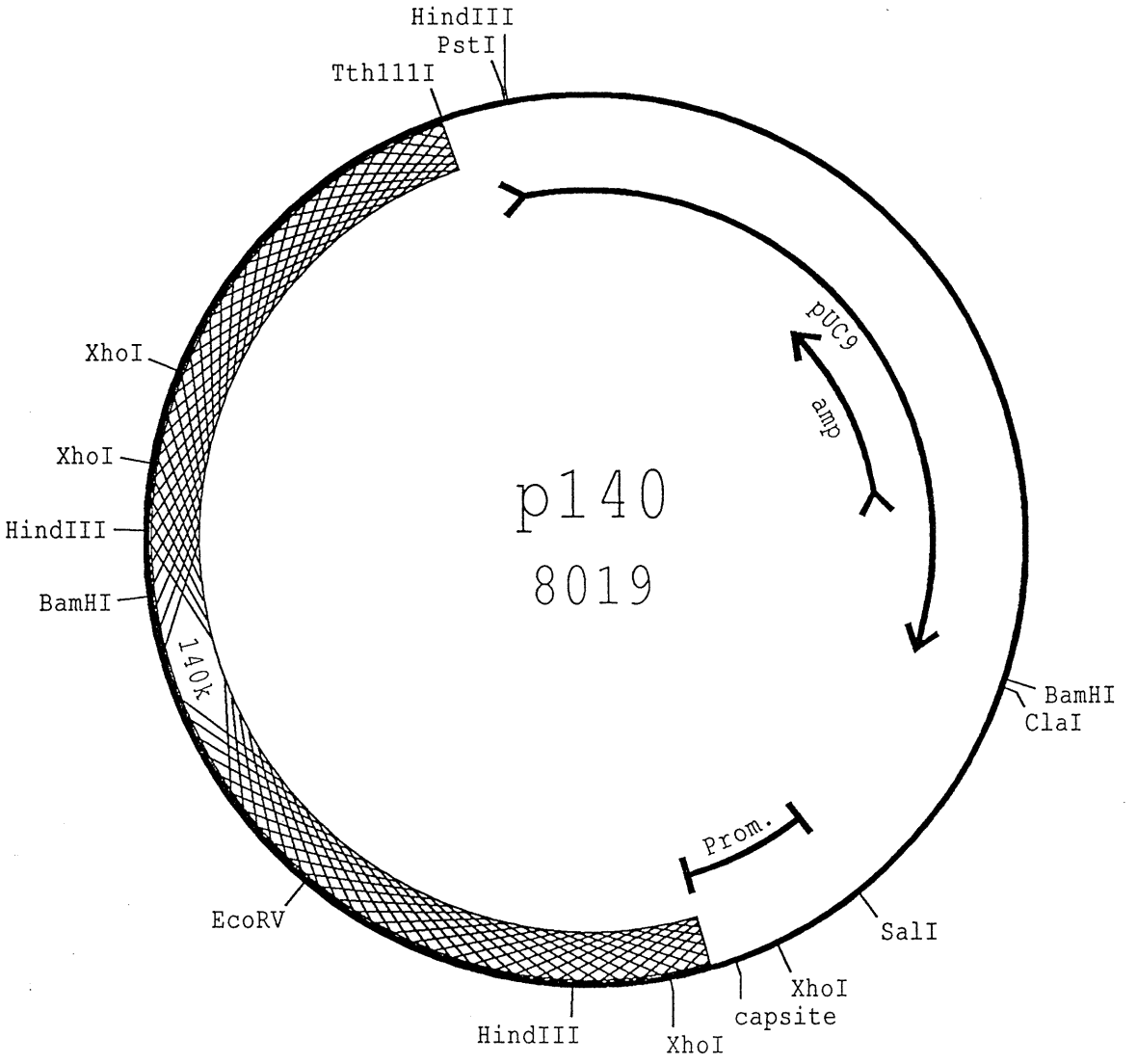
STOW, N. D., MURRAY, M. D. & STOW, E. C. (1986). Cis-acting signals involved in the replication and packaging of herpes simplex virus type 1 DNA. In *Cancer Cells*, vol. 4, pp. 497–507. Edited by M. Botchan, T. Grodzicker & P. Sharp. New York: Cold Spring Harbor Laboratory.

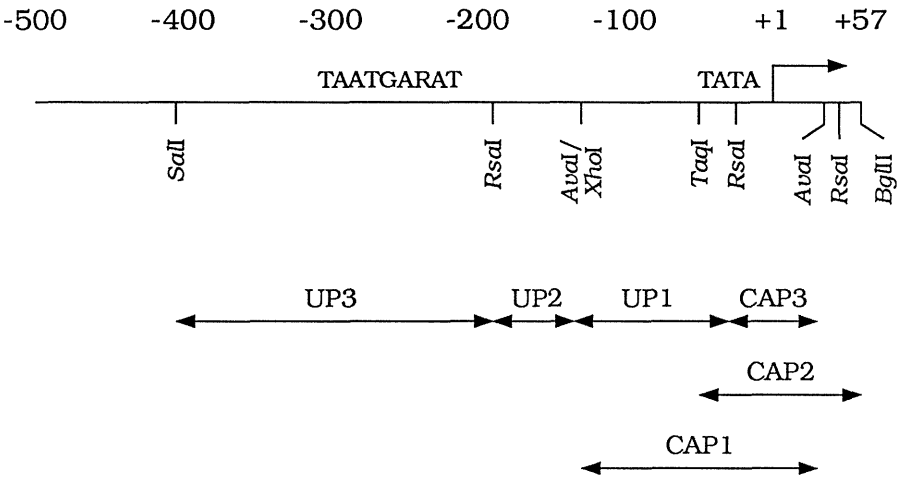
STURM, R., BAUMRUKER, T., FRANZA, B. R. & HERR, W. (1987). A 100kD HeLa cell octamer binding protein (OBP100) interacts differently with two separate octamer-related sequences within the SV40 enhancer. *Genes and Development* **1**, 1147–1160.

TRIEZENBERG, S. J., LAMARCO, K. L. & MCKNIGHT, S. L. (1988). Evidence of DNA : protein interactions that mediate HSV-1 immediate early gene activation by VP16. *Genes and Development* **2**, 730–742.

WATSON, R. J. & CLEMENTS, J. B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. *Nature, London* **285**, 329–330.

(*Received 19 September 1989; Accepted 7 December 1989*)





Probe	Gene 62 Fragment	
	From	To
CAP1	***Xho*I (-131)**	*Ava*I (+43)
CAP2	*Taq*I (-51)	***Bgl*II (+57)**
CAP3	*Rsa*I (-23)	***Ava*I (+43)**
UP1	***Ava*I (-131)**	*Rsa*I (-23)
UP2	*Rsa*I (-188)	***Ava*I (-131)**
UP3	***Sal*I (-410)**	*Rsa*I (-188)